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Purification and Characterization of Phytase Produced by *Aspergillus niger* Isolated from Poultry Farm Soil Using Submerged Fermentation

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ABSTRACT

Phytase is used in feed processing as an exogenous supplement in monogastric animal feed. This study was aimed at determining phytase production capacity of *Aspergillus niger* isolated from poultry farm soil. *A. niger* was isolated from poultry farm soil using phytase screening medium (PSM) containing sodium phytate as substrate. Quantitative estimation of phytase production was carried out by submerged fermentation using 3 x 10^5 spores/100 mL PSM for 5 days at 30°C and 150 rpm. The obtained crude phytase was purified using a combined purification process of ammonium sulphate precipitation and gel filtration chromatography using a 50 x 1.5 cm Sephadex G-75 column. The activity of the partially purified enzyme was assessed by subjecting it to varying pH, temperature, and nutrient conditions. The optimum temperature and pH were 50°C and 6 respectively. The enzyme remained active for over 30 min at 70°C and completely lost activity after 10 min at 80°C. No activity was observed at 100°C. The phytase produced with glucose as carbon source had higher activity (0.087 U/mL) than that produced with sucrose (0.018 U/mL). The *A. niger* isolate used in this study was unable to utilize ammonium sulphate as a source of nitrogen (no activity). This study establishes the potential of indigenous fungi for industrial phytase production.

***Keywords*:** Phytase, *Aspergillus niger*, Industry, Submerged fermentation.

Introduction

Phytase (EC 3.1.3.8) is an enzyme capable of hydrolysing phytic acid (myo-inositol hexakisphosphate) - an indigestible form of phosphorus that is found in grains and oil seeds.[1] Two broad groups of phytase known based on optimum pH requirements are the histidine acid phosphatase and alkaline phytase. The first group has an optimum pH around 5.0 while the second have an optimum pH around 8.0.[2] In industry, the enzyme has been mostly applied to animal feed processing, where it is employed as an exogenous supplement in the feed of monogastric animals.[3] Non-ruminant animals lack phytase, and are therefore unable to utilize phytate-bound phosphorus present in plant feeds. Phytase can be derived from various sources; however, microorganisms have more potential for producing relevant industrial enzymes[4] including phytase commercially.[5] The use of microbes in industrial enzyme production is due to ease of cultivation and manipulation of cultural conditions in the minimum space and time. *Aspergillus niger* is ubiquitous in nature, has minimal nutritional requirements and generally regarded as safe (GRAS) which may possibly be responsible for its ability to produce a number of important industrial enzymes.[6] It is also the most commonly employed organism for the production of phytase in solid-state fermentation (SSF) on a commercial scale.[9] Species of the genus *Aspergillus* have been reported to produce the most active extracellular enzyme with the most suitable characteristics of both pH and temperature stability.[7]

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The choice of submerged fermentation in this study is due to the ease of product recovery associated with the technique.[8] In solid state fermentation technique, several substrates including soybean meal[9] and triticate residues[10] have been used. However, *Aspergillus* species are able to utilize a wide range of substrates, some species of fungi may show specificity for a particular substrate e,g *Aspergillus flavus* which is specific for sodium phytate.[10] Following the increasing demand for phytase, therefore, it is becoming necessary to produce phytase in a cost-effective manner using microorganisms and a more effective fermentation technique. Therefore, the aim of this study was to evaluate the phytase production potential of an indigenous *Aspergillus niger* using submerged fermentation technique.

Materials and Methods

Sample collection
Soil samples (100 g) were collected from ten different spots (at 10 g per spot) of a poultry site in, Alakuko, Lagos State (6°27'14.65" N 3°23'40.81" E), Nigeria in April, 2019, for isolation of *A. niger*.

Screening and selection of phytase producing fungi from poultry soil
Soil (1 g) was suspended in 10 mL of 0.85% NaCl solution. One milliliter (1 mL) of the soil suspension was plated out onto a phytase-screening medium (PSM) containing (D-glucose -15.0 g, sodium phytate -3.0 g, NH_4NO_3 -5.0 g, $MgSO4.7H_2O$- 0.5 g, KCl- 0.5 g, $FeSO4.7H_2O$- 0.01 g, $MnSO_4.4H_2O$- 0.01 g and Agar- 15.0 g in 1 L.[11] The plates were incubated at 30°C for five days[12]. Isolate growing on screening medium with zone of hydrolysis was selected as phytase producing.

Identification of fungal isolates
Selected isolate, showing zone of hydrolysis was subjected to Lactophenol cotton blue test and identified based on their morphological and microscopic characteristics referenced to fungi compendium.[13]

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Phytase production via submerged fermentation
The selected fungal isolate were quantitatively screened for the production of phytase by submerged fermentation. Five day old fungi isolate was used to collect fungal spores. The spore suspension was prepared by adding 2-3 drops of Tween 80 to a test tube containing 10 mL sterile distilled water. The solution was poured on the surface of the fungi isolate in PDA plates. The surface of the fungi was scraped gently using sterile scalpel. The resulting solution was poured into a clean test tube using a pre-sterilized funnel and Whatmann No 1 filter paper. It was then centrifuged at 4000 rpm for 6 min and the supernatant discarded. To the sediment, 10 mL sterile distilled water was added in the second test tube and shaken gently. The fungal spores contained in 1 mL of the suspension was counted using a counting chamber and viewed under the microscope. One mL of spore suspension containing 3 x 10^5 spores was inoculated into 100 mL phytase screening broth (PSB) (PSM without agar) and incubated in a shaking water bath at 30°C for 5 days at 150 rpm. After fermentation for 5 days, fungal cells were separated from the medium using Whatman No 4 filter paper and cell-free filtrate was used for phytase assay.

Phytase assay
The activity of the phytase was determined by incubating 1 mL of the crude phytase at 37°C for 30 min in 1 mL 0.2 M sodium acetate buffer (pH 5.5) which contained 0.5% sodium phytate. The reaction was stopped by adding 1 mL of trichloroacetic acid (15% [w/v]). After the addition of 2 mL of a colouring reagent (3.66 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 1.6 mL of concentrated H_2SO_4 in 50 mL of distilled water), to the chilled sample on ice, the mixture was incubated for 10 min at 30°C. The amount of phosphate released was measured using a Genesys UV-VIS spectrophotometer at 750 nm.[12] Protein quantification was carried out according to the method of Lowry *et al*.[14] The colouring reagent was prepared freshly per use.

Purification of phytase
Ammonium Sulphate Precipitation
Crude phytase was partially purified by precipitation using 70% ammonium sulphate. The ammonium sulphate-crude phytase mixture was incubated overnight at 4°C with gentle stirring. The resulting solution was centrifuged at 21,000 g for 20 min to obtain precipitated proteins in pellet form. The pellet was then dissolved in 10 mL of 0.1 M acetate buffer pH 6.0 and dialyzed using a pre-acetylated cellophane tubing against 2-3 changes of same buffer solution for 24 h, to remove salt from the concentrated enzyme.[15,16] The partially purified/concentrated phytase sample was assayed for phytase activity as previously described by Lee at al.[12] and protein content was determined according to the method of Lowry *et al*.[14]

Gel filtration chromatography
Sephadex G-75 column of (50×1.5 cm) was packed with Sephadex G-75 gel and glass wool placed in the lower part of the column. The Sephadex G-75 was prepared by suspending 16.7 g of the gel in 250 mL of the phosphate buffer pH 7.0 (following manufacturer's instruction of 1 g in 15 mL), then boiled in a water bath for 5 min for swelling and degasing. The gel was allowed to settle for 18 h. The column was washed with phosphate buffer pH 7. After column equilibrium, 5 mL of ammonium sulphate phytase fraction was poured gently on the surface of the gel, and eluted with Tris-HCL buffer pH 7.8 at 20 mL/h flow rate. Aliquots of 2 mL fractions of the eluent were collected, then the optical density (at 280 nm) and phytase activity (U/mL) were determined for each fraction.[17,18] The phytase activity was determined as previously described by Lee *et al*.,[12] and protein content was determined according to the method of Lowry *et al.*[14]

Effect of carbon and nitrogen sources on phytase production
To determine the effect of temperature on phytase activity, carbon source was changed from glucose to sucrose and nitrogen source was changed from NH_4NO_3 to $(NH_4)_2SO_4$ in the PSB. Phytase activity was determined following the descriptions by Lee *et al*.[12]

Effect of temperature on phytase activity
To determine the effect of temperature on activity of the phytase produce, phytase assay was carried out as previously reported by Lee *et al*.[12] However, incubation temperature was varied between at 30°C, 40°C, 50°C, 60°C and 70°C for 30 min each.

Effect of pH on phytase activity
To determine the effect of temperature on activity of the phytase produce, phytase assay was carried out as previously reported by Lee *et al*.[12] However, substrate was prepared by dissolving 0.5 g of phytic acid in 0.2 M sodium acetate buffer of varying pH between pH 3 and 7.

Thermal stability of phytase
To determine the temperature stability of the phytase, the phytase was initially subjected to a temperatures of 70°C, 80°C and 100°C for 5, 10, 20 and min each. In each case, phytase activity was determined following the description of Lee *et al*. [12]

Results and Discussion

This study revealed the presence of a fungi sp capable of producing phytase by utilizing sodium phytate as substrate. Microscopic examination by Lactophenol cotton blue technique suggested that the selected phytase producing isolate was *A. niger* (Table 1). This study reported the isolation of *A. niger* which is able to degrade sodium phytate by producing the enzyme; phytase. Other studies have previously reported the production of phytase by members of the genus *Aspergillus*,[9,12,15,16] and particularly from species *A. niger*.[12,19,20] Additionally, *A. niger* is able to utilize a wide range of substrates for phytase production unlike *Aspergillus flavus* which shows specificity for sodium phytase only.[10] We report here, the production of phytase after five days of incubation as corroborated by Neira-Vielma *et al*.[10] and Qasim *et al*.[11]This prolonged fermentation time may be as a result of the inducible nature of the enzyme, hence a prolonged lag phase might be required by the fungi to produce the enzyme. Generally, fungi species have been reported to be slow growers requiring about 2- 5 days for optimal growth depending on the nutrient medium in which they are grown.[21] Quantitative evaluation of phytase production showed that the phytase produced by the isolate had a total activity of 342.8 units/mL and 186.5 mg/mL of protein (Table 2). Upon purification by ammonium sulphate precipitation, there was a reduction in the total activity and total protein content to 186.6 U/mL and 21.6 mg/mL respectively. However, we observed a continuous increase in specific activity of the enzymes from 2.5 FTU/mL to 8.6 U/mL for ammonium sulphate fraction and 17U/mL for gel filtration fraction (Table 2). These values were higher when compared with the specific phytase activity of crude extract (1.13 U/mL), microfiltration fraction (1.15 U/mL), ultrafiltration fraction (1.93 U/mL) and DEAE Sepharose fraction (8.38 U/mL) reported by Muslim *et al*.[1] when solid state fermentation (SSF) was employed. This confirms the limitations of SSF in commercial enzyme production as previously highlighted by[6]. However, Tariq *et al.*,[22] reported phytase activity of 68.88±2.55 and 274.99±10.14 FTU/mL using submerged fermentation method and sodium phytate as substrate which is way higher compared to what was reported in this study. Purification by gel filtration, showed a distinct elution peak as presented in Figure 1. Characterization of the phytase produced by the isolate showed that glucose was the most preferred as carbon source with a higher activity (0.087 U/mL) than phytase produced with sucrose (0.018 U/mL). The isolate used in this study, was unable to utilize ammonium sulphate as a nitrogen source (Figure 2). Optimum temperature was ascertained at 50°C (Figure 3) while optimum pH was 6 (Figure 4). The *A. niger* in this study was unable to utilize ammonium sulphate as nitrogen source for phytase production hence no activity was recorded. This corroborated reports by Pedri *et al.*,[23] where 100% ammonium sulphate was shown to decrease the production of all enzymes tested whereas supplementation of ammonium sulphate with soybean resulted in optimum enzyme production. In their study, Pedri *et al*[23] reported that treatment 1 which consisted of 100% ammonium sulphate was inactive in the production of manganese peroxidase until the 30th day.

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Table 1: Microscopy of phytase producing fungi isolated from soil sample

Isolate code	Nature of hyphae	Asexual spore	Pigmentation	Suspected organism
FB	Septate	Spores borne on sporangium	Brown	*A. niger*

Table 2: Purification table of phytase obtained from *Aspergillus niger* (FB) isolated from poultry soil

Protein Step	Total Activity (Unit/mL)	Protein (mg/mL)	Phytase Activity (Unit/mL)	Yield (%)	Purification Fold
Crude Extract	342.8	134.6	2.5	100	1
Ammonium Sulfate Precipitation (70%)	186.5	21.6	8.6	54	3.4
Sephadex G-75 Fraction	25.6	1.5	17	7.5	6.8

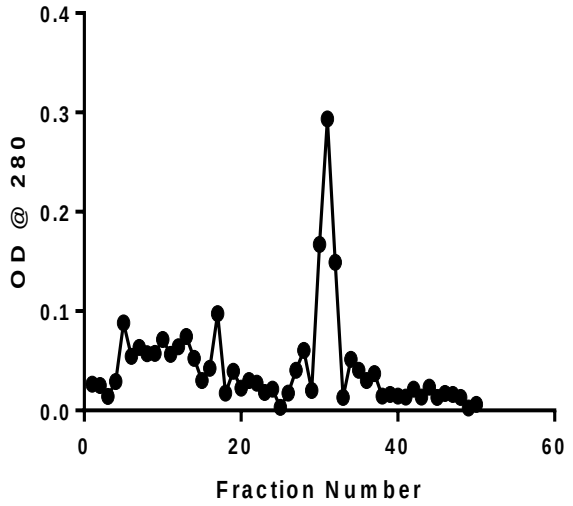

Figure 1: Chromatogram of Phytase producing *Aspergillus sp* (Sample FB) isolated from poultry soil in Lagos State using a Sephadex G 75 (50cm X 1.5cm) colum.

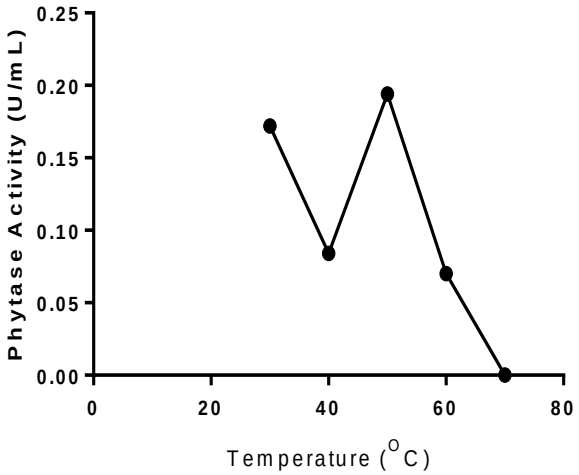

Figure 3: Effect of temperature on activity of phytase produced by *Aspergillus* sp (FB) isolated from poultry soil

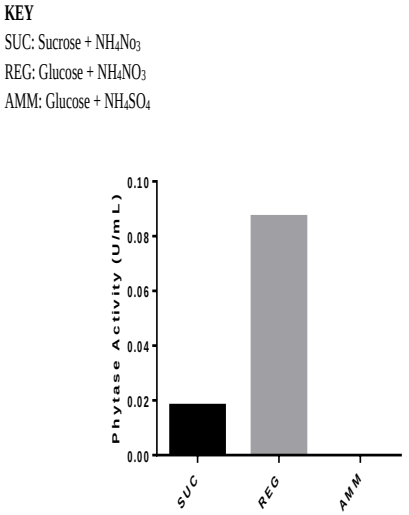

Figure 2: Effect of different carbon and nitrogen sources on the production of phytase produced by *Aspergillus niger* (FB) isolated from poultry soil

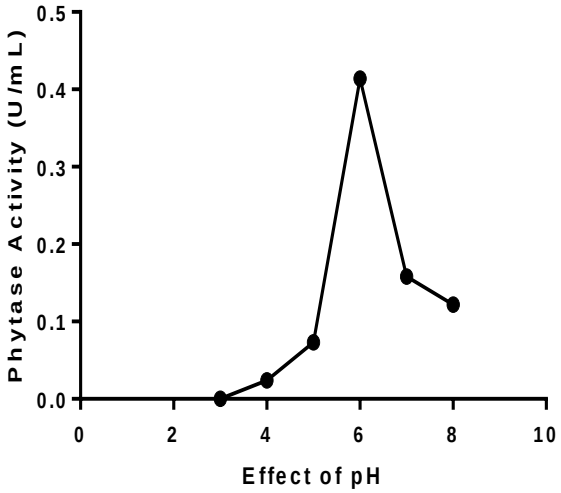

Figure 4: Effect of pH on activity of phytase produced by *Aspergillus* sp (FB) isolated from poultry soil

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Typically, organic sources of nitrogen are reported to greatly improve enzyme production than inorganic source, hence the better result observed in treatment supplemented with soybean. For carbon source, enzyme production with glucose was higher (0.087 U/mL) compared with sucrose (0.018 U/mL). Glucose is a simple sugar hence utilization of glucose by microorganisms may be easier and require less energy (ATP). For initial growth and effective metabolic action, the fungi may require carbon source in a form that is easily accessible.[24] Previous study has reported glucose to be the most effective source of carbon for *Myceliophthora thermophile*, *Pseudomonas aeruginosa* and *A. flavus.*[25] For the thermal stability assessment, the enzyme withstood 30 min of heating at 70°C (Figure 5), 10 min at 80°C (Figure 6). However, there was no activity recorded when the enzyme was subjected to a temperature of 100°C. The optimum temperature for the phytase produced was observed to be 50°C, Lee *et al*.,[12] reported an optimum temperature of 50°C for a phytase produced by a fungus of the genus Aspergillus. The phytase in this study lost all activity after 10 min of heating at 80°C. Although phytase is regarded as a thermo-tolerant enzyme, this result is similar to the reports of Neira-Vielma *et al*.,[10] where the enzymes lost 90.7% of its activity after heating at 80°C.

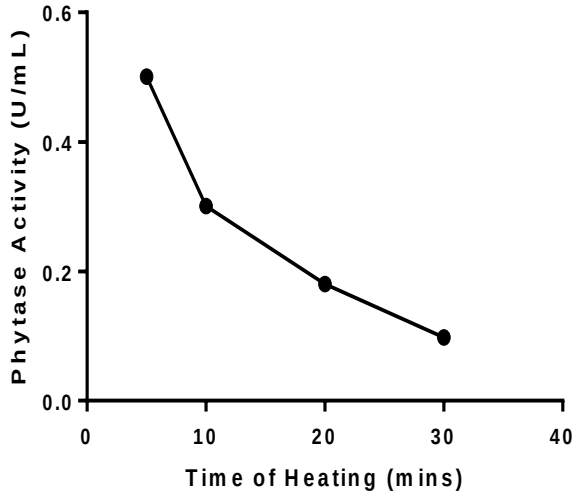

Figure 5: Thermal stability at 70°C of phytase produced by *Aspergillus* sp isolated from poultry soil

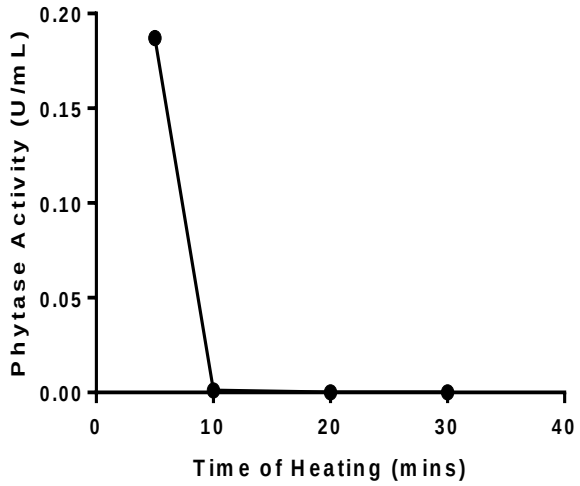

Figure 6: Thermal stability at 80°C of phytase produced by *Aspergillus* sp (FB) isolated from poultry soil

Conclusion

This study confirms the production of phytase by *Aspergillus* species isolated from poultry farm soul. The optimum conditions for activity of the phytase has been ascertained at 50°C, for temperature and 6 for pH. The phytase was able to withstand 5 minutes of heating at 80°C which is a suitable property for inclusion of the phytase as animal feed additive.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them

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