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Abigail I. Ogbonna
Department of Plant Science and Technology, University of Jos, Nigeria.

Isaac A. Onyimba
Department of Science Laboratory Technology, University of Jos, Nigeria.

Aleruchi Chuku
Department of Microbiology, Federal University Lafia, Nasarawa State, Nigeria.

Patience O. Nwadiaro
Department of Plant Science and Technology, University of Jos, Nigeria.

Chike I. C. Ogbonna
Department of Plant Science and Technology, University of Jos, Nigeria.

Festus C. Onwuliri
Department of Plant Science and Technology, University of Jos, Nigeria.

Correspondence:
Abigail I. Ogbonna
Department of Plant Science and Technology, University of Jos, Nigeria.

Growth assessment and amylase production by *Aspergillus niger* and *A. terreus* isolated from soils of *Artemisia annua* l. Plantation

Abigail I. Ogbonna, Isaac A. Onyimba, Aleruchi Chuku, Patience O. Nwadiaro, Chike I. C. Ogbonna, Festus C. Onwuliri

Abstract
Fungi associated with soils of decomposing *Artemisia annua* L. process waste in an *A. annua* Plantation were studied. Two of the isolates, *A. niger* and *A. terreus* which had high frequencies of occurrence were assessed for their growth rates over an incubation period of 168hrs using Czapek Dox and Sabouraud Dextrose Agar media. Their abilities to produce glucoamylase of biotechnological importance using submerged fermentation (SmF) were studied. The two isolates grown in basal medium containing starch as sole source of carbon and were harvested at 24 hour intervals over a period of 168hours. *A. niger* and *A. terreus* were found to have colony diameters of 5.2cm and 5cm after the 7th day of incubation. The highest glucoamylase potential at pH 5.03 was demonstrated by *A. terreus,* with peak enzyme activity of 0.375mmol^{-L} while that of *A. niger* was 0.281mmol^{-L} on the 6th day of incubation. These two fungal species could be useful in the degradation of biological wastes.

Keywords: Growth Assessment, Soil, Amylase production, *Aspergillus niger*, *A. terreus*, *Artemisia annua.*

1. Introduction
Fungi have proven to be an important source of industrial enzymes and due to their diversity; they have been recognized as source of enzymes with useful and/or novel characteristics [1, 2]. Glucoamylase (GA) is a hydrolyzing enzyme. It can degrade amylose and amylopectin by hydrolyzing both α-1, 4 and α-1, 6 glucosidic links of starch to produce glucose [3, 4]. Hence glucoamylase can convert starch completely to glucose and have found applications in many industries [5, 4, and 6]. It is used for the production of glucose and fructose syrups from liquefied starch [7]. It is also employed in baking, juice and beverage making, pharmaceuticals, and numerous fermented food production industries [8], textile, leather, paper, detergents industries and bioconversion of solid wastes [9]. Due to its increasing demand, the production technique of glucoamylase and α amylase has been studied extensively. Amylase production has been reported from several fungi, yeasts, bacteria and actinomycetes isolated from natural habitat such as soil and organic wastes. Soil provides a heterogeneous and complex environment for all soil inhabitants [10].
Among the large number of filamentous fungi capable of producing useful enzymes, the genus *Aspergilli* are particularly interesting due to their ease of cultivation, feasibility of mass culture and ease of genetic manipulation. They are also known for high production of extracellular enzymes with potential industrial exploitation.
In the present study an attempt has been made to screen the indigenously isolated *Aspergillus* species from soil of *Artemisia annua* L. plantation for amylase production which could be employed in biodegradation of *A. annua* process waste in the plantation. The biodegradation process could help in the reduction of spontaneous fire outbreak as well as to enrich the humus content of the plantation soil.

2. Materials and methods

2.1 Isolation of fungi from soil

Fungal colonies were isolated from soil samples collected from *Artemisia annua* plantation soil enriched for amylase producing microorganisms by soil plate method described by [11] using Potato Dextrose Agar medium. The inoculated Petridishes were incubated at 25± 2° C for 5days. Six different fungal isolates differentiated on the basis of cultural and morphological characteristics were obtained after incubation. The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 25± 2° C for 5days in order to obtain pure fungal colonies. The isolates were identified as *Aspergillus fumigatus, A. niger, A. terreus, Cladosporium cladosporioides, Penicillium Chrysogenum* and *P. citrinum* using standard identification manuals [12, 13]. The isolates were maintained on Potato Dextrose Agar slants supplemented with 0.5ml gentamycin (40mg/ml) in order to suppress the growth of bacteria and were kept at 4ºC. Based on the frequency of occurrence of the fungal isolates, *Aspergillus niger* and *A. terreus* were selected for further studies.

2.2 Growth rate studies

In performing growth rate studies on the isolates, colony diameter and mycelia weight measurements were used.

2.2.1 Colony diameter measurement

For the estimation of colony diameter of the fungal isolates, modified method of [14] was employed. A sterile cork borer of pore size of 5.0 mm diameter), was used to bore a hole enclosing a disc of the pure culture of the respective pure fungus maintained on Potato Dextrose Agar plates. One disc of each fungus was aseptically transferred and placed at the center of sterile, freshly prepared Sabouraud and Czapek-Dox agar plates. This experiment was done in triplicates. The average diameters of the growing colonies were measured at intervals of 24 for 120 h using a calibrated transparent ruler and the values recorded.

2.2.2 Mycelia weight measurement

Modified method of [14] was used for the mycelia weight measurement. Conidia of each of the fungus were harvested using sterile cork borer and three discs transferred into a sterile test tube and diluted with equal volume of sterile distilled water. The content of the test tube was agitated vigorously to form a homogenous mixture. One milliliter of the spore suspension was inoculated unto Petri dishes containing already prepared Potato Dextrose agar medium. Seven Petri dishes were used for each of the isolates and were done in triplicates. The Petri dishes were incubated in the dark at 25° C without agitation. Cultures were harvested at 24 h intervals over a 168 h period. A set of each replicate was removed at 24h interval, the mycelia washed and dried in the oven at 80° C for 2 h, and carefully weighed. The average weight increase of mycelia per day was recorded.

2.3 Enzyme Assay on the Selected Fungal Isolates

2.3.1 Preliminary Screening for Amylase Production Using Plate Assay

Preliminary screening was done using the modified method of [15] by inoculating 5mm mycelia discs from the edge of an actively growing 4-day old fungal isolate on starch agar (containing peptone, 1%; KH_2PO_4, 0.5%; agar 2% and 1% (w/v) starch (HiMedia) which served as a carbon source. The medium was supplemented with 0.5ml gentamycin (40 mg/ml) to suppress bacterial growth. The starch agar plates were incubated at 25 ºC for four days after which they were flooded with lugol's iodine solution (Iodine 0.2 g, Potassium Iodide-0.4g, Distilled water-100 ml) for two minutes. Control experiment was also set up using basal salt agar plates without the inducing substrate (starch). The plates were observed for a clear zone of hydrolyzed starch against a blue background of unhydrolyzed starch. The experiment was replicated thrice. The diameters of the clear zones were measured and the means were recorded as the measure of amylase activity.

2.3.2 Secondary Screening for Amylase Production by Submerged State Fermentation

For the enzyme assay, modified method of [16] and [17] as was described by [18] was adopted. The pure cultures of each fungus were grown in separate 250 ml Erlenmeyer flask containing 100 ml of production medium (NH_4NO_3, 1%; KH_2PO_4, 0.2%; $MgSO_4.7H_2O$, 0.2%; $FeSO_4.7H_2O$, 0.001% and soluble starch, 2%; pH 6.0) and incubated at 25ºC for 5 days under static condition. Enzyme activity was assessed at 3, 6, and 9 day intervals using cell free culture filtrate of each of organisms. Boiled enzyme extracts was used as blank and D-glucose as standard. The glucose concentration was determined by DNS method, as described by [19]. The color developed was measured at at 625nm using Jenway spectrophotometer. The experiment was done in triplicates.

2.3.3 Effect of Nitrogen Source on the Concentration of Reducing Sugar Produced

In addition further investigation was carried out using Starch medium supplemented with 1% Yeast extract using modified method of [20] to determine the effect on the concentration of reducing sugar produced. For the enzyme assay, 100ml of basal medium already containing 1% starch was prepared in six (6) 250ml Erlenmeyer flask. A volume of 1% yeast extract was added to the medium. A weight of 5mm mycelial plugs of the test fungi (*A. niger* and *A. terreus*) were inoculated respectively. The flasks were incubated at 25º C for 9 days under static condition. Enzyme activity was assessed at 3, 6, and 9 day intervals using cell free culture filtrate of each of organisms. Boiled enzyme extracts was used as blank and D-glucose as standard. The glucose concentration was determined by DNS method, as described by [19]. The color developed was measured at at 625nm using Jenway spectrophotometer. The experiment was done in triplicates.

3. Results

3.1 Isolation of Fungi from Soil

Six filamentous fungi were isolated from soils of *Artemisia annua* plantation and were identified as *Aspergillus fumigatus, A. niger, A. terreus, Cladosporium cladosporioides, Penicillium Chrysogenum* and *P. citrinum* based on their cultural and morphological characteristics of their sporulating structures. Details of these isolated fungal structures are presented in Table 1. The fungal isolates are also shown in Figures 1a- 1f.

Table 1: Cultural and morphological characteristics of the fungal isolates

Organism identified	Hyphae	Cultural characteristics	Sporrulating structure	Morphology of conidiophore
Aspergillus fumigatus	Septate.	At first white, then bluish-green to gray, very powdery due to massive conidia production.	Conidiophore terminated in dome shaped vesicle. Uniserriate, Conidia borne on the phialides, only on upper two- thirds of vesicle.	Conidiophores Short and smooth
A. niger	Septate	Mycelia whitish at first and then sometimes with yellow margins. Turns black and powdery due to conidia production.	Biserriate phialides covers the entire globose vesicle to form a radiate head.	Conidiophore smooth or finely roughened
A. terreus	Septate	Orange-brown or Brown with age. Reverse is light brown	Biserriate phialides borne on hemispherical vesicle. Conidial heads strictly columnar	Conidiophores short and smooth
Cladosorium cladosporioides	Septate	Grayish to olivaceous-brown. Velvety, powdery with age due to abundant conidia.	Conidia is ellipsoidal or lemon-shaped. Ramoconidia present at base of conidial chain.	Conidiophores are long and smooth, without sympodial elongations and swellings.
Penicillium chrysogenum	Septate	Whitish at first then turns pale green blue.	Phialides flask-shaped, bearing subglobose smooth conidia.	Conidiophores up to four stage branched, smooth-walled. Have secondary sterigmata.
P. citrinum	Septate	Blue green in colour, leathery.	Conidia smooth walled, produced in columns.	Conidiophores smooth,

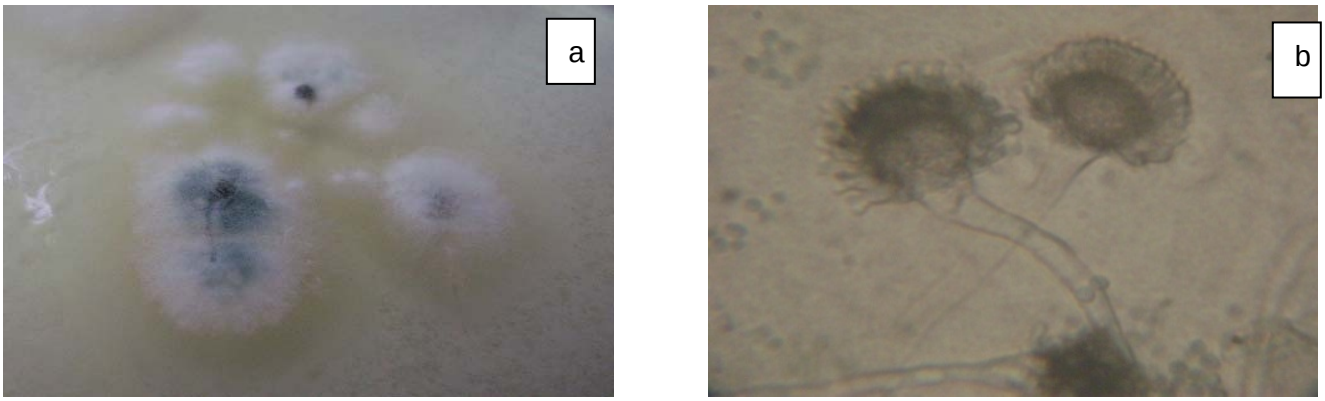

Fig 1a: *Aspergillus fumigatus*:
(a) Colony on Potato Dextrose Agar
(b) Structure showing the conidigenous cells

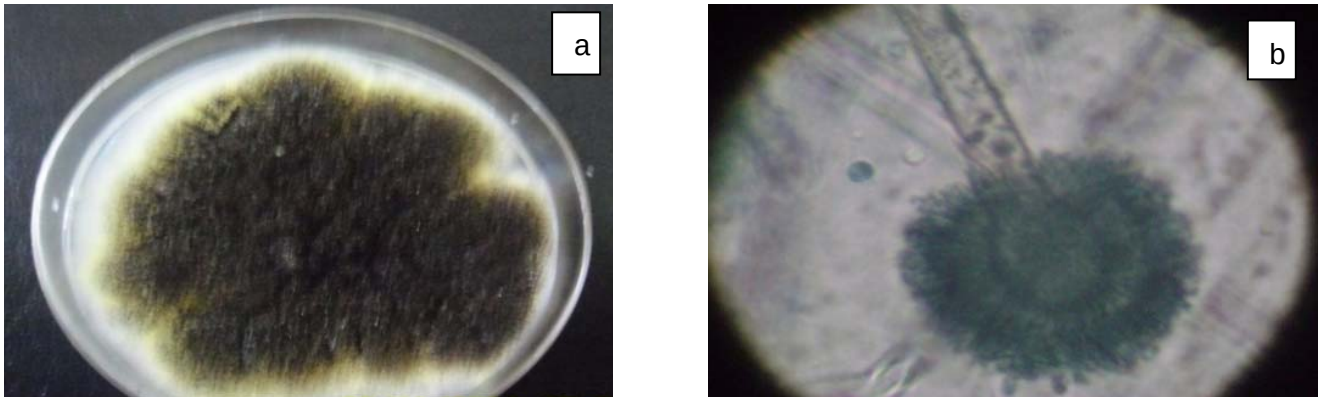

Fig 1b: *A. niger*:
(a) Colony on Potato Dextrose agar plate and
(b) Structure showing the conidiophore and the conidial head

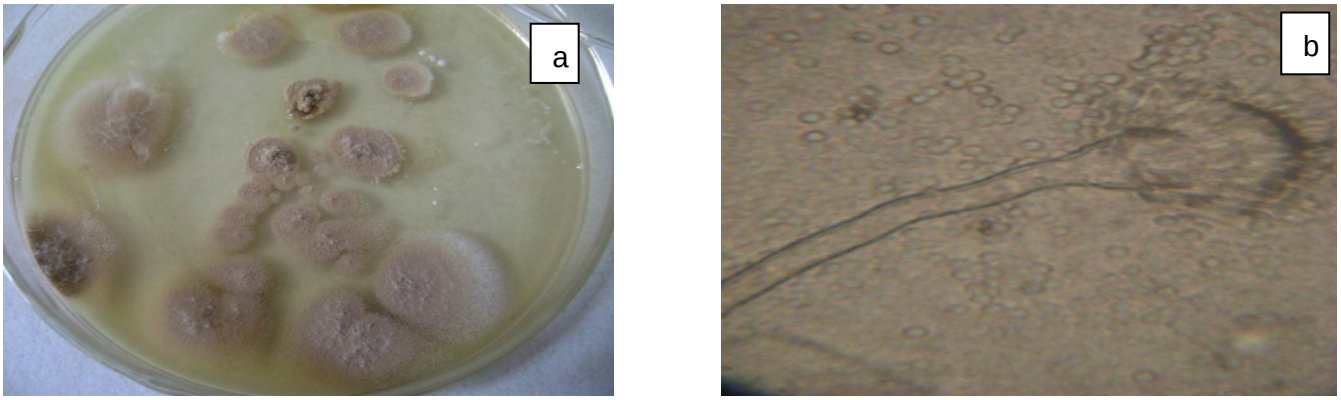

Fig 1c: *A. terreus*
(a) Colony on Potato Dextrose agar plate and
(b) Structure showing the conidiophore and the conidial head

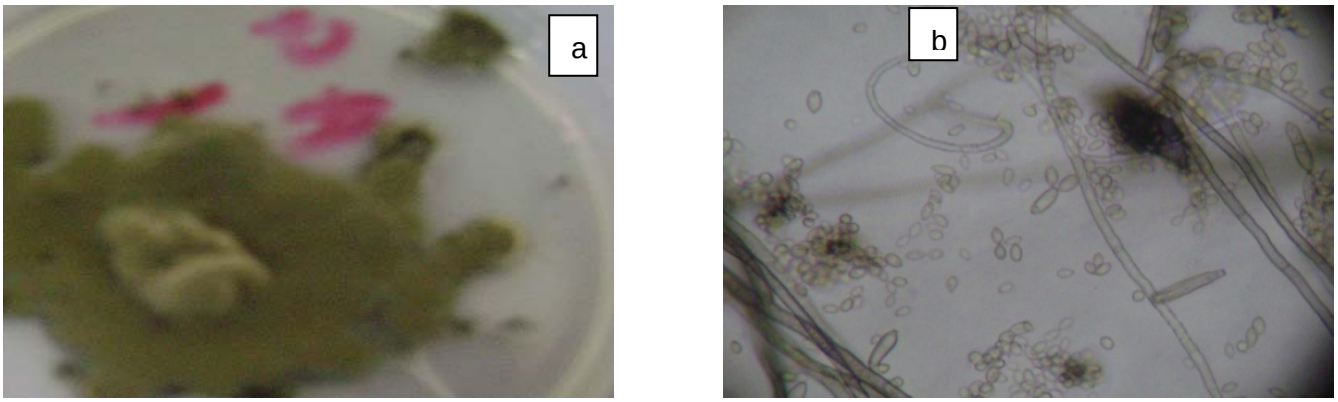

Fig 1d: *Cladosporium cladosporioides*:
(a) Colony on Potato Dextrose agar plate
(b) Structure showing the conidiophores, conidia and ramiconidia

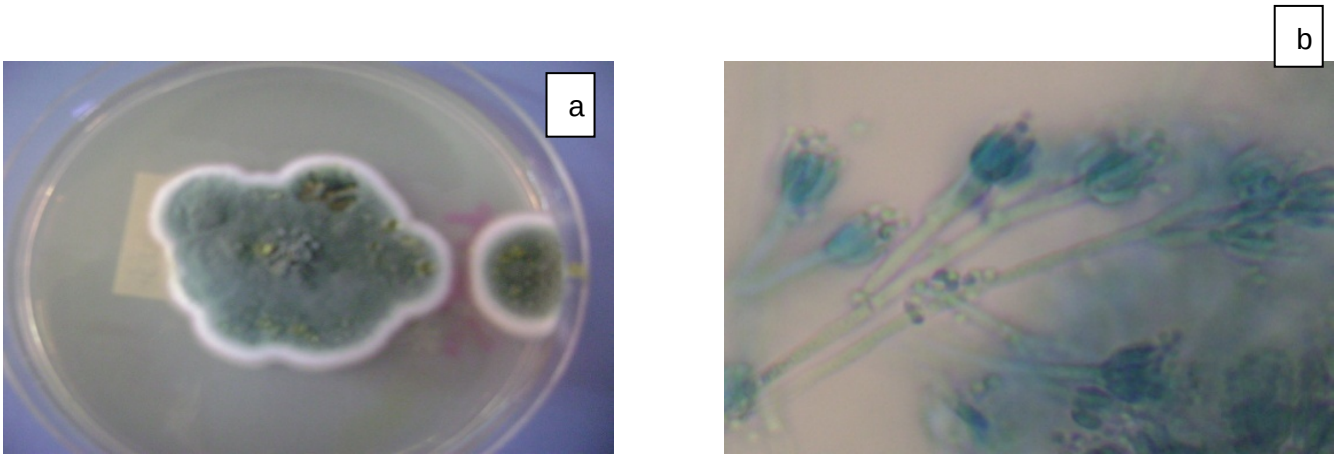

Fig 1e: *P. chrysogenum*
(a) Colony on Potato Dextrose agar plate
(b) Structure showing the branched conidiophores, stipe and the conidial head

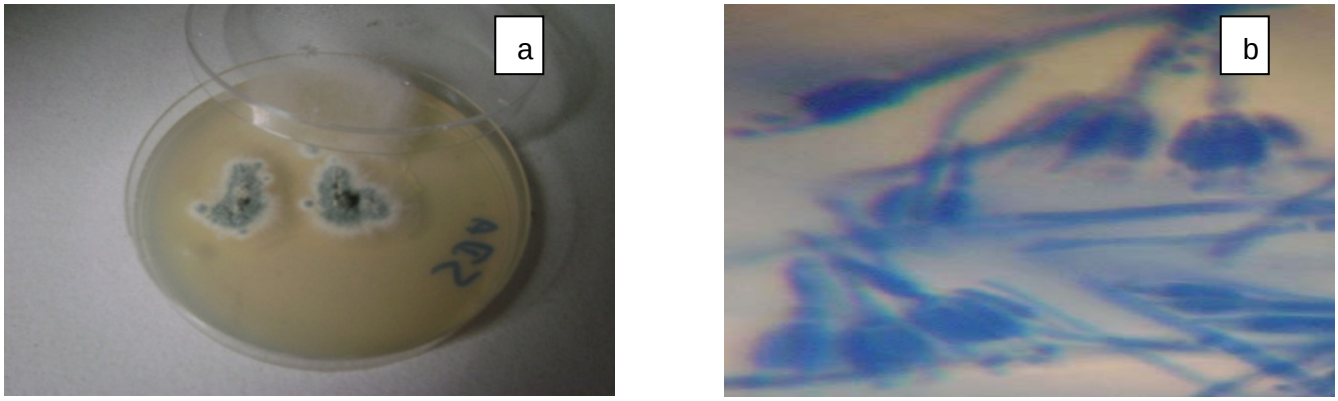

Fig 1f: *P. citrinum*
(a) Colony on Potato Dextrose agar plate
(b) Structure showing one stage branched conidiophores, stipe and the conidial head

3.2 Growth Rate Studies

3.2.1 Colony Diameter Measurement

The media (Sabouraud Dextrose agar and Czapek-Dox Agar) used for the growth rate studies on the test fungi were found to have supported their growth (Figures 2a and 2b). The test fungi grew rapidly on both media. The fastest growth on the Sabouraud Dextrose agar was obtained for *A. niger* that initiated growth within 24 hours of incubation with colony diameter of 1.2cm and recorded colony diameter of 5cm by the 7th day of incubation. Initiation of growth on Czapek Dox agar medium was observed in *A. niger* after 48 hrs. However, *A. terreus* initiated growth on both media (SDA and CZA) on the second day (48hrs) of incubation with colony diameter of 1.5cm on SDA and1.0cm on CZA, attaining 5cm after 7 days incubation period on SDA and 4.0cm on CZA (Figures 2a and 2b).

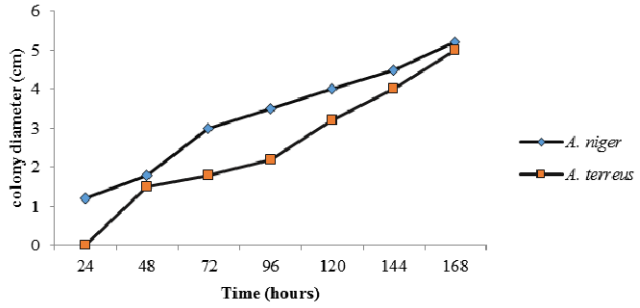

Fig 2a: Colony Diameter of *A. niger* and *A. terreus* on Sabouraud Dextrose Agar medium

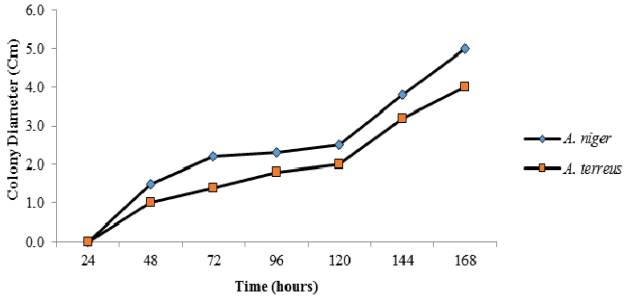

Fig 2b: Colony Diameter of *A. niger* and *A. terreus* on Czapek Dox Agar medium

3.2.2 Mycelia weight measurement

The mycelia weights of the culture filtrates of the isolates harvested at 24 h intervals over a period of 168 h as shown in Figure 3 indicated that there was increase in the mycelia weight of the isolates within the first twenty-four hours which increased progressively until the 5th day (120 hours) that was observed as peak (2.5 mg/ml) for *Aspergillus terreus* and the mycelia weight then declined. *Aspergillus niger* had its peak (3 mg/ml) on the 6th day (144 hours) and the mycelia weight then declined afterwards.

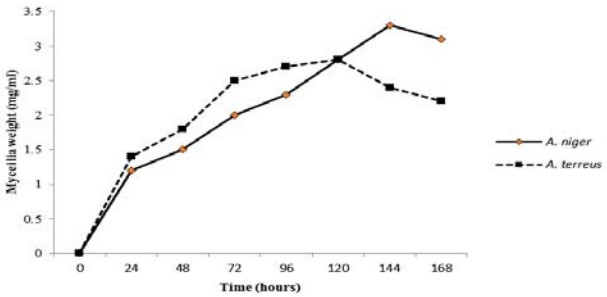

Fig 3: Mycelia Weight of *A. niger,* and *A. terreus* at 24-hour Intervals

3.3 Preliminary screening for amylase production

3.3.1 Amylolytic Activity of the Test Fungal Isolates Using Plate Assay

Amylolytic activities of the selected fungal isolates on the soluble starch agar were depicted by the presence of halo zones of clearing of the soluble starch agar after flooding with Lugol's iodine. *Aspergillus niger* was the best hydrolyzer of the soluble starch with highest zone of clearing of 55 mm and was grouped as strongly amylolytic (+++), *A. terreus* with zones of clearing of 43mm was grouped as moderately amylolytic. The details of the results are shown in Table 2. The analysis of variance showed that there were significant differences (P≤0.05) in the amylolytic activities of the fungi tested.

Table 1: Amylolytic activity of the fungal isolates

Test organism	Mean diameter of clearing(mm)	Activity level
A. niger	55 ± 0.00^{g}	*+++
A. terreus	43 ± 1.00^{d}	++

Figures in the same column having the same superscript are not significantly different (p≤ 0.05) ++ = moderately amylolytic, +++ = strongly amylolytic.

3.3.2 Secondary Screening for Amylase Production

The test fungi demonstrated enzyme activity on the basal medium containing soluble starch substrate as sole carbon source. The highest glucoamylase (amylolytic) potential at pH 5.03 was demonstrated by *A. terreus* and then followed by *A. niger* with peak enzyme activity (0.375 mmol-^{L} and 0.281 mmol-^{L}) respectively on the 6th day of incubation as shown in Figure 4.

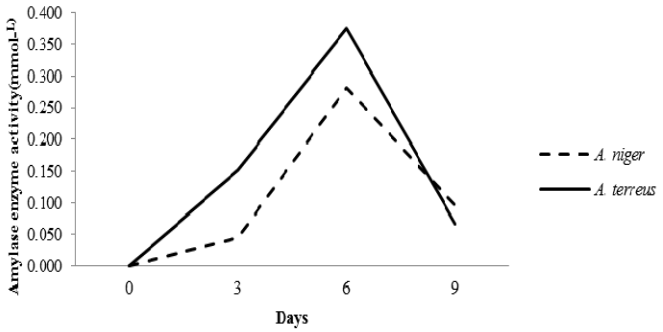

Fig 4: Enzyme assay of *A. niger* and *A. terreus* over a period of 9 days

3.3.3 Effect of Nitrogen Source (yeast extract) on the Concentration of Reducing Sugar Produced

Nitrogen sources have a great effect for microbial growth and in the production of extra cellular enzymes. The optimum fungal growth as well as glucoamylase production was found in the mixture of starch and yeast extract as nitrogen source for the 9 days of incubation (Figure 5). The highest enzyme liberation at pH 5.92 was observed for *A. terreus* with peak enzyme activity of 0.756 mmol-^{L} on the 9th day of incubation. *A. niger* had its peak enzyme activity of 0.394 mmol-^{L} on the 6th day of incubation.

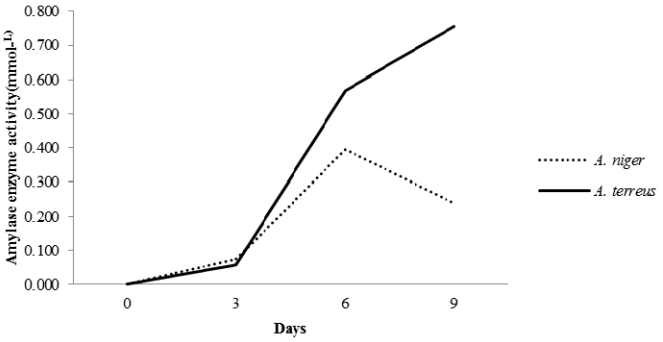

Fig 5: Effect of yeast extract on amylolytic activity by *A. niger* and *A. terreus* over a period of 9 days.

4. Discussion

Fungal strains were isolated from soils enriched for amylase producing microorganisms using soil plate method. Among these *Aspergillus* species especially *Aspergillus niger* was found to be frequently isolated. *Aspergillus niger* and *A. terreus* were recorded from all the soil samples. Their common occurrence could possibly be due to their high sporulating nature and also coupled with their ability to grow well and fastidiously on laboratory media [21].

The growth of the organisms on different media (Sabouraud agar and Czapek-Dox agar) shows the versatility of the organisms to utilize different carbohydrate sources. However, these organisms utilized the carbon sources at different rates as indicated by their different rates of growth (Figure 1). Nwodo-Chinedu *et al*. (2005) reported that *A. niger*, belong to the genus *Aspergillus* which has been documented as source of the most prevalent airborne fungi. The increase in the mycelia weight shows that the medium supports the growth of the organisms but at different rates which was depicted by the different peaks values observed in the fungi studied. The mycelia weight after reaching the peak value in both organisms declined steadily (Fig. 2). This is not surprising since there was no inflow of nutrients into the culture medium throughout the period of incubation. The result of this work is similar to the findings of Nwodo *et al*. (2010) in their work on assessment of growth and cellulase production of wild-type microfungi.

Screening of the fungal isolates for amylase production was carried out in starch agar plates followed by iodine test. The two test fungi, *A. niger* and *A terreus* showed maximum hydrolysis zone but *A. niger* had the highest zone of hydrolysis of 55 mm (Table 2). *A. niger* has been known as a good hydrolyzer of starch [23, 24].

The test fungi demonstrated enzyme activity in submerged fermentation (SmF). The fungi utilized the media for its growth and secreted various secondary metabolites including amylases into the medium. The enzyme quantity expected to increase with increase in fungal growth within the period of incubation. The crude extract from media was therefore harvested at the interval of 3 days up to 9 days. The cultivation time allows maximum growth of fungi and product formation to a certain degree in a fermentation broth. The results revealed increasing trend of enzyme activity for both tested fungal isolates up to the 6th day of incubation and then decline as shown in Figure 3. This could be as a result of increase in concentration of certain toxic wastes and depletion of nutrients in fermentation media which leads to decreased fungal biomass and enzymes production. It could also be as a result of high viscosity of the fermentation medium, which decreases the oxygen supply to the microorganisms. High viscosity leads to retardation in cell division, resulted in low production metabolism and amylase production.

It is pertinent to note that *A. terreus* producing 0.375 mmol-^{L} demonstrated greater potential in the production of glucoamylase than *A. niger* with 0.281 mmol-^{L} in submerged fermentation (Figure 3). *A. niger*. However, *A. niger* performed better during the plate assay with halo zone of 55mm diameter against *A. terreus* with halo zone of 43mm diameter (Table 2). Ali *et al*. (1989) reported *A. terreus* cultured on rice bran as a good producer of amyloglucosidase. Amylase enzyme production has been reported in *Aspergillus* species including *A. niger* by several authors [26, 27, 28, 29].

The effect of yeast extract as nitrogen source on enzyme production was studied and it was observed that the nitrogen sources had a great effect for microbial growth and in the production of extra cellular enzymes. The growth and concentration of the enzyme liberated had a significant increase when starch and yeast extract were used in combination than when starch was used separately (Figure 4). Nitrogen sources have a great effect for microbial growth and in the production of extra cellular enzymes [30, 31].

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