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PRODUCTION AND CHARACTERIZATION OF AN *Aspergillus niger* GS_1S_6 PHYTASE CULTIVATED IN PHYTIC ACID-RICH AGRICULTURAL SUBSTRATES

Olubusola Odeniyi, Leonard Itaba*

Address (es): Olubusola Odeniyi,
University of Ibadan, Faculty of Science, Department of Microbiology, Ibadan, Nigeria, phone number: +234 802 519 8741.

*Corresponding author: busolaodeniyi@gmail.com

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ABSTRACT

In animal farming, improvement of growth performance through successful nutrient uptake and digestibility is critical. Phytases hydrolyze the anti-nutritive phytic acid present in grains to lesser derivatives and release the phosphorus trapped therein. Twenty-eight fungi obtained from poultry droppings, cereal-rich soils and garden soils were screened for phosphate solubilization and phytase production using Pikovskaya agar and phytase screening medium. Out of the fungi, 61% were positive for phytase producing abilities and the most proficient, isolate GS_1S_6 with a solubilization index of 121%, was identified as *Aspergillus niger* GS_1S_6. Optimum phytase production was achieved at a fermentation period of 120 h, pH 5.5, glucose, $(NH_4)_2SO_4$ using 72 hour old fungal inoculum. Mineral supplementation of natural agricultural substrates enhanced phytase production (1000U/L) the most in milled sesame seed medium. The ~31-38 KDa partially purified and characterized enzyme demonstrated optimum activities at 55ºC and pH 6.0, while cationic inclusions reduced phytase activities. The K_m and V_{max} were low (1.308 mM and 0.077 mM/mL/min) from Linear-weaver plot with increasing substrate concentration implying that *A. niger* GS_1S_6 phytase may efficiently mineralize phytic acid and therefore hold great prospect for its commercialization.

Keywords: Phytic acid; Phytase characterisation; *Aspergillus niger;* Submerged fermentation, Agro-based polymeric substrates

INTRODUCTION

Phytic acid is a major form of phosphorus storage in plants and a constituent of all plant seeds with about 1-5% occurring in many cereals and oilseeds. They account for 60-90% of the total phosphorus in seeds (**Afinah *et al*. 2010; Kalsi *et al*. 2016**). Traditionally, phytic acid is considered by as an anti-nutrient due to its ability to chelate and form protein-mineral-phytic acid complexes with divalent minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} **(Afinah *et al*. 2010; Aziz *et al*. 2015**). Thus, concern continues to trail the implication of phytic acid on certain vulnerable segment of monogastric and agaric population (fishes, poultry birds, pigs and humans) that lacks the required hydrolytic enzymes capable of disassembling these complexes in their gut since intestinal absorption requires that minerals remain in their ionic state in order to maintain normal mineral homeostasis **(Bae *et al*. 1999; Noureddini & Dang, 2008)**. Hence, the continuous release of these faecal matter into soil and water bodies raise the amount of insoluble phosphorus in such environments, raising the pollution index therein **(Tariq *et al*. 2017)**.
Phytases catalyse the sequential hydrolysis of phytic acid to inorganic phosphate and myo-inositol hexaphosphate derivatives **(Singh & Satyanarayana, 2011; Kumar *et al*. 2019)**. In animal feed industry, especially for monogastrics, the eco-friendliness of phytases in the degradation of phytic acid has made them one of the most desired feed additives **(Bae *et al*. 1999; Wang *et al*. 2013)**. Phytase supplementation in feed maximizes nutrients uptake in the animal thus minimizing the phatate ultimately released into environment through the undigested feed within faecal matter **(Robinson *et al*. 2002; Vashishth *et al*. 2017)**. Despite the unique role that poultry farming holds in animal husbandry, the current high cost of commercial phytase continues to task nutrition, enzymology and microbiology researchers to find new microorganisms which can efficiently utilize cheaper agro-based substrates to produce phytases for application in animal feeding.
This work therefore isolated phytase producing fungi from cereal-rich soils and poultry waste. In addition we determined the production and enzyme characteristics of a fungal phytase produced in a submerged fermentation medium containing different underutilized agro-based 'waste' materials as fermentation substrates either devoid of, or supplemented with essential nutrients.

MATERIAL AND METHODS

Sample collection and isolation of fungi

Agro-based substrates (orange peel, sesame seed and watermelon seeds) were obtained from agro-produce vendors at Bodija market in Nigeria. The substrates were oven dried at 45ºC, milled and passed through a 60 mesh (250µm) sieve. Samples of poultry droppings and soils (from untilled farmland and cereal-storage/vending areas) were collected for use in microbial isolation. The soil samples were collected at a depth of 2-8cm into Ziplock bags while poultry droppings were collected from broiler, layer and turkey pens and appropriately labeled. Samples (10g) each was suspended in 90mL of sterile distilled water and then serially diluted (1:9) up to the 8^{th} dilution. From each dilution, fungi were isolated by pour-plating 1mL of the different dilutions using Potato Dextrose Agar **(Harrigan & McCance, 1976)** amended with 100 µg mL^{-1} of streptomycin to inhibit bacterial growth. The plates were incubated at 27±2 °C for 5 days and selected fungi were sub-cultured onto agar slants and stored at 4ºC until further use.

Screening for phosphate solubilization and phytase producing microorganisms

Phosphate solubilization ability of 5 day old fungal isolates was determined by culturing 7mm fungal discs on Pikovskaya medium (PKV) and incubating for seven days at 27±2°C **(Pikovskaya, 1948)**. The formation of clear, translucent halos around inoculated fungal cultures was indicative of phosphate solubilization by such isolates. Further screening for the ability of the PKV-positive fungi to solubilise phytic acid was achieved by inoculating them on a compounded Phytase Screening Medium (PSM) of **Nautiyal, (1999)** and incubating for 7days. Phytic acid solubilization was detected by the formation of zones around the inoculated positive phosphate-solubilising fungi and phytase activity was confirmed using the double counter-staining assay **(Bae *et al*., 1999)**. Briefly, presumptive-positive fungi growing on PSM plates were flooded with 2% w/v cobalt chloride solution for 5 minutes and discarded, then counter-stained with equal volumes of a mixture of 6.25% ammonium molybdate solution and 0.42% ammonium vanadate solution for another 5 minutes. Thereafter, the Solubilization Index (SI) was calculated for the cultures which persistently exhibited distinct zones of clearance around the cultures after the double counter-staining **(Patki *et al*., 2015)**:

$$SI = \frac{(\text{Colony diameter} + \text{Halo diameter})X\ 100}{\text{Colony diameter}}$$

Isolates which showed ≥50% solubilization efficacy were considered as good phytase producers and stored appropriately at 4°C on PDA. The fungus with the highest SI was selected for further work and was identified using cultural, morphological and microscopic characteristics.

Phytase production

Phytase was produced using submerged fermentation by cultivating two 7mm agar plugs of the selected fungus (which exhibited the highest Solubilisation Index) in autoclaved and cooled 25 mL of Phytase Production Broth (PPB) containing (g/L): Glucose, 10; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; $MnSO_4$, 0.01; sodium phytic acid 0.5 with the pH adjusted to 5.5 **(Qasim *et al*. 2016)** and incubated at 27±2 °C for 7days. The production broth was centrifuged at 4000 *g* for 15 minutes and the phytase activity quantified as described under assay for phytase activity.

Effect of cultural parameters and medium components on phytase production

The selected fungus was cultivated in PPB over 7 days and assayed at 24 hour intervals to determine the optimum phytase production period. The effects of inoculum age (48, 72, 96, 120 and 144 hours old fungal cultures), pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) carbon (fructose, lactose, maltose, sucrose and starch), and nitrogen ($NaNO_3$, NH_4Cl, NH_4NO_3, peptone and yeast extract) were determined **(Wang *et al*., 2004; Singh & Satyanarayana 2012; Sareen, 2014)**. Under the production conditions earlier described, each control carbon or nitrogen source variable (glucose or $(NH_4)_2SO_4$) were substituted for using the stated materials. Another control setup, devoid of the respective carbon and nitrogen source, was used to determine the effect of the absence of a carbon/nitrogen source on phytase production.
Milled sesame seed, watermelon seed and orange peel (1%w/v) were mixed in distilled water, autoclaved and used as sole substrates for phytase production in a 25ml flask culture cultivation. After cooling, each flask was inoculated with two 7mm fungal agar plug and incubated at 27±2°C. In another setup, the milled substrates were supplemented with all the medium components of PPB: (g/L) 0.1 $MgSO_4$, 0.2g KCl, 0.0001 $MnSO_4$, 0.0001 $FeSO_4$ and 0.5 $(NH_4)_2SO_4$ but devoid of glucose and sodium phytic acid. Inoculated phytase production broth was used as control. After incubation, the broths were centrifuged and assayed for phytase activity **(Singh & Satyanarayana 2012; Qasim *et al*., 2016)**.

Assay for phytase activity

Phytase activity was determined using 0.5 mL enzyme mixed with 2 mL of 0.1% sodium phytic acid (in 0.1M of pH 5.5 acetate buffer) for 10min at 37°C. The reaction was stopped with 2mL freshly prepared acetone-acid-molybdate stop solution. The orthophosphate released from the reaction setup was quantified by measuring the absorbance at 355nm and the values extrapolated from a standard curve prepared with K_2HPO_4. One unit of phytase activity was defined as that which liberated 1 µM phosphate per minute under the assay condition **(Choi *et al.* 2001)**.

Partial purification of fungal phytase and enzyme characterization

Partial purification of produced phytase using the best production conditions was accomplished by the method of **Roy *et al*., (2012)**. Cell free supernatants were subjected to ammonium sulfate precipitation (0-40% and 40-80% saturation) incubated overnight at 4°C, centrifuged afresh and the precipitate desalted using a dialysis bag suspended overnight at 4°C in a glass beaker containing sodium acetate buffer (pH 5.5) with continuous mixing using a magnetic stirrer. The molecular weight, phytase activities and total protein contents of the partially purified samples were determined **(Lowry *et al*. 1951; Laemmli, 1970)** .
Partially purified phytase enzymes were characterized by determining the effect of pH (3-9), temperature (25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C), and different metal ions inhibition of enzyme activity: Ca^{2+}, Co^{2+}, Cd^{2+}, Li^{+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+} and Fe^{2+} on phytase activity **(Roy *et al.* 2012; Sareen, 2014)**. Effect of increasing substrate concentration by addition of fixed quantity of enzyme (0.5 mL) to 2 mL varied concentration of assay mixture 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6% of phytic acid in acetate buffer was also investigated to determine the maximum velocity (V_{max}) and Michaelis-Menten constant (Km) for the substrate **(Lineweaver & Burk, 1934)**.

Statistical analyses

Studies were performed in triplicates and all data were analyzed using One-Way ANOVA ($p < 0.05$).

RESULTS

Screening for phytase and effect of cultivation conditions on phytase production

Twenty-eight fungi with phytase producing abilities were obtained in this work and the fungal count in the samples studied varied between 10^5 and 10^6 TFU/g sample (Table 1). Broiler poultry droppings (BPD) sample had the highest fungal load (1.6×10^6). Phytic acid solubilization was observed in 61% of the isolates (*n-28*) while the counterstaining assay confirmed fourteen fungi (50%) as phytase producers by eliminating the false-positive fungal producers initially observed before counterstaining. The solubilization index was highest (121%) in isolate GS_1S_6 and least in fungal isolate $TPDP_{10}$ (30%).
Isolate GS_1S_6 was therefore selected for phytase production studies. On PDA, the colonies of isolate GS_1S_6 presented as flat and compact mycelium with cream-brown basal mycelium which progressively became entirely dark brown with a cream/yellowish observe. By the fourth day, the culture was covered by dense layer of intense black conidial heads with powdery texture. Microscopically, the fungal morphology revealed hyphae bearing conidiphores on which strings of circular spores were suspended.
When the impact of the extent of incubation on phytase production was investigated, a significant amount of phytase was produced at 120 hours post inoculation (Figure 1a) beyond which the production dwindled. In Figure 1b, the inoculum age supporting the maximum production of phytase by *A. niger* GS_1S_6 (900U/L) was recorded when 72 hour old fungal discs were used for cultivation and significantly different from the numerically close value (800 U/L) recorded using a 96 hours old culture.
From the results of phytase production in adjusted pH media (Figure 1c), phytase production gradually was found to increase as the pH increased from pH 3 to 5 and then peaked at a pH of 5.5 (1100 U/L). After this, there was a steady decline in enzyme production. *Aspergillus niger* GS_1S_6 utilised simple sugars (glucose and fructose) with glucose producing highest phytase compared to other sugars used (Figure 1d). The use of more complex sugars resulted in lower quantities of phytase. *Aspergillus niger* GS_1S_6 produced phytase in medium devoid of carbon or nitrogen source and the least phytase (119.6 U/L) was produced in a medium devoid of a carbon source. When ammonium sulphate was used in the cultivation medium as a source of nitrogen, the highest phytase production (580 U/L) was observed (Figure 1e).

Table 1 Solubilization index and probable identity of phytase-positive fungal Isolates

Sample Source	CFU/g Sample	Isolate code	% S.I	Probable identity
Cereal rich soil $(CRS)_1$	1.3×10^6	CRS_1C_1	58±0.08	*Aspergillus* sp.
		CRS_1C_2	ND	*Fusarium* sp.
		CRS_1C_3	77±0.05	*Mucor* sp.
		CRS_1C_4	55±0.07	*Penicillium* sp.
		CRS_1C_5	ND	*Fusarium* sp.
		CRS_1C_6	ND	*Fusarium* sp.
		CRS_1C_8	ND	*Penicillium* sp.
		CRS_1C_9	ND	*Mucor* sp.
Cereal rich soil $(CRS)_2$	9.0×10^5	CRS_2C_7	51±0.04	*Rhizopus* sp.
		CRS_2C_{10}	110±0.03	*Aspergillus* sp.
Broiler poultry droppings (BPD)	1.6×10^6	$BPDP_1$	ND	*Mucor* sp.
		$BPDP_2$	92±0.07	*Aspergillus* sp.
		$BPDP_3$	ND	*Penicillium* sp.
		$BPDP_4$	98±0.04	*Aspergillus* sp.
Turkey poultry droppings (TPD)	1.4×10^5	$TPDP_5$	ND	*Trichoderma* sp.
		$TPDP_6$	71±0.40	*Aspergillus* sp.
		$TPDP_7$	ND	*Trichoderma* sp.
		$TPDP_9$	ND	*Aspergillus* sp.
		$TPDP_{10}$	30±0.02	*Rhizopus* sp.
Layer poultry dropping (LPD)	1.0×10^6	$LPDP_8$	66±0.06	*Mucor* sp.
Garden Soil $(GS)_1$	1.0×10^6	GS_1S_1	ND	*Aspergillus* sp.
		GS_1S_2	55±0.02	*Mucor* sp.
		GS_1S_3	ND	*Penicillium* sp.
		GS_1S_6	121±0.06	*Aspergillus* sp.
		GS_1S_8	44±0.03	*Aspergillus* sp.
Garden Soil $(GS)_2$	8.0×10^5	GS_2S_4	ND	*Aspergillus* sp.
		GS_2S_5	68±0.01	*Penicillium* sp.
		GS_2S_7	ND	*Mucor* sp.

Key: BPD= Broiler poultry droppings, CRS= Cereal rich soil, GS= Garden Soil, LPD= Layer poultry dropping, TPD= Turkey poultry dropping, ND = Not Detected, S.I= Solubilization Index.

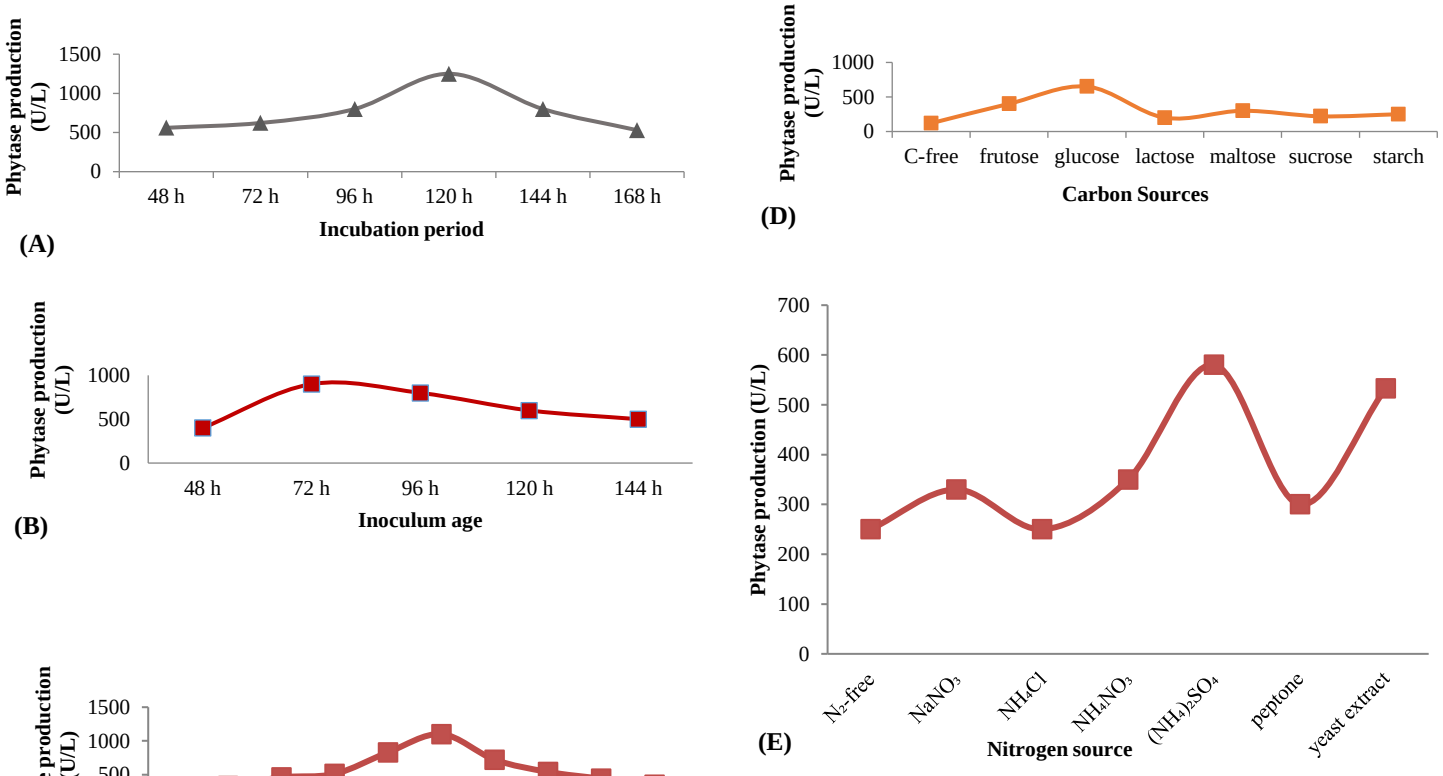


Figure 1 Time course of (A), effect of inoculum age (B), medium pH (C), carbon (D) and nitrogen sources (E) on phytase production by *Aspergillus niger* GS_1S_6

The sole use of agricultural substrates in sterile broth without additions of mineral components resulted in sesame seed supporting the highest phytase production (700 U/L) by *A. niger* GS_1S_6 while the least phytase production (310 U/L) was

recorded in the orange peel medium (Figure 2). The control phytase production broth (containing both glucose and sodium phytate) supported a phytase production of 980 U/L by the *A. niger* GS_1S_6. However, the inclusion of the mineral phytase production broth (devoid of both glucose and sodium phytate) to the agro-substrates medium boosted phytase production in all the media used. The mineral-supplemented sesame seed-containing medium demonstrated the overall highest phytase activity (1000 U/L) followed by the watermelon supplemented mineral medium which recorded 720U/L. Using the control phytase production broth void of both glucose and phytic acid, the *Aspergillus niger* GS_1S_6 still produced phytase (330 U/L).

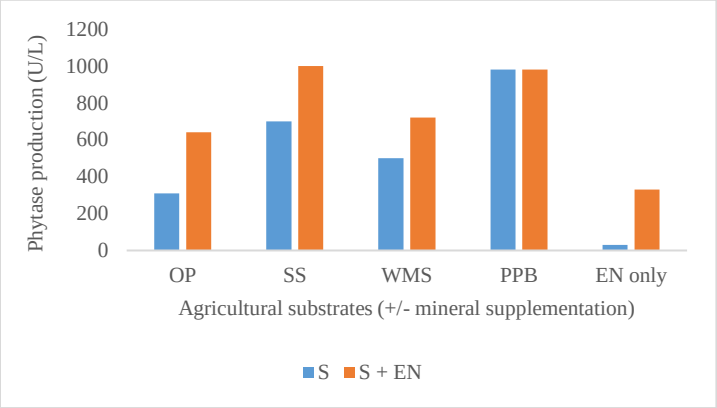


Figure 2 Phytase production by *Aspergillus niger* GS_1S_6 in broth media devoid of, and supplemented with essential nutrients using orange peel, sesame seed and watermelon seeds as sole substrates.

Phytase Characterisation

The phytase produced from a sesame seed-essential nutrient culture broth was used for enzyme characterization studies. With sequential purification, the phytase activity increased (Table 2) and the *A. niger* GS_1S_6 phytase had a specific activity of 0.211 U/mg after the dialysis of the ammonium sulphate precipitated enzyme. The protein content of the crude phytase was 5.476 mg/mL and this further reduced in the dialysed ammonium sulphate precipitated phytase (4.945 mg/mL).

The crude phytase (Lane 2) had two protein bands, both within the 31 and 38KDa molecular weight mark. However, one band (located between 31 and 38KDa) was observed in the partially purified *A. niger* GS_1S_6 phytase (Figure 3).

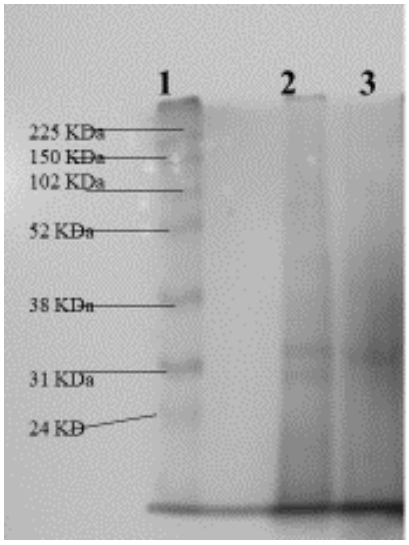
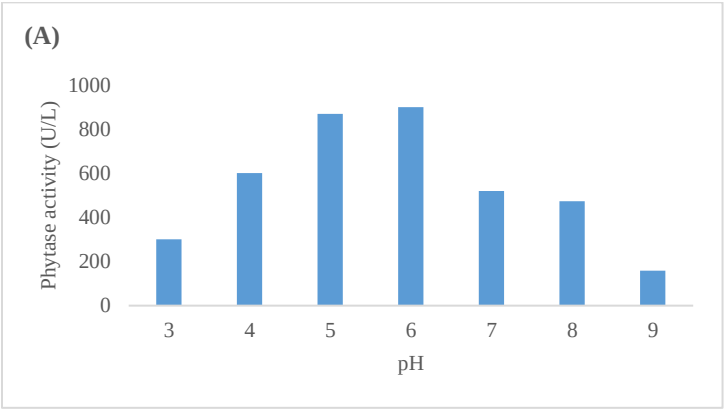


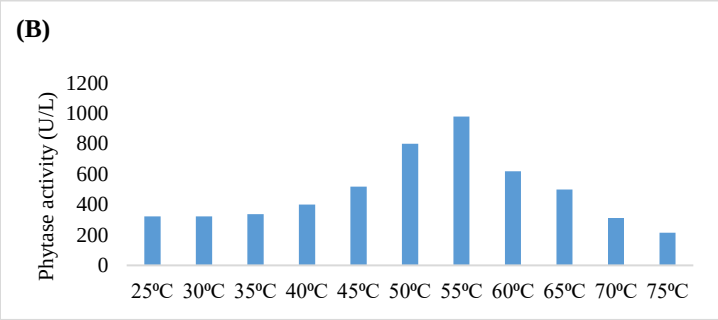
Figure 3 Electrophoretogram of the crude and partially purified *Aspergillus niger* GS_1S_6 phytase.
Key: *1~Molecular weight bands; 2~Crude phytase, 3~$(NH_4)_2SO_4$ precipitated and dialysed Aspergillus niger GS_1S_6 phytase*

Table 2 Partial purification of *Aspergillus niger* GS_1S_6 phytase

Purification steps	Total activity (U)	Total protein (mg/mL)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude phytase	81.826	547.595	0.1494	1.000	100.0
$(NH_4)_2SO_4$ precipitatation	95.555	530.731	0.180	1.205	116.8
Dialysed enzyme	104.118	494.503	0.211	1.409	127.2

A narrow pH range for phytase activity was observed between 5.0 and 6.0, having an optimum activity at pH 6 (899.82 U/L) beyond which a steady decline was observed from neutral to slightly alkaline regimes (Figure 4a). The optimum temperature for phytase activity was observed at 55ºC (980 U/L) and phytases subjected to higher temperatures beyond 55ºC had reduction in phytase activity (Figure 4b). At 65ºC, the residual activity was 51%. All metallic inclusions reduced the phytase activity recorded (Figure 4c). Enzyme activity was most inhibited by Cu^{2+}, which recorded a 17.11% activity and moderately inhibited by Mg^{2+} (80.5%).





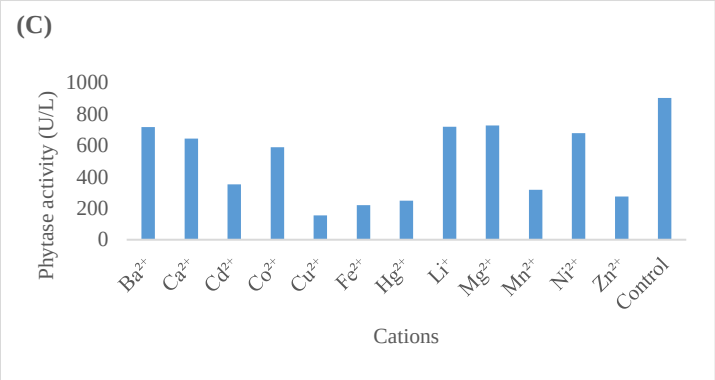


Figure 4 Effects of different (a) pH (b) temperature and (c) cations on the activity of phytase produced by *Aspergillus niger* GS_1S_6

The enzyme kinetics of *A. niger* GS_1S_6 phytase obtained when a fixed quantity of enzyme was used along with varied concentration of substrate (phytic acid) revealed that maximum velocity rate (V_{max}) of *A. niger* GS_1S_6 phytase was attained at 0.077 mM/mL/min while the Michaelis-Menten constant (Km) was 1.308 mM.

DISCUSSION

Fungal isolates with phytase producing abilities were obtained in this work from the samples studied and their load, which varied between 10^5 and 10^6 total fungal units (TFU)/g sample, were similar to other works. Other authors have also reported the isolation of phytase producing microorganisms from similar sources. **Awad *et al.* (2014)** isolated from root nodule soils while **Shivanna & Govindarajulu (2009)** isolated *Aspergillus niger* from soil samples around poultry livestock areas. The highest phytase activities as indicated by the solubilization index were recorded in four *Aspergillus* species (GS_1S_6, $BPDP_2$, CRS_2C_{10} and TPD_6), all of which had solubilization index ≥50%. While the selected isolate was identified as *Aspergillus niger* GS_1S_6 through its colony and microscopic descriptions, other authors also documented similar morphological descriptions for *Aspergillus niger* **(Lee *et al*, 2005; Tariq *et al.* 2017)**. Similarly, **Tariq *et al.* (2017)**, also recorded a maximum solubilization index of 119% in their phytase studies. The counterstaining technique successfully eliminated false positive results caused by the presence of organic acid **(Bae *et al*., 1999; Qasim *et al*. 2016)** and the quantitative screening was an additional confirmatory test to complementary strain selection and development for possible strain commercialization process. Several authors have reported the isolation of phytase producing microorganisms from similar sources. **Awad *et al.* (2014)** isolated phytase producing *Penicillium purpurogenum* GE1 from root nodule soils while **Shivanna &Govindarajulu (2009)**, isolated a phytase producer from soil samples obtained from poultry livestock areas.

In the first five of the seven days of cultivation, The phytase produced increased. The decline observed beyond 120 hours could possibly be attributed to an increase in fungal biomass which might have resulted in depletion of available nutrients in the medium, thus affecting the production of phytase **(Mittal *et al*., 2012)**. Inoculum age, being a function of microbial growth, is known to affect enzyme production. At 72 hours, fresh fungal cultures are at a rapid growth phase which might directly continue in the new medium into which the fungus is introduced for cultivation.

Phytase production was low at extremely acidic pH, peaked at pH 5.5 and reduced beyond pH 5.5. A similar observation was reported by **Gargova &Sariyska (2003)**, who documented the optimal phytase production at pH 5.0, while other authors reported an optimum pH of 5.5 for *Aspergillus* sp. L117 **(Lee *et al*, 2005)**. The ranges of carbon sources utilized for mycelial growth and enzyme production of different fungi vary widely. A diversity of sugars can be used as suitable carbon sources for the proliferation of fungi **(Odeniyi *et al*. 2009)**. Simple sugars (glucose and fructose) supported phytase production more than the disaccharide and polysaccharides used in the production medium, an advantage for the microorganism, since disaccharides and other complex carbohydrates would require an initial hydrolysis into monomeric units or their derivatives, before entering the various metabolic pathways (**Praveen & Arun, 2013)**. In nature, both the organic and inorganic forms of nitrogen are available to fungi, but how these fungi utilize the various nitrogen sources is contingent on their specific response towards different nitrogenous substances. The high phytase production observed in this study when ammonium sulphate was used may owe to the fact that ammonium nitrogen needed no modification before entering the synthetic pathway **(Praveen & Arun, 2013)**.

Phytase production by *A. niger* GS_1S_6 was recorded in all the agro-material based fermentation medium (orange peel; sesame seed and watermelon seed), with or without mineral supplementation. The addition of mineral constituents to the substrates in the medium resulted in higher phytase activities. Studies have reported phytase production through submerged fermentation in medium containing wheat bran, a cheap agro-residue, which is however, also used as a component of animal feed **((Mittal *et al*., 2012; Sreedevi & Reddy, 2012)**. In many underdeveloped nations the preservation of perishable crops is almost non-existent, a condition which the Food and Agriculture Organization of the United Nations have researched extensively upon, worldwide. Some crop parts such as watermelon seeds and sesame seeds are considered underutilized **(Biswas *et al*. 2017)**. In Nigeria, watermelon, for example, is disposed of immediately fruit rot sets in and the seeds within are wasted. **Olasupo & Okorie (2019)**, however, reported that sesame seeds still find some use in traditional condiment production. Since watermelon seeds currently have no documented competing economic use in Nigeria, these may be considered as a cheap source for phytase production considering its 72 and 73.5% phytase production relative to that from sesame seed and phytase production broth, respectively. **Qasim *et al.* (2016)** reported similar findings for *Aspergillus tubingensis* SKA and suggested that enhancement in phytase production could be linked to improvement in the nutritional value of the fermentation medium as depicted in the mineral supplementation results. The ability of *Aspergillus niger* GS_1S_6 to produce phytase in medium devoid of both phytic acid and glucose, shows its constitutive nature. **Idriss *et al*. (2002)** reported a phytase constitutively secreted by a *Bacillus amyloliquefaciens* independent of the nutrient composition which had its highest phytase produced in a medium devoid of any form of phosphorus.

When the phytase produced using sesame seed-essential nutrient culture broth was subjected to precipitation and dialysis, the resulting enzyme had a higher activity and yield compared to the crude while its final protein content reduced compared to the crude. The molecular weight of the partially purified *A. niger* GS_1S_6 phytase was between 31 and 38KDa. **Tariq *et al.* (2017)**, documented phytases from *Aspergillus* species with molecular weights between 35 and 107.82 KDa and reported that the *A. niger* which produced the highest phytase recorded the least molecular weight. The optimum pH for *A. niger* GS_1S_6 phytase activity was between 5.0-6.0 beyond which the activity declined as it moved from neutral to slightly alkaline regimes. The pH optima of some microbial phytases have been reported in the ranges of 4.5-5.5 **(Roy *et al*. 2012)**. The phytase of an *Aspergillus foetidus* was reported to have an optimum pH of 5.5, similar to what obtained in this work **(Ajith *et al*. 2019).** The optimum pH of phytase activity was similar to that at which the production medium of the organism also produced the highest yield. The phytase was optimally active within the temperature range 50ºC and 60ºC. **Vohra & Satyanarayana, (2003)**, reported that for most fungal and yeast phytases, the optimum temperature was usually 50-70°C. The phytase activity was inhibited by Cu^{2+}, Cd^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+}. Metal ions play an important role in enzymatic catalytic activities, however, many phytases do not require metal ions for maximum catalytic activity **(Fu *et al.* 2008)**. The inhibitory effect of mineral ions on phytase activity might be attributed to the strong chelating capacity of phytic acid, resulting in an insoluble metal-phytic acid complex that reduce the bioavailability of phytic acid for the enzyme due to the glycosylation folding of the enzyme structure **(Wang *et al*., 1980; Afinah *et al*., 2010)**. The

metal ion inhibition of the *A. niger* phytase was analogous to the observation of **Segueilha *et al*. (1992)**, who documented a reduction in phytase activity by 90% using 5 mM Fe^{3+} with *Pichia anomala*. The enzyme activity of a phytase from *Shigella* sp. CD2 was inhibited in the presence Fe^{2+}, Zn^{2+} and Cu^{2+} **(Roy *et al*. 2012)**.

The K_m recorded was lower than those reported by **Elkhalil *et al.* (2011)** in an *Aspergillus* sp. A low phytase K_m allows the maintenance of sufficient substrate degradation leading to higher transformation **(Nagashima *et al*., 1999)**.

CONCLUSION

A phytase-producing *Aspergillus niger* GS_1S_6 was isolated from soil and glucose, $(NH_4)_2SO_4$, 5 days incubation, pH 5.5 and inoculum age of 72 hours were most suitable for phytase production. Phytase production was constitutive and the characteristics of the phytase obtained using different underutilized agro-based materials/'waste' as fermentation substrates in mineral replete/devoid systems revealed a partially purified enzyme which was most active at pH 5.5, 55°C and required no metal ion inducement. Alternative medium composition, especially in the form of oil-based seeds with and without mineral supplementation can be enlisted to economically produce phytase with commensurate activities instead of synthetic media. To the best of our knowledge this is one of the first works to utilize watermelon seed as a cost effective agro-material for phytase production thus solving the challenges of agro-waste management and reducing the cost of enzyme production of phytase which is held as a major drawback to phytase commercialization.

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