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Isolation and Identification of Cellulytic Fungi from Agrowastes and Sawmill Soils

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Authors' contributions

This work was carried out in collaboration between both authors. Author ETC designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author ADJ managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Five agricultural wastes and sawmill soil were investigated for fungi capable of producing cellulase. Isolation was carried using serial dilution techniques and isolates were screened for cellulase production on carboxymethyl cellulose-containing agar plates. Copious isolates were further subjected to submerged cultivation for quantitative evaluation of cellulase biosynthesis. Highest fungal load was $6.67\pm0.33\times10^6$ sfu/g (Sawmill soil) and the least was $2.33\pm0.33\times10^6$ sfu/g (yam peels). A total of sixty seven fungi were identified from the samples. The isolates were of fifteen fungal species namely *Mucor mucedo, Aspergillus niger, A. repens, A. flavus, A. parasiticus, Articulospora inflata, Gonatobotrys simplex, Gyrothrix circinata, Dendrospora erecta*, *Penicillium notatum, P. italicum, Varicosporium elodea, Gonatobotryum apicolatum, Mucor racemosus* and *Rhizopus nigricans. Aspergillus* had the highest occurrence (36.11%), while the least occurrence was *Varicosporium* and *Gonatobotryum* (2.78%). Highest cellulase activity ratio was exhibited by *Aspergillus niger* (1.90) and the least by *Aspergillus repens* (1.04) on plate screening. The highest cellulase producer among selected isolates under submerged growth was *Gonatobotrys simplex* (1.2143±0.02 U/ml), followed by *Aspergillus niger* (1.1429±0.01 U/ml) and the least by *Aspergillus parasiticus* (0.8265±0.01 U/ml). Submerged protein content was highest in *Aspergillus niger* culture

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and least in *Penicillium notatum.* The study has revealed array of cellulytic fungi from agricultural wastes and sawmill soil.

Keywords: Cellulytic fungi; agricultural wastes; cellulose; sawmill soil; submerged cultivation.

1. INTRODUCTION

Agricultural wastes, not only serve majorly as sources of environmental pollutions, but are also natural repositories of bio-tools especially fungi of industrial interest for lignocellulytic enzymes production. Agrowastes being the most abundant renewable material produced on earth, are accumulated through agricultural practices and agro-allied based industries such as breweries, paper and pulp, textile and timber industries [1]. These wastes generally are composed of polysaccharides such as cellulose and hemicellulose. Cellulose is a linearly condensed polymer consisting of D-anhydroglucopyranose joined by β-1, 4-glycosidic bonds [1]. Cellulose, a β (1→4)-linked glucose polymer, is considered to be the primary product of photosynthesis and the most abundant renewable carbon resource in nature [2].

Among agrowastes and sawmill soil inhabiting fungi, are heterogeneous physiological group of thermophilic cellulytic strains that grow in heaped masses of sawdust, piles of agriculture and forestry products and other accumulations of organic matter where the warm, humid and aerobic environment provides the basic conditions for their development. Usually, the strains constitute various genera in the Phycomycetes, Ascomycetes and Deutromycotina (fungi imperfecti) with a temperature range between 20°C to 62°C for their growth [3]. They are capable of producing hydrolytic enzyme cellulase and are thus named cellulytic fungi. Thermophilic fungi grow at elevated temperatures that offer faster growth rates as compared with mesophiles [4]. These fungi have a powerful ability to degrade polysaccharide constituents of biomass like cellulose and are the potential source of cellulytic enzymes with scientific and commercial interest that could meet extreme parameters of industrial and environmental applications. They can make the process more economical due to their thermostable enzymes, high rate of cellulolysis and ability to saccharify under non-aseptic conditions [5].

Cellulases are the group of hydrolytic enzymes that are capable of degrading all types of lignocellulosic materials [6]. Cellulase complex comprise a family of at least 3 groups of enzymes endo-(1,4)-*β*-D-glucanase (EC 3.2.1.4) exo-(1,4)-*β*-D-glucanase (EC 3.2.1.91), and *β*-glucosidases (EC 3.2.1.21) [7]. The exoglucanase (CBH) acts on the ends of the cellulose chain and releases *β*-cellobiose as the end product; endoglucanase (EG) randomly attacks the internal *O*-glycosidic bonds, resulting in glucan chains of different lengths; and the *β*-glycosidases act specifically on the *β*-cellobiose disaccharides and produce glucose [7]. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials [8]. Cellulase and other lignocellulytic enzymes have significant applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture [7].

The habitat of isolation has a strong influence on the performance of a microorganism in fermentation [9] and the properties of its enzyme could reflect the qualities or physicochemical functions of the habitat. This study therefore bio-prospect some selected agrowastes and sawmill soils for cellulytic fungal biotools of characteristic novelties in cellulase zymosynthesis.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in this study were of analytical grade. Urea was procured from HmbG Chemicals (Hamburg, Germany) and $CaCl_2.2H_2O$ by Emory Laboratory Reagents. $ZnSO_4.7H_2O$, sodium acetate and sodium nitrate by Merk (Germany). Phenol, NaOH and $(NH_4)_2SO_4$ were supplied by Fisher Scientific (USA). $FeSO_4.7H_2O$, K_2HPO_4 and KH_2PO_4 were supplied by GENE Chemicals; $CoCl_2.6H_2O$, $MgSO_4.7H_2O$ and $MnSO_4.6H_2O$ by Progressive Co, HmbG (Germany). Carboxyl methylcellulose, (CMC, low viscosity with degree of substitution 0.78) and potato dextrose agar were purchased from Sigma Chemical Company (St. Louis, MO USA).

2.2 Agricultural Wastes

Five selected agricultural wastes namely, orange peels, yam peels, sawdust, banana peels and pineapple peels were obtained from farm fields, domestic sources and sawmill in Akure, Nigeria. The samples were blended and milled mechanically with electric blender and sieved with a 40 mm mesh size sieve to obtain uniform particle size.

2.3 Sawmill Soil Collection

Soil samples were collected employing sterile hand trowel and polythene bags [10]. The soil was dug out up to 0-10 cm depth and immediately scooped into sterile polythene bags using the hand trowel. The samples were collected from 10 spots in the sawmill site and then mixed together in order to obtain a representative sample.

2.4 Isolation and Enumeration of Fungal Load

For the isolation and determination of fungal load from different sources, 1.0 g of solid sample was suspended in 9 ml of sterilized 0.85% normal saline (NaCl). The solution was vortexed for 60 second and one percent (v/v) of the solution was further serially diluted. One (1.0) millilitre of the diluents was plated in molten sterile Malt extract agar (MEA) and Potato dextrose agar (PDA) and the plates were incubated at 28±2.0°C for 72 hours. Colonies obtained within each plate after incubation were sub-cultured with repeated streaking on sterile Malt extract agar plate and incubated for 72 hours at 28±2.0°C to obtain pure cultures [11]. The isolates were identified in the Microbiology Research Laboratory, Federal University of Technology Akure, Nigeria; according to [12] based on cultural characteristics (colour, shape of colony, surface and reverse pigmentation and texture of the colony) as well as microscopic structures (septate or nonseptate hyphae, structure of hyphae and conidia). The fungal isolates were maintained on agar slant containing PDA at 4°C and sub-cultured at 28±2.0°C for active mycelium at regular intervals.

2.5 Plate Screening of Isolates for Cellulase Production

Primary screening of the isolates was conducted by plating pure cultures onto agar plate containing carboxymethyl cellulose containing streptomycin (100 µg/mL) described by Mandles and Weber as reported by [13]. The plate was incubated at 28±2°C for 5 days. The plates were thereafter stained with 0.1% Congo red dye for 30 min after the incubation period elapsed, followed by destaining with 1M NaCl for 15 min. The colonies with clear zones are indications of cellulase activity were observed and the ratio of diameter of clear zones to colony was determined.

2.6 Cellulase Production Time-profile of Isolates

The effect of cultivation time on cellulases production by the *Aspergillus* species was carried out in production medium up to 120 h. Cellulase production was measured at 24, 48, 72, 96 and 120 h [14] according to the standard assay procedure of [13] as previous described. Then, the period corresponding to highest enzyme production was noted.

2.7 Preparation of Conidial Suspension

Spore suspension of the *Aspergillus* species was prepared by serial dilution method from 5 days old culture with phosphate buffer (pH 7.0) according to [15]. The spore suspension of each strain was prepared by adding sterilized phosphate buffer (pH 7.0) into a 5 days old slant and with aid of sterile inoculating loop; spores were dislodged from mycelia and mixed thoroughly by voltexing. Inoculum size was determined with haemocytometer as described by [16]. Ten microlitres of homogenous spore solution was pipetted into one of the two counting chambers of the haemocytometer. Thereafter, spores in each of the four 0.1 mm^2 corner squares of the haemocytometer were counted, recorded and the average was calculated.

2.8 Cellulase Submerged Cultivation System

The highest cellulase-producing fungal species selected at each interval of treatment by primary screening (results were not shown) were further screened under submerged (shaken condition) state fermentation. Enzyme production was performed in 250 mL Erlenmeyer flask containing 50 mL of basal medium described by Mandles and Weber as reported by [13]. The medium composition (per litre of distilled water) was given as follow: urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4

2.0 g, $CaCl_2$ 0.3 g, $MgSO_4.H_2O$ 0.3 g, peptone 1.0 g, Fe_2SO4. H_2O 0.005 g, $MnSO_4.H_2O$ 0.00016 g, $ZnSO_4.H_2O$ 1.4 mg, $CoCl_2$ 0.002 g and carboxymethyl cellulose (CMC) 10.0 g. The pH of the medium was adjusted to 6.5 with pH meter Model 20 Denver Instrument (Systronics Limited, India) prior sterilization. Then, 100 mL of the liquid medium was measured into 250 mL Erlenmeyer flask and sterilized by autoclaving at 121°C for 15 min. This was cooled and inoculated with 10 discs of 8 mm diameter of the fungus from culture plate using sterile cup borer. The flasks were incubated at 28±2°C for 5 days on a rotary shaker (Gallenkamp, Model RS-12, Singhla Scientific Industries, India) at 120 rpm. Sterile basal medium supplemented with carboxymethyl cellulose without fungus served as a control. Crude enzyme preparation was obtained by centrifugation at 6,000 rpm for 20 min at 4°C using refrigerated ultracentrifuge (Model KBM-70, Centurion Scientific Limited, Germany). The supernatant was used as crude extracellular enzyme source [17].

2.9 Cellulase Activity of Submerged Cultures

Enzyme activity of supernatant collected at the end of submerged state fermentation was determined using Spectrophotometer (Lab-Tech Digital Colorimeter) by the method of [13]. The reaction mixture contained 0.5 mL of culture supernatant (crude enzyme) and 0.5mL of 0.5% (w/v) carboxymethyl cellulose as substrate prepared in 0.5 M sodium acetate buffer pH 5.5. The control tube contained the same amount of substrate and 0.5 ml of the enzyme solution heated at 100°C for 15 min to inactivate the enzyme. Both the experimental and control tubes were incubated at 50°C for 30 min. At the end of incubation period, tubes were removed from water bath (Lamfield Medical England Model DK-600, Labnics Equipment, United Kingdom), and the reaction was terminated by addition of 3.0 mL of 3, 5-dinitrosalicylic acid (DNSA) reagent per tube. The tubes were incubated for 5 min in a boiling water bath for colour development and were quenched by cooling on ice rapidly. The activity of reaction mixture was measured against a reagent blank at 540 nm. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentration of glucose. One unit of cellulase activity was defined as amount of enzyme producing 1 micromole of glucose per minute under the experimental conditions.

2.10 Reducing Sugar Estimation

The amount of reducing sugar in culture filtrate was determined by the method of [18]. A measure of 0.5 mL of the crude enzyme was added to 0.5 M sodium acetate buffer (pH 5.5). The reactions mixture was incubated at 37°C for 20 min. The reaction was terminated by adding 1 mL of DNSA and heated at 50°C for 30 min and then cooled. One millilitre of distilled water was added to the solution. The reaction mixture was allowed to stand for 15 min at room temperature and the absorbance was measured with spectrophotometer at 540 nm. The amount of reducing sugar released was read from standard curve of glucose in one minute under the experimental condition. Amount of reducing sugar released by control sample and buffered substrate solution were compared with test enzyme sample read from the glucose standard curve to give corresponding values for estimation of reducing sugar released at 540 nm.

2.11 Protein Estimation of Submerged Cultures

Protein in the medium was determined by the method of [19] with bovine serum albumin (BSA) as standard. To determine the soluble protein, culture supernatant prepared previously was also used (serially diluted) and folin-phenol reagent prepared as follows. Reagent A: Contained 2% sodium carbonate in 0.1 mL sodium hydroxide (NaOH). This was prepared by adding 0.4 g of sodium hydroxide into 100 mL distilled water. Then 2.0 g of sodium carbonate was added into the solution. Reagent B: Contained 0.5% $CuSO_4.5H_2O$ in 1% Rochelle's salt (Na-K Tartarate). This was prepared by weighing 0.5 g of copper sulphate into 100 mL of Rochelle's salt solution. Reagent C: 1 mL of reagent B was added to 50 mL of reagent A. Reagent D: 10 mL of distilled water was added to 50 mL of phenol reagent. From 10^4 serially diluted extract, 0.3 mL each was pipetted into test tubes, 3 mL of Reagent C was added to each test tubes and the solution was thoroughly mixed and allowed to stand for 10 min. A 0.3 mL of reagent D was added to each tube and mixed properly and allowed to stand for 30 min. The absorbance was measured with a spectrophotometer (Lab-Tech Digital Colorimeter) at 660 nm. The amount of protein (µg/mL) was read from the standard curve of bovine serum albumen (B.S.A).

2.12 Statistical Evaluation

Data obtained were subjected to Analysis of Variance (ANOVA) of statistical package for social sciences (SPSS) software version 17 (Microsoft Corporation, USA) at 95% confidence interval. Data presented as Mean±SE were obtained from triplicate determinations. Means were separated using Duncan's New Multiple Range Test and differences were considered significant at P≤0.05.

3. RESULTS

3.1 Fungal Loads and Isolates from the Samples

Data presented in Fig. 1 showed the fungal counts from each of the agricultural wastes and the sawmill soil. Sawmill soil gave $6.67\pm0.33\times10^6$ sfu/g and recorded the highest number of fungal population, while yam peels had the least fungal counts of $2.33\pm0.33\times10^6$ sfu/g. A sum total of sixty seven fungal isolates was isolated from the agricultural wastes and sawmills soil (Tables 1). All the isolates were of fifteen fungal species namely *Mucor mucedo, Aspergillus niger, A. repens, A. flavus, A. parasiticus, Articulospora inflata, Gonatobotrys simplex, Gyrothrix circinata, Dendrospora erecta*, *Penicillium notatum, P. italicum, Varicosporium elodea, Gonatobotryum apicolatum, Mucor racemosus* and *Rhizopus nigricans* (Tables 1). Fig. 2 shows the frequency of occurrence of each genus out of the eleven genera. *Aspergillus* had the highest occurrence (36.11%), while the least occurrence was *Varicosporium* and *Gonatobotryum* (2.78%).

3.2 Primary Screening of Fungal Isolates for Cellulase Production

The primary screening for fungal cellulase producers was based on the clear zones formed on agar plate of basal medium. Formations of zones of clearance among the isolates were significantly different ($P < 0.05$). However, a total of 9 fungal isolates with the highest clear zones of cellulase activity at 30±2°C (Table 2) were selected. The highest zone of cellulase activity expressed as ratio of clear zone to colony diameter was recorded by the isolate *SWTS0X* (1.90), while the least was with isolate *SMS41-R* (1.04).

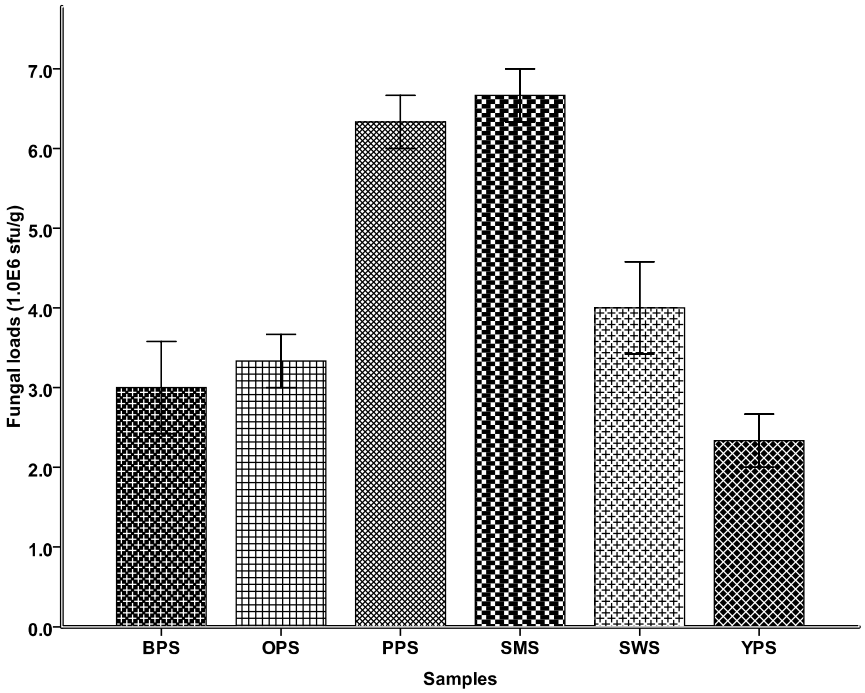

Fig. 1. Fungal load of agricultural wastes and sawmill soil

Keys: BPS: Banana peels; OPS: Orange peels; PPS: Pineapple peels; SMS: Sawmill soil; SWS: Sawdust and YPS: Yam peels

Table 1. Fungal isolates obtained from the selected agricultural wastes and sawmill soil

Sources	Code	Description	Isolates
YPS OPS BPS	*WXMS0r* *ORP07r* *BWrSMi8*	Aseptate hyphae with large diameters. Sporangiophores are erect, branched or unbranched, bearing aglobose sporangia, containing numerous spherical or ellipsoidal spores. Rhizoids are absent. Aerial mycelia are loose cotton wool-like which at first appeared white/grey and later become darker as sporangia are produced. Sporangia are visible as black pin heads over the aerial mycelia.	*Mucor mucedo*
SWS SMS	*SWtS0u* *SMS04-Rk*	White, dense and compact mycelium, Septate, conidiophores are hyaline, slender and appeared sparingly branched. Dull white fluffy cotton-like mycelia. Conidia with 3 slender branches on slender main axis	*Articulospora inflata*
SWS SMS YPS	*SWtS0x* *SMS04-R* *WXMS0y*	Black conidiophores. Colonies consist of a compact white or yellow basal felt covered by a dense layer of large brown to black globose conidial heads which become radial tending to split into several loose columns with age. Conidiophores are smooth-walled, hyaline or turning dark towards the vesicles. Conidial heads are biseriate with the phialides borne on brown, septate nuclei. Conidia are globose to subglobose, dark brown to black and rough-walled.	*Aspergillus niger*
SMS	*SMS02H*	Conidiophores erect, sometimes tall, septate, simple or sparingly branched, percurrent with terminal and intercalary, inflated, denticulate cells were bearing conidia simultaneously; conidia (botryoblastospores) borne singly on the teeth, 1-celled, hyaline, ovoid to subglobose.	*Gonatobotrys simplex*
SMS SWS	*SMS04-G* *SWtS0M*	Conidia slightly curved, narrowly ellipsoid; branched, curved setae present, circinate or wavy	*Gyrothrix circinata*
SMS SWS	*SWS05*-E *SWtS0S1*	Pale brown mycelium. Branches of conidia widely divergent, irregular septate mycelium; conidiophores simple, slender, hyaline; conidia apical, single branched, several celled, each consisting of one main axis with several secondary and tertiary branches arising irregularly, hyaline.	*Dendrospora erecta*
OPS	*SWTS045*	Yellowish green to dark green	*Penicillium notatum*

PPS SWS	*PWiS033* *SWtS045*	hyphae. Conidiophores arising from the mycelium singly or less often in synnemata, branched near the apex, penicillate, ending in a group of phialides; conidia (phialospores) hyaline or brightly colored in mass, 1-celled, mostly globose or ovoid, in dry basipetal chains.	
SMS	*SMS04-Ro*	No distinction between coniophores and conidia. Conidiophores are simple, sparingly branched near the apex, bearing apical conidia. Conidium constists of a main elongated with 2 or 3 laterals on one side. Each lateral septate are branched, pinkish and fluffy in appearance.	*Varicosporium elodea*
YPS BPS SWS	*WXMS0t* *BWrSKt1* *SWtS05a*	Aseptate hyphae with large diameter,thick and cotton wool-like, pin head-like visible sporangia. Growth is usually rapid with stolon development and aerial mycelia usually fill the entire plate.	*Rhizopus nigricans*
SWS SMS	*SWtS01c* *SMS40-N*	Conidiophores dark, tall, stout, upright, typically simple, septate, forming a head of dry conidia on an inflated terminal cell, proliferating to form successive conidiogenous nodes; conidia (botryoblastospores) dark, 1-celled, ovoid to short cylindrical. G. apiculatum bears conidia in branched chains of several conidia	*Gonatobotryum apiculatum*
SWS SMS	*SWtS011* *SMS41-R*	White mycelia, turned brownish to gray with age and became heavily speckled with the appearance of sporangia, sporangia contained ellipsoidal to subglobose sporangiospores, Sporangiophores were short branched, sometimes recurved with encrusted walls, globose in shape. Columella is obovoid, ellipsoidal, cylindrical-ellipsoidal, and slightly pyriform, usually with truncate base and light brown in colour. Chlamydospores were barrel-shaped when young, subglobose and yellowish in colour in old cultures	*Mucor racemosus*
OPS PPS BPS SWS	*ORP70t* *PWiS0C1* *BWrS11* *SWtS01*	Greenish yellow mycelia growth, an upright conidiophores that terminates in a davate swelling bearing phialides at the apex or radiating from the entire surface; conidia are 1-celled and globose	*Aspergillus flavus*
OPS	*SMS08-C*	Dark green mycelia growth with	*Aspergillus parasiticus*

PPS SMS	*PWiS041* *SMS03-Rt*	colourless stipes, Conidial heads mostly uniseriate, vesicles were spherical and uniseriated; conidia distinctly rough	
OPS BPS SMS	*ORP33m* *SMS04Rt3* *SMS04-Rt*	Yellow mycelium with black spores. Septate hyphae bearing conidiophores	*Aspergillus repens*
OPS PPS BPS	*ORP05X* *ORP05X* *BWrS09*	Blue mould growth Septate mycelium bearing Single conidiophores which are branched near the apex ending in phialides that carries the conidia	*Penicillium italicum*

Keys: BPS: Banana peels; OPS: Orange peels; PPS: Pineapple peels; SMS: Sawmill soil; SWS: Sawdust and YPS: Yam peels

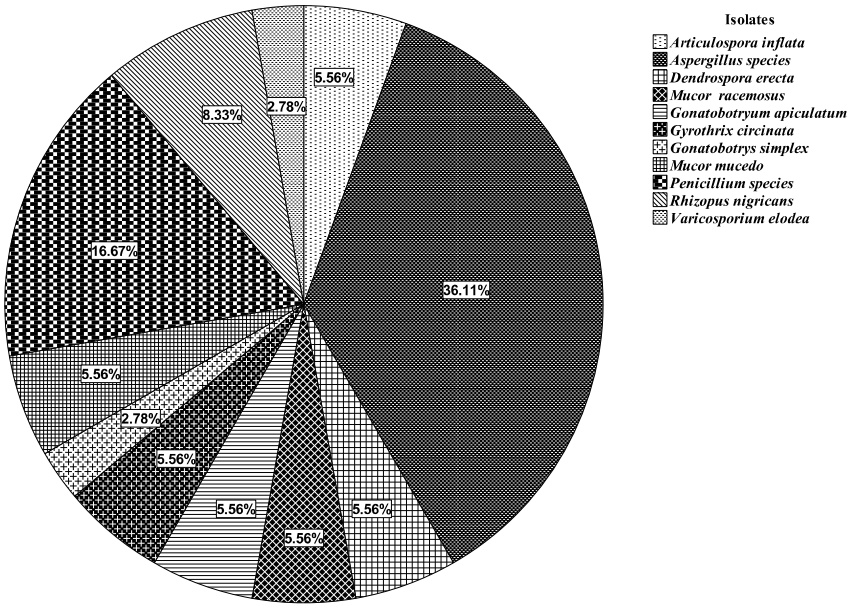

Fig. 2. The frequency of occurrence of fungal isolates obtained from the agro wastes and sawmill soil

Table 2. Selected fungal isolates based on expression of cellulase activity as ratio of clear zone to colony diameter on basal medium

Source	Isolate	Colony (mm)	Clear zone (mm)	Ratio of clear zone/colony
Sawmill soil	*SWtS0x*	30	57	1.90
Sawdust	*ORP70t*	31	52	1.68
Banana peels	*SMS04-Rt*	26	39	1.50
Sawmill soil	*SMS03-Rt*	29	50	1.72
Pineapple peels	*ORP05X*	29	32	1.10
Sawdust	*SWtS0S1*	42	54	1.29
Sawmill soil	*SMS02H*	39	46	1.18
Orange peels	*SWtS045*	51	55	1.08
Sawmill soil	*SMS41-R*	56	58	1.04

Keys: SWtS0x: Aspergillus niger; ORP70t: Aspergillus flavus; SMS04-Rt: Aspergillus repens, SMS03-Rt: Aspergillus parasiticus, ORP05X: Penicillium italicum, SWtS0S1: Dendrospora erecta, SMS02H: Gonatobotrys simplex, SWtS045: Penicillium notatum and SMS41-R: Mucor racemosus

3.3 Determination of Cellulase Activity and Protein Content of Submerged Cultures

Furtherance to quantitative determination of cellulase production, cellulase activities range from 0.8265±0.01 to 1.2143±0.02 U/ml among selected isolates. Productions were significantly different under the fermentation condition ($P<0.05$). Highest cellulase activity was exhibited by isolate SMS02H (1.2143±0.02 U/ml), followed by isolate SWtS0x (1.1429±0.01 U/ml) and least in SMS08-C (0.8265±0.01 U/ml) (Table 3). Protein production ranged from 0.3731±0.01 to 0.1092±0.01 mg/ml (Table 3.) and was significantly different ($P<0.05$). The highest protein content was found with isolate SMS04-R, but the least was recorded by isolate *SWtS045.*

3.4 Effect of Incubation Period on Cellulase Production by Submerged Cultures

The samples were withdrawn from flasks at different fermentation times; 24, 48, 72, 96, and 120 h for the time course production of cellulases. Evidently, cellulase yields by the wild strains were substantially different with respect to incubation time ($P<0.05$). The effect of different incubation periods on cellulase production by the selected isolates were presented in Fig. 3. Cellulase activities were expressed in terms of specific activity for the incubation periods. From the results, it was found that cellulase activity increased with increase in incubation period and reached maximum production peak at 72 h of incubation for isolates SMS03Rt (8.9260±0.002 U/mg), SMS08-C2 (8.0982±0.001 U/mg), and ORP70t (3.8082±0.000 U/mg). Highest cellulase activity was also recorded at 96 h for SWtS0S1 (8.1118±0.000 U/mg), SMS02H (8.4707±0.002 U/mg), SWtS045 (8.2827±0.003 U/mg), SMS41-R (6.6673±0.000 U/mg), SMS04Rt (4.6529±0.000 U/mg), ORP05X (5.0357±0.000 U/mg) and SWtS0x (3.1040±0.000 U/mg). Lowest cellulase activity was recorded at 24 h for isolates SMS03Rt (1.9968±0.014 U/mg), ORP05X (1.4708±0.000 U/mg), SMS08-C2 (82.1099±0.000 U/mg), SWtS0S1 (2.8876±0.003 U/mg), SMS02H (1.8688±0.005 U/mg),

4. DISCUSSION

One of the major approaches to biotechnological researches into agrowastes has been driven by the need to isolate and identify microorganisms which are overproducers and/or are producers of novel exoenzymes that could breakdown organic wastes much more rapidly. In addition, is their ability to withstand unfavourable parameters of the intended industrial or environmental applications [20]. The microorganisms should possess all the properties that are required for cost-effective implementation in achieving these noble tasks [21]. In achieving this goal, various environments are been exploited for potential candidate microorganisms that possess the desired traits. According to [9], habitat of isolation has a strong influence on the performance of microorganisms in fermentation. The properties of a particular microbial enzyme are also presumed to reflect the quality and physicochemical characteristics of the microorganism's environment. This study is a quest into selected agrowastes and sawmill-soil for fungal biotools of characteristic novelties in cellulase zymosynthesis. Isolation of microorganisms for enzyme production from soils (soil from rice mill and soil under decaying wood [22], agro-wastes [11], decaying organic soil [23] and abattoir waste [24]), has been reported.

Table 3. Cellulase production by different fungal isolates under submerged system

Isolates	Cellulase activity (µmol/min/ml)	Protein content (mg/ml)
Aspergillus niger	$0.7619±0.01^{e}$	$0.3731^{e}±0.01$
Aspergillus flavus	$0.7166±0.00^{cd}$	$0.2912^{d}±0.01$
Aspergillus repens	$0.7256±0.02^{d}$	$0.2457^{d}±0.01$
Aspergillus parasiticus	$0.5510±0.01^{a}$	$0.1046^{a}±0.01$
Penicillium italicum	$0.6780±0.02^{b}$	$0.2411^{d}±0.02$
Dendrospora erecta	$0.6757±0.01^{b}$	$0.1729^{c}±0.00$
Gonatobotrys simplex	$0.8095±0.01^{f}$	$0.1456^{bc}±0.01$
Penicillium notatum	$0.6599±0.01^{b}$	$0.1092^{a}±0.01$
Mucor racemosus	$0.7007±0.01^{c}$	$0.1183^{ab}±0.01$

Values are presented as Mean±S.D (n=3). Means with the same superscript letter(s) along the same column are not significantly different (P<0.05)

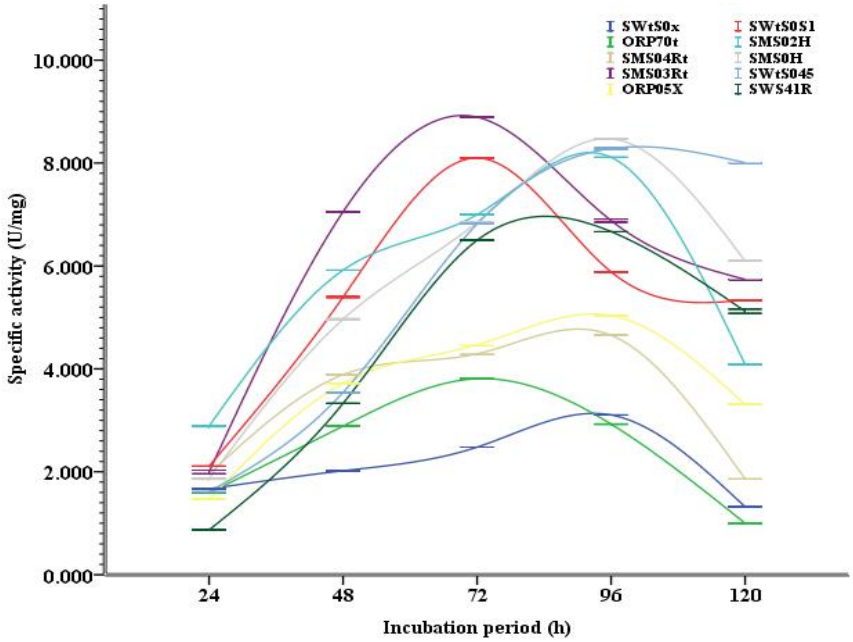

Fig. 3. Cellulase production time-profile of fungi from agricultural wastes and sawmill soil
SWtS0x: *Aspergillus niger, ORP70t*: *Aspergillus flavus, SMS04-Rt*: *Aspergillus repens, SMS03-Rt*: *Aspergillus parasiticus, ORP05X*: *Penicillium italicum, SWtS0S1*: *Dendrospora erecta,* SMS02H: *Gonatobotrys simplex, SWtS045*: *Penicillium notatum* and *SMS41-R*: *Mucor racemosus*

Of all samples studied, sawmill soils had highest fungal counts, while yam peels had the least fungal counts. The highest fungal bio-loads from sawmill soil could be attributed to large availability of different substrates for fungal growth. Also, it could be indications that sawmill soils have been experiencing long term depositions of sawdust and wood dust that might have encouraged the growth of different fungal species. This subscribes to the report of [25], that lignocellulosic substrates such as sawdust, wood chips and bark, which are composed of natural biopolymers (cellulose, hemicellulose and lignin) are good substrates for fungi and are available in various forms in sawmill soil. The physicochemical parameters of the sawmill soils could also be suitable for the growth of diverse groups of fungi. According to [21], low moisture content of soils which is prevailing in sawmill soil often favours the growth of fungi as dominant cellulytic biota. The high fungal loads may be due to lack of efficient management and discharge of agricultural wastes (sawdust) into the environments [26]. Ability of the fungi to produce series of extracellular enzymes required for depolymerising different recalcitrant polysaccharide biomass in sawmills soil could be ascribed to the high fungal loads in the soil. The low fungal counts observed in yam peels could be as a result of level of nutrients available for fungal growth. Aside this, the isolates can be said to be transient microorganisms surviving only in the absence of inhibitory substances [26] for a short period of time in the yam peels. The variations in the fungal loads from different agrowastes depend on the ability of microorganisms to colonize, metabolize and obtain necessary nutrients from the agrowastes as well as the prevailing environmental conditions and physicochemical properties of the ligninocellulosic biomasses and sawmill soils. This also in line with [27], that chemical constituent and physicochemical property of lignocellulosic biomass makes them suitable receptacles for cellulytic fungi.

The fungal genus with highest percentage occurrence was *Aspergillus* (*Aspergillus niger, A. repens, A. flavus, A. parasiticus*) in the samples studied. This could be adduced to their ability to utilize diverse sources of biomass as substrates for growth. In addition, it could reflect the ability of *Aspergillus* to produce different extracellular enzymes complex required to degrade cellulosic materials efficiently. The low occurrence of *Varicosporium* and *Gonatobotryum* could be

attributed to their inefficient abilities to degrade and derive nutrients from lignocelluloses. Furthermore, it could be due to inability to produce one or more ligninocellulytic enzymes required in enzyme complex necessary for degradation of agricultural polysaccharide components. The fungal occurrences and isolated species concur with the findings of [28,27]. They found that *Aspergillus* species had highest occurrence (36.11%), while *Penicillium* species showed moderate occurrence (16.67%) among soil isolates.

The documentation of fungi capable of cellulase production from five agrowastes and sawmill soil subscribes to [11], who isolated mannanases-producing fungi from agricultural wastes. Soils generally are end receiver of all accumulations of organic matter and heaped masses of plant material, piles of agriculture, and forestry products that usually provide appropriate physiological and nutritional conditions for fungal growth especially the cellulytic biota [3]. The suitability and ease of agricultural wastes and sawmill soils to home cellulase-producing fungi could typically be due to their high hemicelluloses and cellulose contents. This is similar to report of [29]. Enzyme production by fungi using different agricultural wastes reported for *A. niger* [13], *A. fumigatus, A. nidulans* and *A. humicola* [30], substantiates the cellulase-producing capability of the agrowastes and sawmill soil fungi.

The initial screening of isolates for cellulytic properties was conducted based on the clear zones formed on carboxymethyl cellulose agar plates. Formation of clear zones on carboxymethyl cellulose containing agar plates indicated the ability of the isolates to produce extracellular cellulase. Fungi isolated from different agricultural wastes and sawmill soil unveiled different activity ratio on carboxymethyl cellulose agar plates. This suggested that the isolates have different cellulase producing capabilities. Plate screening based on clear zones formation of cellulases produced by microorganisms had been early reported for *Penicillium funiculosum, P. simplicissimum, P. brevicompactum, Stachybotrys chartarum*, *Aspergillus repens, Mucor racemosus, Penicillium fellutanum, Epicoccum nigrum, Gliomastix murorum var. murorum,* and *Paecilomyces lilacinus* [31], *Aspergillus niger* [9] and psychrophile [22]. The differences in diameter of clear zones formed by the isolates could be as a result of the ability of cellulase produced to diffusion through the medium and/or direct functionality of their genetic makeup to secrete cellulase with high diffusion speed [17,32]. The fungi in addition, are known to produce an array of extracellular enzymes, including cellulases, hemicellulases, pectinases, chitinases, amylases, proteases, phytases and mannases into their cultivation media [33].

Quantitative evaluation of cellulase produced by isolates under submerged state conditions could be an impetus to the ability of the fungi. The prodigious accumulation of extracellular enzymes elaborated by microorganism into its culture medium is a critical aspect of enzyme biotechnology. More also, it could be a crucial trademark of the microorganism or its enzyme usefulness for industrial application. The significant variability in the amount of cellulase biosynthesized by the isolates signifies that production rate hinges on genetic traits of the microorganisms and the fermentation parameters. This was also supported by [17, 32].

The effect of incubation period on cellulase production by the wild strains was estimated for 120 hours. Highest biosynthesis of enzyme was observed after 72 hours of incubation in *A. flavus, A. parasiticus;* and 96 h in others, beyond which production substantially decreased. Maximum cellulase activity for *A. niger* after 72 h and 96 hours of incubation was earlier reported by [34,35] respectively. Highest zymosynthesis of cellulase by *A. flavus* grown on orange peels after 96 hours of incubation was reported by [32]. The drop in cellulase activity of the fungi after 72 h or 96 h of incubation as the case may be, might be due to depletion of nutrients, and accumulation of by-products in the fermentation medium. This was in accordance with [11,36].

Besides the aforementioned, the medium components were initially at their maximum concentrations, more available for fungal metabolism, and thus make a rapid rise in enzyme biosynthesis. Perpetuations of cultivation time led to complete use up of substrate components by fungi which could inhibit enzyme secretion pathways. Repression of enzyme production as a result of total substrate consumption by microorganisms was earlier reported by [17]. Other related factors, such as the nature of the microorganism and physiological conditions of the media, were also considered to be important during enzyme production [36]. Multi-factorial influences of parameters such as carbon source, pH,

temperature, and aeration and growth time are cruxes for cellulases yield [30].

5. CONCLUSION

The searches for suitable microorganisms that possess robust biotechnological potentials continue; agricultural wastes and sawmill soils proved to be ideal environments for cellulase-producing fungi. The species of fungi obtained can thus be exploited for industrial production of cellulase after further studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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