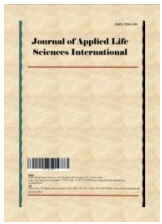

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Screening of Locally Isolated *Penicillium Species* from the Soil for Amylase Production

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SPEJ and JT designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors MU, MRS, JDG and ICA managed the analyses of the study. Authors FSO, AKA and JOO managed the literature searches. Authors SAO and NAK performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Background: A number of microorganisms are capable of degrading amylase and only a few of these microorganisms produce significant quantities of enzymes capable of completely hydrolyzing the amylase. Fungi are the main amylase-producing microorganisms.
Aims: The present study targeted to screen *Penicillium* species isolated locally from soil for amylase production.
Materials and Methods: In this research, fungal species belonging to genera penicillum species were isolated from soil and screened for their ability to degrade cellulose (lactose, fructose, sucrose and dextrose) for enzyme activity. Enzymatic fungi were evaluated after 7 days for the production of amylase enzymes by lactophenol microscopic examination. The concentrations for 7 days of

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production were formulated by dilution method as 0.148 mg/ml, 0.313 mg/ml, 0.303 mg/ml, 0.127 mg/ml, 0.161 mg/ml, 0.079 mg/ml and 0.099 mg/ml respectively.
Results: The maximum amylase production was obtained on the 2nd day of production which was 0.313 mg/ml and minimum production was obtained on the 6th day of production which was 0.079 mg/ml. Optimal pH was obtained at pH of 6 and temperature of 45°C with concentrations 0.587 mg/ml and 0.090 mg/ml.
Conclusion: The results of this study implied that a high concentration of enzymatic fungi could be obtained locally from the soil and the *Penicillium* isolates hold varied industrial production of amylase.

Keywords: Amylase; Penicillium; isolation; physicochemical parameters.

1. INTRODUCTION

Amylase is an enzyme that catalyzes the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Foods contain large amount of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar [1].

Most studies on α-Amylase production using fungi have been restricted to some mesophilic fungal species; and efforts have been made to define culture conditions and to choose strains of superior fungus to produce in large quantities [2]. *Penicillium* and *Aspergillus* are the two major fungal sources utilized for amylase production [3]. Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized in to exo-acting, endo-acting and debranching enzymes. Among the amylases, b-amylase is exo-acting whereas α-amylase is endo-acting enzyme. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermoacidophilic bacteria [4].

There are so many foods, pharmaceutical, brewing and baking industries in Nigeria that utilize amylase for their production process. However, amylase enzymes are yet to be produced commercially in Nigeria, making the cost of procurement high as a result of importation. Problems associated with the production of this enzyme in developing countries such as Nigeria is the high cost of production. Isolation and identification of some microorganisms with best amylase activity could contribute a lot for the discovery of novel potential amylases for different industrial and biotechnological applications. Therefore, isolation and screening of thermophilic fungi from soil samples are significant to discover novel industrial enzymes. There is a need to isolate and screen amylase producing fungi from starch rich soil associated with high temperature environment [5].

Amylase being one of such enzymes produced especially on starchy foods. Various moulds are commercially used for enzyme production, and it is believed that the ability of these moulds to grow and proliferate, and subsequently cause spoilage will depend on their ability to produce the requisite hydrolytic enzymes to breakdown the fermented foods components. Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccahrides such as starch into simple sugar constituents by degrading 1-4 linkage of starch. Amylases are of great importance in fermentation and food industries for hydrolysis of starch and other related oligosaccharides. Although amylase can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand [5]. For this reason, this present study was designed to isolate *Penicillium* species from different soils for the production of amylase, with view to isolate Penicillium species from different soil for the production of amylase, to produce amylase, and to determine the amylase activity.

2. MATERIALS AND METHODS

2.1 Sample Collection

Exactly 100 g of four refuse dump site soils were obtained at a depth of 5 cm and 10 cm at four different points in February, 2020 around Nigerian Institute of Leather and Science Technology, Kaduna, Nigeria, and made into composite samples. These soil samples were collected in clean polythene bags. The samples were then appropriately labeled and immediately transported to the laboratory for further investigation.

2.2 Sample Preparation

The soil samples were sun-dried with heat at 40°C for 7 days prior to isolation of the organism [6], since Singh et al. [7], reported that α-Amylase, is a multi-domain enzyme well-known for its thermostability, which has been the model enzyme to study and elucidate the origins and mechanisms of thermostability and thermal adaptation. The hydrolytic activity of α-amylase went unperturbed up to 65°C and it lost only 4% of catalytic activity at 70°C, whereas complete loss of enzyme activity was observed around 85°C.

2.3 Isolation of Amylase Producing Fungi

Amylase producing fungi was isolated from serial dilution method. Approximately 1g of each soil sample was mixed in 10 ml of distilled water and transferred into test tubes for dilution i.e. 10^{-1} to 10^{-10} and further 0.1ml of sample was inoculated into sterilized nutrient agar media plates and spread on the plates. All the plates were incubated at 37ºC for 24hours. Pure isolates were purified by sub-culturing on Sabouraud's Dextrose agar (Plates 1). A Petri dish (9cm) containing 25 mL of the media was used for the isolation. For observing colony characteristics and growth rate, inoculum was taken from the actively growing margin of 4days culture, grown on SDA. The dish was kept for incubation at 28°C. Radial growth was observed until colony covers the whole Petri dish. All micro morphological data was examined on culture grown on SDA for five days at 28°C. The microscopic examination and measurements of conidiophores and conidia was made from slide preparations stained with lactophenol-cotton blue (Plate 2).

2.4 Production of Amylase and Enzyme Assay

The isolates showing clear zone around them were propagated into Sabouraud's Dextrose broth supplemented with 1% starch medium in shaking incubator (Environmental Incubator shaker, NJ, USA) at 150 rpm at 37°C for 24 hrs. After incubation, the resultant broth was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was recovered and used as source of crude enzyme. Exactly 1 ml of crude enzyme and 1 ml of 1% soluble starch in Sodium phosphate buffer (Sigma-Aldrich, Co., Germany) (pH 6) was then added in a test tube. The test tubes were covered and incubated at 35°C for 10 minutes. Then 2 ml DNS reagent (Sigma-Aldrich, Co., Germany) was added in each tube to stop the reaction and was kept inside boiling water bath for 10 minutes. After cooling at room temperature, final volume was made to 10ml using distilled water. The absorbance was read at 540nm by spectrophotometer against maltose as standard [8].

2.5 Screening of Amylase Producing Fungi

Starch hydrolysis test was carried out for screening of amylase producing fungi. Fungi isolates were streaked on the starch agar medium (IPM Scientific®, Inc., Canada) plates and incubated at 37°C for 24-48 hours. After incubation Iodine solution was flooded on the plates with the help of dropper. The plates were then kept undisturbed for 5-10 minutes and then the iodine solution was discarded from the plates.

2.6 Optimization for Amylase Production

Amylase producing fungi was optimized for amylase production. For optimization of different carbon source (lactose, fructose, sucrose and dextrose), nitrogen sources (potassium nitrate, ammonium sulfate, sodium nitrate, ammonium nitrate, ammonium chloride, Casein, peptone, urea, gelatin and yeast extract), at different pH (4, 5, 6, 7, 8 and 9) to test its ability to induce amylase production in the production medium. The enzyme activity was measured after 24h of incubation [9].

2.7 Production of Alpha-Amylase Using a Chemically Defined Medium

The fermentation media including g/l (5 starch, 0.8 KCl, 0.8 NaCl, 2.0 Na_2HPO_4, 0.1 $FeSO_4$, 0.1 $CaCl_2$ and 2.0 NH_4Cl) were autoclaved, cooled and inoculated with mycelia before incubation at 27°C.

2.8 Determination of Physicochemical Parameters pH

The pH of the produced amylase was determined using pH meter for 2 days (48 hours) interval after it has been standardized using buffer solutions 4, 6 and 7.

2.9 Effect of Temperature on Amylase Activity

This was done according to the modified method of Afiukwa et al. [10]. The effect of temperature

on the crude amylase was assessed by incubating the enzyme at various temperatures between 20°C, 25°C, 30°C, 35°C, 40°C and 45°C for 15 minutes. Amylase activity was then determined.

2.10 Effect of pH on Amylase Activity

This was done according to the modified method of Afiukwa *et al.* [10]. The effect of pH on the crude amylase was assessed by incubating the enzyme for 15 minutes in buffers of varying pH using citrate phosphate buffer (pH 4, 5, 6, 7, 8 and 9). Amylase activity was then determined.

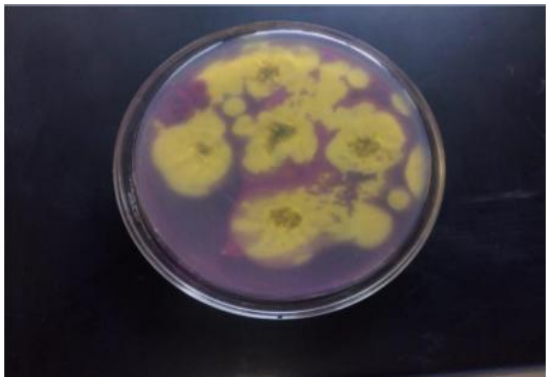

Plate 1. Isolated Organism Flooded with lactophenol

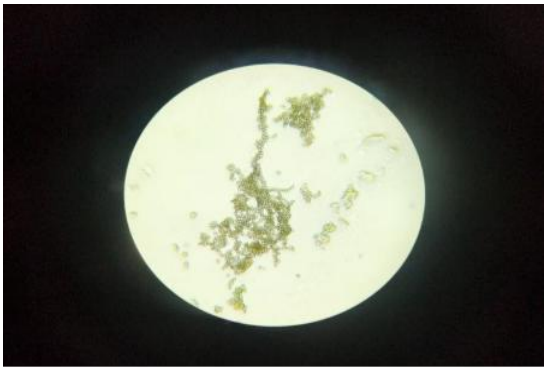

Plate 2. Micrograph Showing Microscopic Examination

3. RESULTS AND DISCUSSION

From the cultural characteristics of *Penicillium species* isolated from the soil sample collected from an open environment, the isolate was found to be flat, filamentous, and velvety, wooly, or cottony in texture, especially on sabouraud dextrose agar (Table 1).

The extent or length of production (yield) of *Penicillium* enzyme for a period of 7 days measured as enzyme activity and also the production rate of the enzyme measured as the ratio of yield to days. Amylase production was maximum at day 2 (0.313 mg/ml) and 3 (0.303 mg/ml) of the production and the increase in amylase activity can be explained to be as a result of availability of amylase, with least yield on day 6 (0.079 mg/ml) (Fig. 1).

From the result observed in Fig. 1, it shows the enzyme activity during production of amylase which indicates the maximum and the minimum values. The chart was plotted as concentration against days of amylase enzyme production. This result shows that on the first day of production, there was maximum activity of enzyme while on the fourth day of production there was minimum activity of enzyme. Therefore this indicates that the higher the enzyme activity, the higher the release of glucose. The characteristics of *Penicillum spp* like colony appearance and sporulation pattern was examined from cultures grown on a media: Sabouraud dextrose agar (SDA) at 28°C for 4days. Colonies of *Penicillum spp* grow rapidly and mature in 5days at 25°C and on potato dextrose agar; the colonies are wooly and become compact in time. From the front, the color is white as the conidia were formed; scattered blue-green or gray-green patches to become visible [11]. These patches may sometimes form concentric rings. They are more readily visible on Sabouraud's dextrose agar compared to other mycological agar. Conidiophores, phialides, and conidia are observed. *Penicillum spp* also produce chlamydospores. Conidiophores are hyaline, branched, and occasionally display a pyramidal arrangement. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides are solitary or arranged in clusters. Conidia (3µm in diameter, average) are one-celled and round or ellipsoidal in shape. They are smooth-or rough-walled and grouped in sticky heads at the tips of the phialides. These clusters frequently get disrupted during routine slide preparation procedure for microscopic examination. The color of the conidia is mostly green [12].

Table 1. Cultural characteristics of *Penicillium species* on sabouraud dextrose agar

Cultural morphology	Microscopy	Inference
Colonies are initially white and blue green, gray green. The plate reverse is usually pale.	Simple or branched Conidiophores. Metulae carries the flask-shaped phialides. Conidia are round, unicellular, and visualized as unbranching chains at the tips of the phialides.	*Penicillium species*

The isolated *Penicillum spp* represent the culturable microorganisms from soil where degrading activities take place. *Penicillum spp* is a genus of fungi in the family Hypocreaceae that is present in all soils, where they are the most prevalent culturable fungi. Fungal species belonging to genera *Penicillium* was isolated from the soil, screened and compared for their ability to degrade amylase. *Penicillum spp* being a saprophyte adapted to thrive in diverse situations, produces a wide array of enzymes. By selecting strains that produce a particular kind of enzyme, and culturing these in suspension, industrial quantities of enzyme can be produced. *Penicillum spp* are highly efficient producers of many extracellular enzymes. They are used commercially for production of amylase and other enzymes that degrade complex polysaccharides. They are frequently used in the food and textile industries for these fungi are used in - bio stoning of denim fabrics to give rise to the soft, whitened fabric stone washed denim. The enzyme are also used in poultry feed to increase the digestibility of hemicelluloses from barley or other crops. For any organism to successfully utilize cellulose in a medium as a carbon source for growth, it must first hydrolyze the cellulose to glucose before utilizing the glucose. Fungi are well known agents of decomposition of organic matter in common and of cellulosic substrate in particular [13]. In industry, these enzymes have found novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc. and extraction of valuable components from plants and improvement of nutritional values of animal feed [14]. Enzyme production from microorganism is directly correlated to the time period of incubation [15]. The present study observed enhanced enzyme activity with the increase in incubation time, but the activity diminishes with time. This concurs with previous study by Aiyer et al. [16], who reported that in some Bacillus species there is increase in incubation time with decrease enzyme activity.

The growth kinetic of fungal enzyme activities against number of days of amylase production was shown (Fig. 1). From the growth kinetic of the isolated *Penicillium* spp, the growth kinetics of the presently isolated *Penicillium* spp started with lag phase right after inoculation. The exponential phase was attained at the late day 1 of the inoculation. Stationary phase started from late 2 days which continued till 3 days and after that growth declined on day 4. There is brief fluctuation in the decline stage from day 4 to the 7th day of the analysis. Growth pattern utilization of carbon sources (lactose, fructose, sucrose and dextrose) varied with the supplement of starch. Low starch concentration favored a first day lag phase for this *Penicillium* spp.

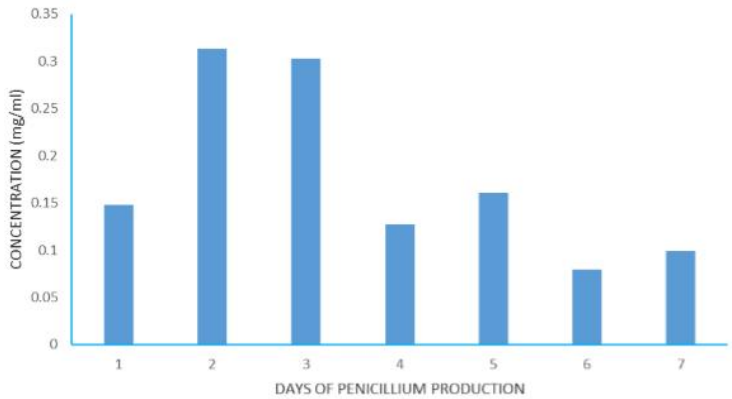

Fig. 1. Kinetic of fungal enzyme activities against days of amylase production

The effect of pH on amylase activity in which the concentration is on the vertical and pH range is on the horizontal was shown (Fig. 2). The optimum pH for amylase production was observed at pH of 7.0 and minimum production was observed at pH of 3.0.

The enzyme activity was reduced on the 4 day of production. This could be due to accumulation of other products in the fermentation media which lead to decrease in amylase activity. The optimum pH for amylase production was observed at pH of 7. This is as a result of maintaining the three dimension shape of active site on enzymes and the change in pH result in the loss of functioning shape of enzyme due to alteration in the ionic bonding of enzyme. The optimum pH for the maximum amylase production in the present study was found to be 7.0 for amylase production of *penicillium species.* This agrees with findings of Gupta et al. [17], who reported that most of the starch degrading microbial strains revealed a pH range between 6.0 and 7.0 for normal growth and enzyme activity.

The optimum temperature on enzyme activity was found to be 45°C. The temperature was maintained between 20°C to 45°C which was within the required optimum temperature range for optimum growth of amylase enzyme (Fig. 3). In the present study, it was observed that 5°C is the optimum growth temperature for the presently isolated *Penicillium* spp and higher temperature (45°C) supported less number of colonies. This could be due to the mesophilic nature of the fungal species. As per earlier report of Aiba *et al.* [18], who reported that high temperature may inactivate the expression of gene responsible for the starch degrading enzyme in microorganisms.

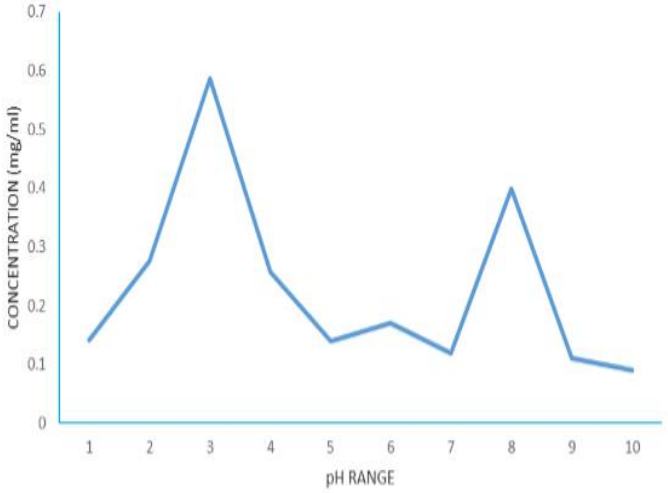

Fig. 2. Effect of pH on amylase activity

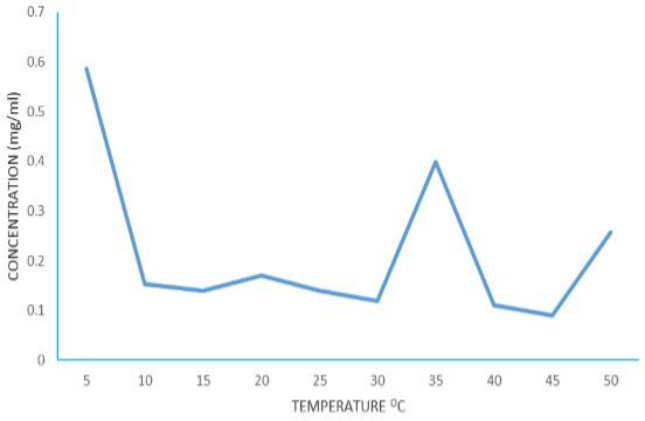

Fig. 3. Effect of temperature on amylase activity

4. CONCLUSION

The results of this study implied that a high percentage of amylase enzymes that can be obtained from isolated *Penicillium species* locally from soil and these isolates hold varied industrial and bioremediation potentials. It can be concluded from this research that a considerable number of enzymatic fungi may be obtained from soil such as *Penicillum spp* being a good enzymatic fungus which secretes pigment into the agar especially Sabouraud dextrose agar (SDA). Microscopically, it has conidiospores, conidia and contains phialides, which are the characteristics of *Penicillium* spp. The maximum amylase activity was found to be 0.313 mg/ml and the minimum was found to be 0.079 mg/ml. The parameters involved in the course of this study include pH and temperature. It was observed that the lower the pH, the higher the concentration. The concentration of the synthesized amylase is directly proportional to the subjected temperature.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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