Production and Characterisation of Amylase by Fungi Isolated from Soil Samples at Gwagwalada, FCT, Abuja - Nigeria

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Abstract: Fifty soil samples were collected from refuse dumps, animal sheds, farmlands, drainage sites and barbers' shops at Gwagwalada Abuja and screened for the presence of amylase- producing fungi spread plate method of inoculation. 1 g of soil sample was dissolved in 10 ml sterilized distilled water. The soil suspension was diluted up to 10^3. About 0.2 ml of the samples were inoculated on already prepared Sabraud's dextrose agar plates. The inoculated plates were incubated at ambient temperature (25 ± 2^0C) for 5 days and were subsequently sub cultured to obtain pure isolates. A total of 51 fungal isolates belonging to two genera and four species of amylase- producing fungi were observed. Sixteen (31.37 %) of the isolates were from refuse dumps, 11 (21.57%) from animal sheds and barbers' shop soils each, while farmland and drainage sites have 10 (19.61 %) and 3 (5.88 %) isolates respectively. *Aspergillus niger* (van Teigh) 17 (33.33 %) was the most abundant species, followed by *Penicillium chrysogenum* (Thom) 13 (25.49 %), *Aspergillus flavus* (Link ex Fr.) 12 (23.53 %) and *Penicillium marneffei* 9 (17.65 %) being the least abundant. The highest amylase activities were recorded in *Penicillium chrysogenum* (16.93±0.22 Amylase unit (Au)/ml), *Penicillium marneffei* (16.02±0.43 Au/ml), *Aspergillus niger* (14.73±0.75 Au/ml) and *Aspergillus flavus* (12.51±1.66 Au/ml).The rate of abundance and the quantity of enzymes produced are significantly different (P = 0.05) for the organisms. These amylolytic fungi have the potential to be a useful tool in biotechnological processes involving starch hydrolysis. The results of this work show that amylolytic activity is relatively widespread among common fungi and may have an important role in starch degradation in natural environment.

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1. Introduction

Fungi are ubiquitous in nature and are used extensively to produce industrial chemicals like citric, gluconic, lactic, and malic acids, (Joseph *et al*., 2008) and industrial enzymes, such as lipases used in biological detergents, (Kumar *et al*., 2008) cellulases used in making cellulosic ethanol and stonewashed jeans, amylases, invertases, proteases and xylanases (Kumar *et al*., 2008). The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used for the treatment of digestive disorder (Sakthi *et al.,* 2012). Alpha amylase has been derived from several fungi, bacteria and actinomycetes, fungal sources are mostly terrestrial isolates such as *Aspergillus* species and *Rhizopus* species. At present *Aspergillus* and *Rhizopus* species are considered to be the most important sources of industrial amylases. Amylases are among the most important enzymes and are of great significance in present – day biotechnology, having approximately 25% of the enzyme market (Sakthi *et al.,* 2012). Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand. Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup, and maltotetraose syrup. In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry (Aiyer, 2005; Radley, 1976).

MATERIALS AND METHODS

Sterilization of glassware

The sterilization of glass wares such as conical flasks, beaker and test tubes after washing with detergent was carried out in hot air oven at 160º C for 2 hours according to the procedure given by Harrigan and McCance (1976).

Sampling site

Gwagwalada is one of the five municipal Councils of the Federal Capital Territory of Nigeria, together with Abaji, Kuje, Bwari, and Kwali; the FCT also includes the City of Abuja. Gwagwalada is also the name of the main town in the Local Government Area, which has an area of 1,043 km²

and a population of 157,770 at the 2006 census. Gwagwalada is where the University of Abuja is located.

Sample collection

A total of fifty (50) soil samples were collected randomly with ten (10) samples each from five (5) different sites in Gwagwalada FCT-Abuja. Samples were collected from fertile land, animal shed, refuse arena, sewage arena and Barber's shop (Sharma and Rajak, 2003). At each location, 20 g of soil were collected from the superficial layer, at a depth of 10 cm. Soil samples were collected in the sterile polythene bags and brought to the Microbiology laboratory of the Department of Biological Sciences, University of Abuja, for the isolation of amylase producing fungi using the method of Sakthi *et al.*, 2012 with some modifications.

Preparation and sterilization of media

Sabraud's Dextrose Agar and Starch Agar were used in this study and they were prepared according to the manufacturer's instructions thus, 65g of SDA was dissolved in 1000ml of sterile water and then sterilized (autoclaved) at 121ºC and pressure of 15Pa for 15 minutes, while Starch Agar was prepared by adding 2% (20g) of starch with 1.5% (15g) of agar in 1000ml of sterile distilled water and then boiled for 30minutes. Sabraud's Dextrose agar was used for the isolation and maintenance of pure cultures of fungi and the starch agar was used for the screening of amylolytic fungi (Sakthi *et al.*, 2012).

Isolation of amylolytic fungi

The fungi were isolated from soil using the spread plate technique (Sakthi *et al.*, 2012). 1 g soil sample was dissolved in 10 ml sterilized distilled water. The soil suspension was diluted up to 10^3. The samples inoculated on already prepared Sabraud's dextrose agar plates. The inoculated plates were incubated at ambient temperature (25 ± 2^0C) for 5 days. Colony developments were observed after incubation period.

Preparation of pure cultures of fungal isolates

The young fungal colonies were aseptically picked up and transferred to fresh sterile SDA plates to obtain pure cultures. The pure cultures on SDA plates were grown at 25 ± 2^0C for 7 days and stored in the refrigerator at 4^0 C until required for further use. The isolates were sub-cultured to obtain young cultures for further studies (Tokhadze *et al.*, 1975).

Identification of amylolytic fungal isolates

Isolates obtained were characterized and identified on the basis of their colonial and morphological characteristics which include macroscopic and microscopic examinations. Among the characteristics used were colonial characteristics such as size, surface appearance, texture, reverse and pigmentation of the colonies (Sharma and Rajak 2003). In addition, microscopy revealed vegetative mycelium including presence or absence of cross-walls, diameter of hyphae, and types of asexual and sexual reproductive structures (Soomro *et al.*, 2007). Slide culture method that minimized serious distortion of sporing structures was used. Appropriate references were then made using mycological identification keys and taxonomic description (Harrigan and McCance, 1976; Samson and Reenen-Hoekstra, 1988).

Viable spore count of amylolytic fungal isolates

The total viable spore number was determined by colony count technique. The spores were suspended in 10ml of distilled water, using a sterile transfer needle and diluted serially up to 10^{10} cells/ml. About 0.1 ml of spore suspension was poured onto sterile Petri-dishes, containing sterile SDA medium and spread uniformly. The inoculated Petri-dishes were incubated at 25 ± 2^0C for 48 hrs. Plates containing 30 to 200 colonies were selected for counting. The spore density was calculated as the count multiplied by the dilution factor (Sakthi *et al.*, 2012).

Screening of fungal isolates for amylase production

Primary screening was done by starch agar Plate method. The isolated fungi were inoculated on the agar plates amended with 2% of starch with 1.5% of agar (Sakthi *et al.*, 2012). The plates were incubated at 25 ± 2^0C for 5 days. The plates were flooded with iodine solution and clear zones around the colonies were observed. The plates that showed a maximum hydrolysis halo on the medium was selected for further investigations.

Determination of amylase potentiality of fungal isolates

A modified medium of Ali *et al.*, (1989) consisting of soluble starch; 2%, peptone; 0.2%, ammonium sulphate; 0.3%, potassium dihydrogen phosphate; 0.1%, magnesium sulphate heptahydrate; 0.03% and calcium chloride; 0.03% (w/v) was sterilized and employed as the substrate for fungal isolates. Spore suspension containing 10^5 spores/ml of 7 days old culture of each isolate was aseptically introduced into each tube of fermentation medium

(Metwally, 1998). Cultures were incubated at 25 ± 2^0C and 40^0C for 7days.

Extraction of amylase enzyme

Two milliliter of 0.1 M phosphate buffer (pH-6.5) was added to cultures, the mixtures were agitated for 30 min at 19^0C and 140 rpm on a rotary shaker. The mixture was filtered using whattman No1 filter paper and the filtrate was centrifuged at 4000 rpm for 10 min. The supernatant was used as the crude enzyme preparation (Mahmoud *et al*., 2007).

Assay of amylase enzyme

Amylolytic products in the supernatant were determined by reading absorbance at 280 nm against basal medium using UV-Spectrophotometer (JENWAY 6305). An increase of 0.100 in the absorbance was considered as equivalent to 1 unit of AU (amylase unit).

Plate assay

The Plate assay was performed using agar plates amended with starch (Sakthi *et al*., 2012). The agar plates were amended with 2% of starch and 1.5% of agar. After the agar solidification, about 10 mm diameter of well was cut out aseptically using a 6 mm cork borer. The well was filled with the culture filtrate and incubated at 40^0C for 72 hours. 1% of iodine solution was over layered on the agar and was observed for zones of hydrolysis around the wells (Tsekova *et al*., 1993). The negative control was maintained by adding sterile water in the separate well. All the examinations were replicated thrice.

Statistical analysis

The results obtained were analyzed using one-way ANOVA and the F- test statistic at P = 0.05.

RESULTS

Isolation rate of amylolytic organisms Isolation rate of amylase producing fungi from five soil samples at Gwagwalada FCT-Abuja shows that *Aspergillus niger* was the highest and higher in soil samples collected from the refuse dump and farm lands (Table 1).

Table1: Isolation rate of amylase producing fungi from soil sample at Gwagwalada Abuja

	Isolation Rate, Number (%)					
Amylolytic fungi	**F.L** (n=10)	**A.S** (n=10)	**B.S** (n=10)	**D.S** (n=10)	**R.D** (n=10)	**Total** (n=50)
Aspergillus niger	4(40)	3(30)	2(20)	2(20)	6(60)	17(33.33)
Aspergillus flavus	3(30)	2(20)	4(40)	0(0)	3(30)	12(23.53)
Penicillium chrysogenum	1(10)	3(30)	3(30)	1(10)	5(50)	13(25.49)
Penicillium marneffei	2(20)	3(30)	2(20)	0(0)	2(20)	9(17.65)
Total	10(19.61)	11(21.57)	11(21.57)	3(5.88)	16(31.37)	51(100)

Keys: F.L=Farm land, A.H=Animal shed, B.S=Barber's shop, D.S=Drainage site, R.A=Refuse dump and n=number of soil sample. Isolation Rate, Number (%)

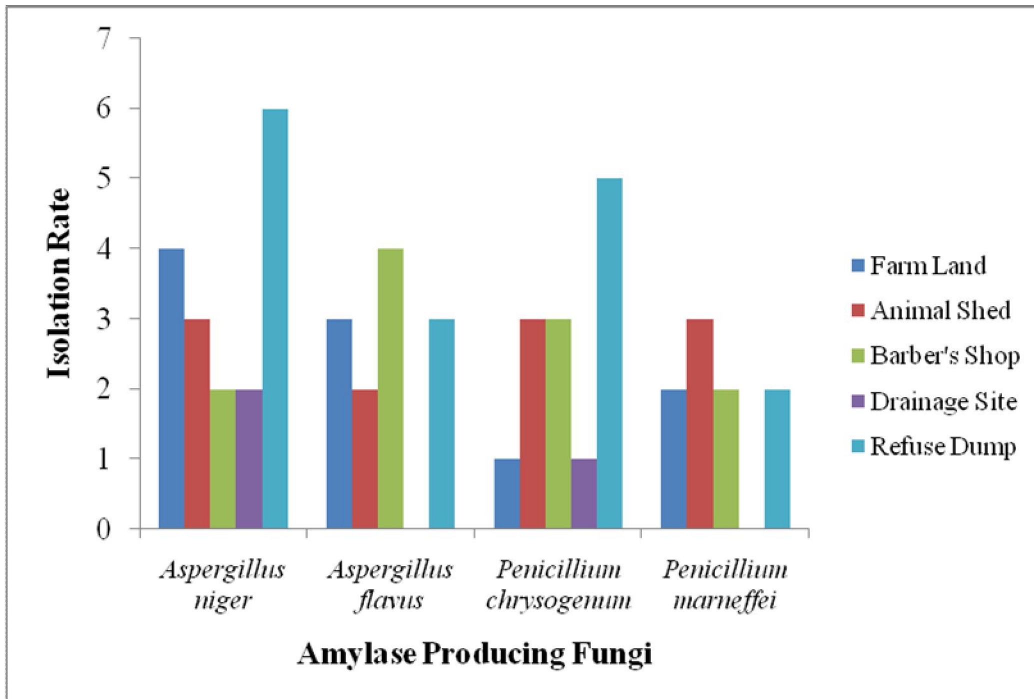


Fig. 1. Isolation rate of amylase producing fungi from soil sample at Gwagwalada Abuja

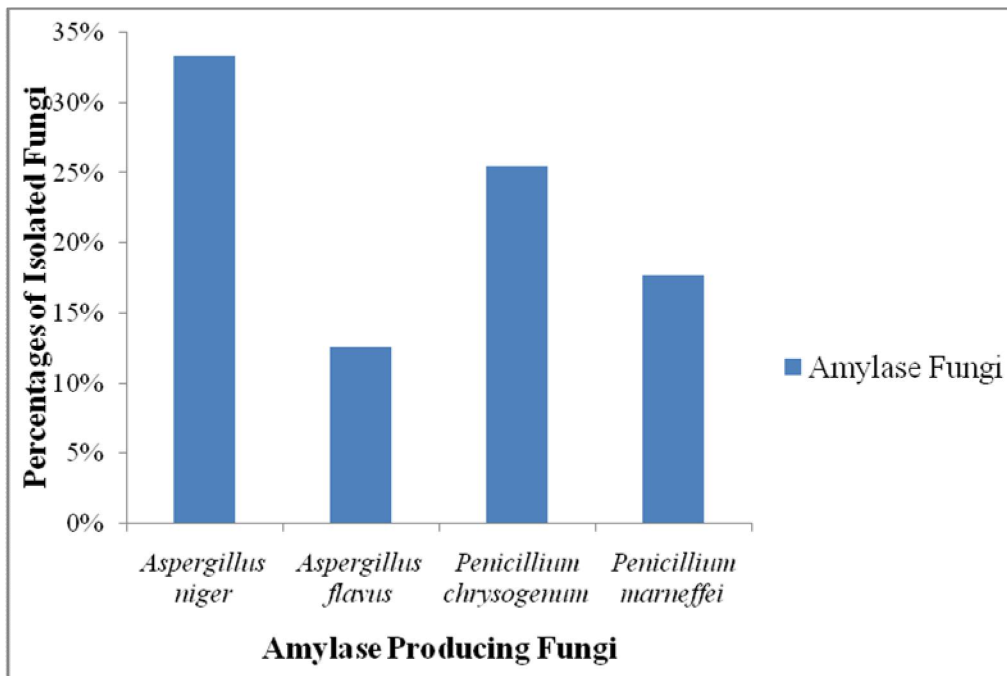


Fig.2. Percentage of Isolation of amylolytic fungi from soil sample at Gwagwalada Abuja

CHARACTERIZATION OF AMYLOLYTIC FUNGI FROM SOIL SAMPLE AT GWAGWALADA ABUJA

Preliminary screening

The results for the primary screening of the fungal strains by starch agar Plate method as described in the materials and methods is shown in Table 3 below.

Table 2: Preliminary Screening of Fungal Isolates for Amylase Production

Fungal Species	Zone diameter after 5 days of incubation (mm)
Aspergillus niger	31.00 ± 4.67
Aspergillus flavus	28.67 ± 2.44
Penicillium chrysogenum	32.33 ± 6.78
Penicillium marneffei	31.33 ± 6.45

Each value represents Mean ± Standard Deviation of three independent determinations

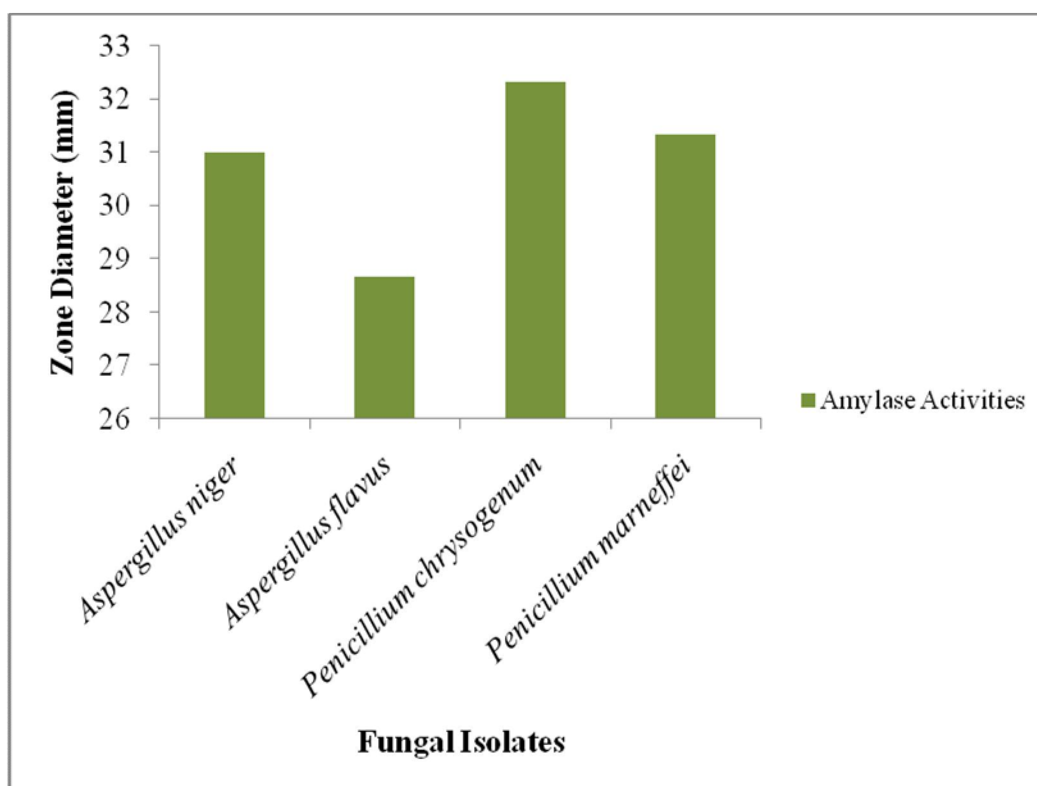


Fig.3. Preliminary Screening of Fungal Isolates for Amylase Production

Plate assay of enzyme amylase.

The result for the determination of the amylase activity of the crude enzyme of the strains of fungi cultivated in basal medium as described in materials and methods. Amylolytic activity of the culture filtrates (Crude enzyme) were confirmed on starch agar Plates and the zone diameter is shown in Table 4.

Table 3: Plate Assay of Enzyme Amylase

Fungal Species	Zone diameter after 72 hours of incubation (mm)
Aspergillus niger	15.00 ± 1.00
Aspergillus flavus	13.00 ± 1.00
Penicillium chrysogenum	22.00 ± 0.50
Penicillium marneffei	16.00 ± 2.00

Each value represents Mean ± Standard Deviation of three independent determinations

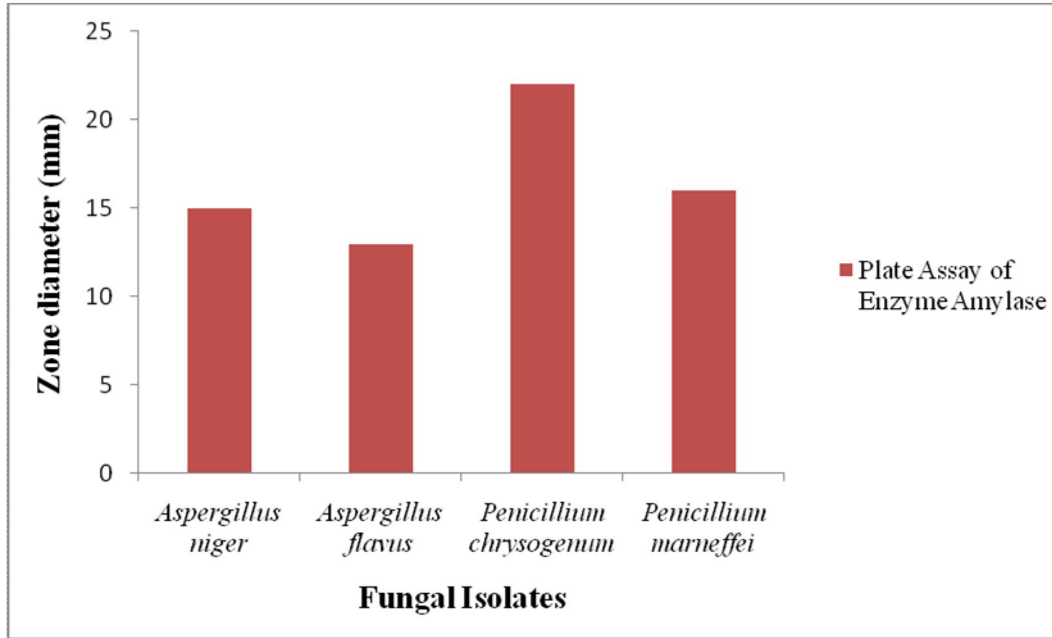


Fig.4. Plate Assay of Enzyme Amylase

Amylase activities of the isolated fungi from soil sample

The quantities of the amylase enzyme produced by the fungi in the basal medium were measured using UV-Spectrophotometer (JENWAY 6305) and the result is shown in Table 5.

Table 4: Amylase activities of the isolated fungi from soil sample in Gwagwalada FCT-Abuja.

Fungal Species	Amylase Activity (AU/mL)	Optimum PH	Optimum Temp(^{o}C)	Optimum Time(days)
Aspergillus niger	14.73±0.75	6.5	40	7
Aspergillus flavus	12.51±1.66	6.5	40	7
Penicillium chrysogenum	16.02±0.43	6.5	40	7
Penicillium marneffei	16.93±0.22	6.5	40	7

Each value represents Mean ± Standard Deviation of three independent determinations

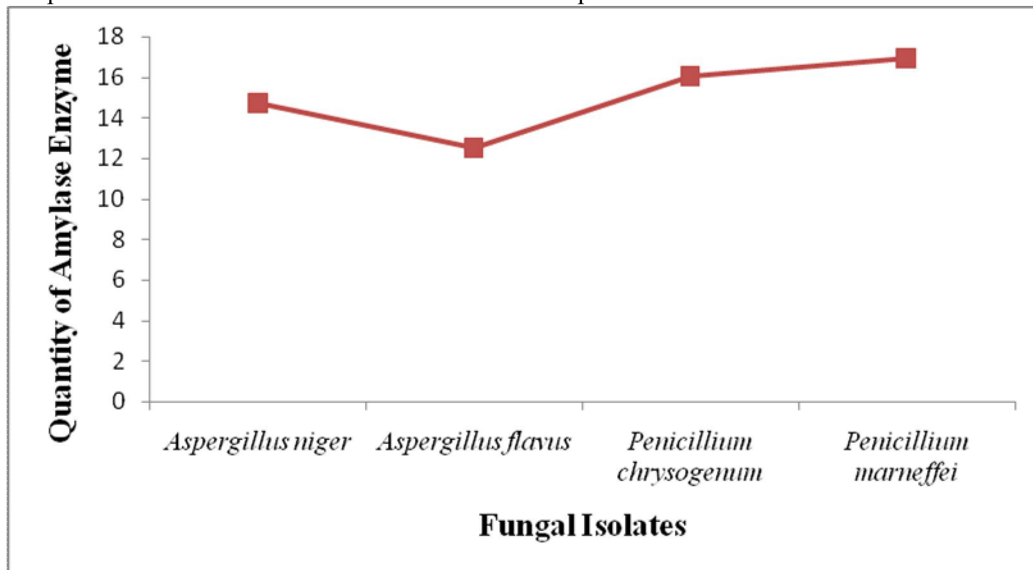


Fig. 5: Amylase activities of the isolated fungi from soil sample at Gwagwalada Abuja

Viable spore count of fungi isolates

The results of the viable spore counts as described in the materials and methods are showed in the Table 6 below.

Table 5: Viable Spore Count of Fungal Isolates

Fungal Species	Density (CFU/ml)
Aspergillus niger	$5.1 \pm 3.40\times10^{12}$
Aspergillus flavus	$4.8 \pm 3.17\times10^{12}$
Penicillium chrysogenum	$3.6 \pm 2.97 \times10^{12}$
Penicillium marneffei	$4.5 \pm 2.23 \times10^{12}$

Each value represents Mean ± Standard Deviation of three independent determinations.

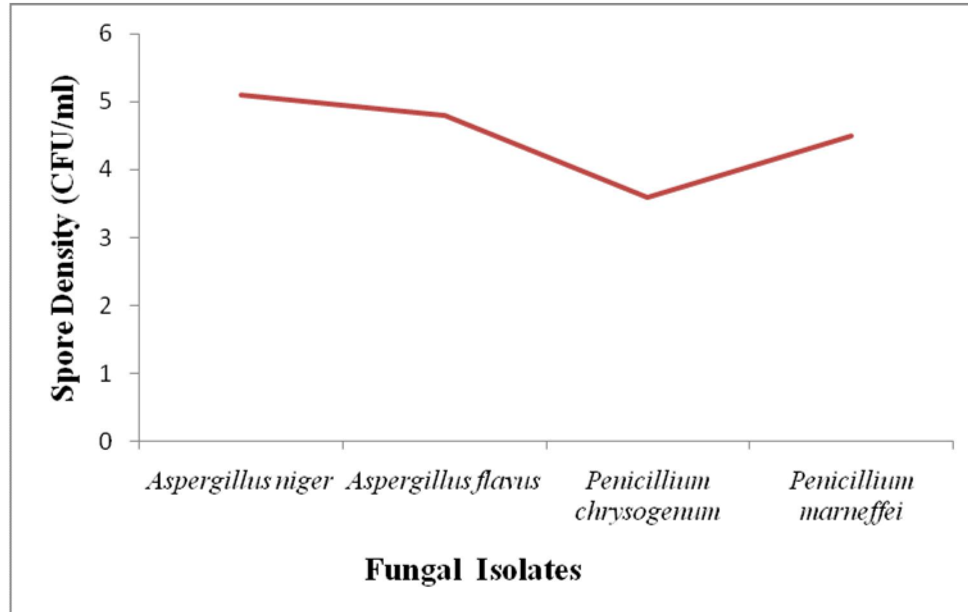


Fig. 6. Viable Spore Count of Fungi Isolates

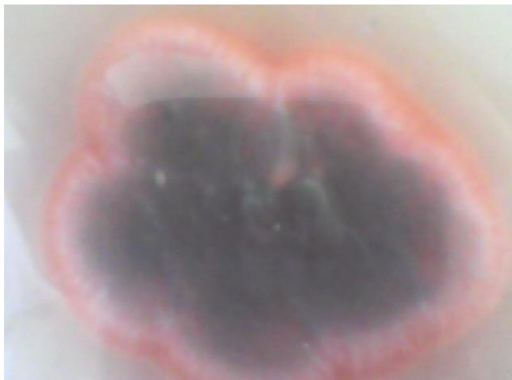


Plate1: *Penicillium marneffei*

Pate2: *Aspergillus niger*

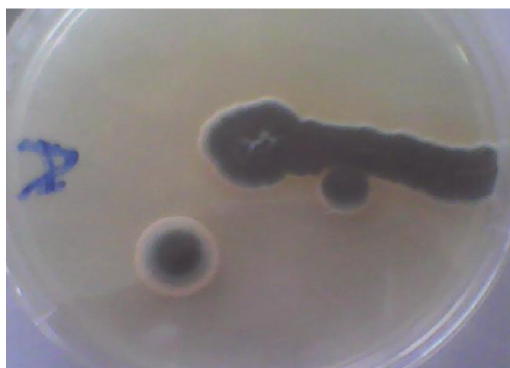


Plate3: *Penicillium chrysogenum*

Amylase fungal strains isolated was identified on the basis of colony morphology, cultural characters, slide culture, pigmentation, morphology of hyphae and their spores as *Aspergillus niger, Penicillium marneffei* and *Penicillium chrysogenum* respectively.

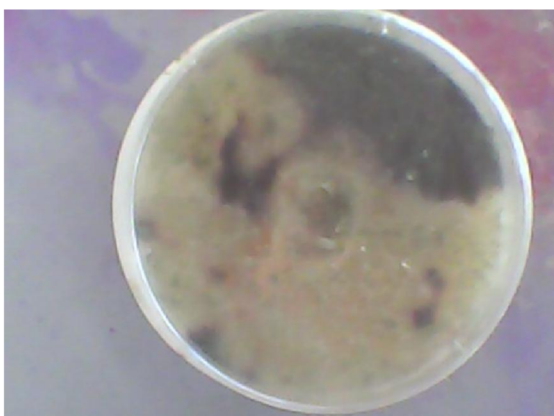


Plate 1: *Aspergillus flavus*

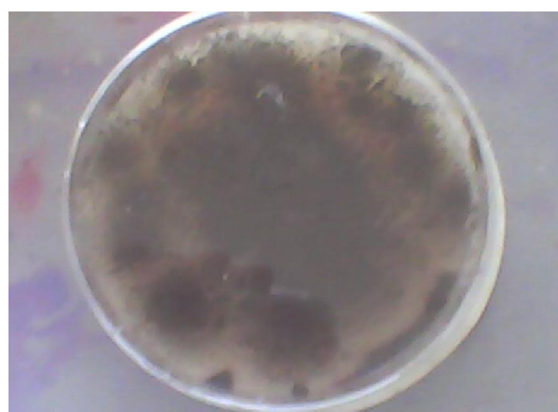


Plate 2: *Aspergillus niger*

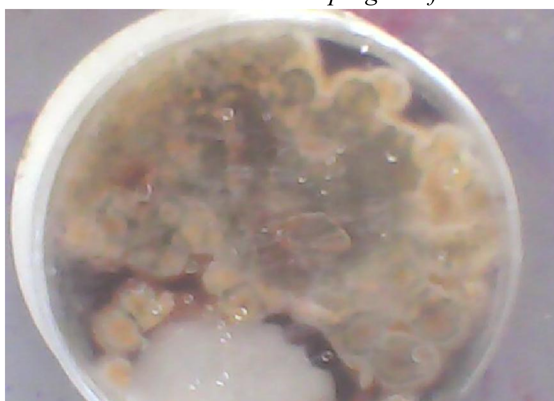
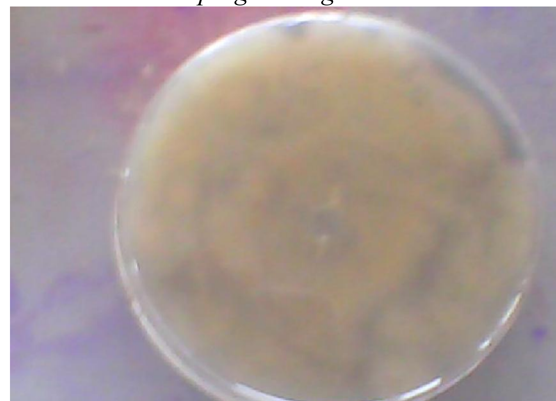
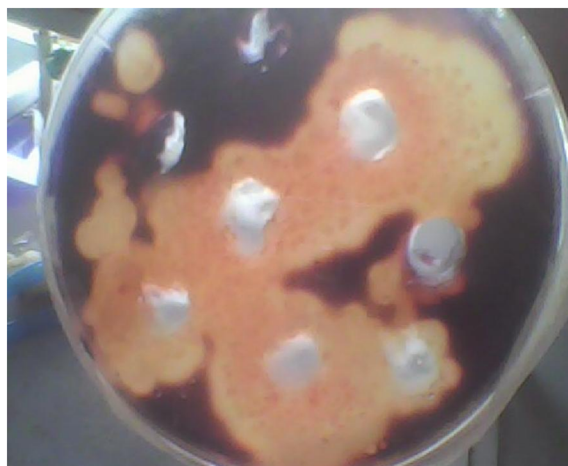


Plate 3: *Penicillium marneffei*



Pate 4: *Penicillium chrysogenum*

Preliminary Screening of Fungal Isolates for Amylase Production



Penicillium marneffei

Plate Assay of Enzyme Amylase Showing Zone Diameter after 72 Hours of Incubation (mm)

DISCUSSIONS

It appears from this study that *Aspergillus niger* (Van Teigh) is the most prevalent amylolytic fungus and also dominant species that was isolated from 17 (33.33%) soil samples of the five different regions, followed by *Penicillium chrysogenum* (Thom) 13 (25.49 %), *Aspergillus flavus* (Link ex Fr.) 12 (23.53 %) and *Penicillium marneffei* (Hubert) 9 (17.65 %) being the least prevalent (Table 1 and Figure 1). Studies revealed that amylolytic microorganisms are present in soils and agricultural wastes. Adeniran and Abiose (2009) reported amylolytic potentiality of fungi isolated from some Nigerian agricultural wastes. In this study, starch enhanced adequate production of amylase by the fungal isolates; alpha amylase can hydrolyze starch and is a key enzyme in metabolism of fungi which utilize starch as carbon and energy sources. This study revealed that *Penicillium chrysogenum* and *Penicillium marneffei* are the most active amylolytic fungi isolated contrary to the report by Adeniran and Abiose (2009) that *Aspergillus flavus* demonstrated the greatest potential in the production of amylase. Research on amylase has progressed very rapidly over the last five decades and potential industrial applications of the enzyme especially in solid waste management have been identified. *Aspergillus flavus* and *Penicillium marneffei* are good producer of amylase but cannot be regarded as “Generally Regarded As Safe” (GRAS) organism due to the possibility of liberation of aflatoxin by *Aspergillus flavus* which limits its application for commercial α-amylase production (although can be used for degradation of starch base waste in the environment as reported by Geweely and Ouf, 2011) while *Penicillium marneffei* causes opportunistic infection in HIV patients. The degradative enzymes produced by *Aspergillus* spp and *Penicillium* spp are capable of breaking down complex starch in nature, and may be responsible for the biodegradation of starch substance in polluted habitats. In this study, *Aspergillus niger* also produced amylase in submerged medium, and therefore regarded as good source of amylase. *Sakthi et al*., (2012) also reported the evaluation of amylase activity of the amylolytic fungi *Aspergillus niger* using cassava as substrate. Major impediments to exploit the commercial potential of amylase are the yield, stability and cost of amylase production. Although amylase production by microbes have been extensively studied by many researchers. A thorough review of literature on microbial amylolytic shown that some of the fungi which were active in the characterization had been mentioned by some authors. But there is still dearth of information about the ability of *Penicillium marneffei* to produce amylase enzymes (being reported for the first time). However, the present study revealed that *Penicillium chrysogenum, Penicillium marneffei, Aspergillus niger* and *Aspergillus flavus* are good producers of amylase enzyme.

CONCLUSION

As a result of fungi ability to produce enzyme amylase, therefore fungi may be responsible for biodegradation of starchy substances in the environment.

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