

**Purification and Characterization of Phytase Enzyme Extracted from *Aspergillus niger* SF58 and its Application in Cereal Dephytinization**

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## ABSTRACT

Phytases are the group of enzymes that catalyze phytate hydrolysis and release a utilizable form of inorganic phosphorus. So, they are used to improve the nutritive value of animal feed. The current study aims to purify, characterize the phytase enzyme, and to study its application in cereal dephytinization. Molecular identification of the fungal isolate was achieved using the 18S rRNA and phytase activity was determined using phytic acid as substrate. Phytase from *Aspergillus niger* SF58 was purified using dialysis and Sephadex G 100 column chromatography to get enzyme recovery and purification fold of 72.74% and 2.303, respectively. The purity of purified phytase was confirmed through SDS-PAGE technique which showed a one protein band corresponding to phytase activity at 22 kDa. The optimum reaction time for phytase activity was revealed to be 15 min, whereas the optimal temperature and pH values were found to be 60°C and pH 5.5. The purified enzyme was able to retain 46.93% of its initial activity after 4 months of storage at 4°C. Metal ions ( $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{+2}$ ) acted as activators for the purified phytase, whereas  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Al}^{3+}$  served as inhibitors. The purified phytase showed the highest substrate specificity toward wheat bran, followed by the wheat flour, soy-bean, and corn-flower, respectively. The use of *Aspergillus niger* SF58 phytase in dephytinization of wheat bran and soybean indicated the possibility of applying the enzyme to increase the nutritional values of feed and food additives.

**Keywords:** Phytase, Purification, Characterization, Dephytinization.

## Introduction

Phosphorus is an essential macronutrient utilized by the entire living organisms to biosynthesize diverse molecules, including phospholipids, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and adenosine triphosphate (ATP). The utilization of phosphorus by microorganisms is depending on some specific enzymes, like phosphatases, which are able to hydrolyze phosphorus-containing molecules. Such enzymes are responsible for mineralization of organic phosphate, once the level of free inorganic phosphate becomes low in the soil.<sup>1</sup>

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are a special class of acid phosphatases that catalyze the hydrolysis of phytate to less phosphorylated *myo*-inositol derivatives (in some cases to free *myo*inositol) and releasing of inorganic phosphate.<sup>2</sup> These enzymes act to hydrolyze phytic acid, the main form of the accumulated phosphorus in most of cereal oil seeds, grains, and plants, to release inorganic phosphate.<sup>3</sup> In the last few years, industrial applications of enzymes have increased, where they are used in detergents, pharmaceuticals, food, feed and other products.<sup>4,5</sup> Phytase enzyme is considered as one of the most important industrial enzymes, so it was the candidate enzyme for a large number of researches. Supplementation of animal feeds with phytase reduces the anti-nutritive effects of phytic acid and lowers the cost of feed diets by reducing the need for inorganic phosphate additives.

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Since agastric and monogastric animals do not have phytase enzyme in their gastrointestinal tract and cannot digest the phosphorus which is present as phytate, so it is necessary to supplement their diets with inorganic phosphorus.<sup>6,7</sup>

Cereals, oil seeds and legumes are grown over 90% of the harvested area of the world and act as the major source of nutrients in the animal kingdom. Phytic acid is considered as an important constituent of these crops.<sup>8,9</sup> It is known as a very stable molecule as a result of its chemical structure, which differs from the other organophosphate molecules in its high phosphate content which causes a highly negative charge over a wide range of pH.<sup>10</sup> At the normal physiological conditions, phytate works as a chelating agent for essential minerals such as iron, calcium, zinc and magnesium. As a result of phytate chelating activity, it reduces the biodigestibility of minerals present in the digestive tract of monogastric animals, leading to environmental problems. Since monogastric animals such as pigs lack phytase enzyme, they excrete large amounts of phosphorus in the environment in the form of phytate, leading to pollution by this element.<sup>11</sup> Additionally, phytate binds non-selectively to proteins and amino acids and thus inhibits the digestive enzymes like trypsin and  $\alpha$ -amylase, causing a reduction of protein digestibility in animals. Phytic acid binds to amino acids such as histidine, arginine and lysine, forming protein-phytate complexes. These complexes are insoluble and resistant to enzyme hydrolysis and thus reduce the efficiency of protein utilization. Also, phytic acid can increase endogenous amino acid loss due to increased secretion of digestive enzymes and mucins, and reduced re-absorption of the endogenously secreted amino acids in the small intestine.<sup>12</sup> Therefore, the enzymatic hydrolysis of phytic acid is very desirable as phytase acts to cleave the phosphate groups that are present in phytic acid mineral complexes and liberate the minerals as available soluble form.<sup>13</sup>

The selection of accurate techniques in enzymes manipulation is essential for the prevention of enzyme inactivation. Lastly, the utilization of ultrafiltration processes to purify and concentrate enzymes and protein samples was increased as a primary step of

purification to exclude other proteins from the solution, on the basis of their cutoff molecular weight.<sup>5</sup> Additionally, the biochemical categorization of enzymes is an imperative stage for their efficient exploitation in various biotechnological applications.<sup>1</sup> The present study aims to purify and characterize the phytase enzyme produced by an isolated fungal strain, and study its application in cereal dephytinization.

## Materials and Methods

### Sources of soil samples

Ninety-nine fungal isolates were isolated from thirty-nine soil samples. These different soil samples were collected from different regions at El godiada (21), Minia El kameh (13), and, Zagazig (5), Sharkia Governorate, Egypt.

### Isolation and screening of fungal isolates for phytase production

Fungal isolates present in different soil samples were isolated via the dilution plate count method. Isolation and maintenance of fungal isolates were carried out using potato dextrose agar (PDA) as isolation medium.<sup>14</sup> All the isolated fungi were screened qualitatively for extracellular phytase production using phytase screening medium (PSM) with some modifications.<sup>15,16</sup> The PSM contained (g/l): Sodium phytate, 3.0; D-glucose, 15.0; MgSO<sub>4</sub>·7 H<sub>2</sub>O, 0.5; NH<sub>4</sub>NO<sub>3</sub>, 5.0; KCl, 0.5; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.01; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01; CaCl<sub>2</sub> 1.0; Agar, 15.0; pH, 7.0. All the isolated fungi were incubated at 30 °C and the formation of clear zones as a result of sodium phytate hydrolysis was measured.

### Molecular identification of the most potent phytase producer isolate

The fungal isolate that exerted the greatest clear zone of sodium phytate hydrolysis in the previous section was identified molecularly using the 18S rRNA. DNA was extracted and purified with the protocol of GeneJET™ genomic DNA purification kit (Thermo, USA). The purified DNA was used immediately in PCR reaction that contained Maxima® Hot Start PCR Master Mix (2x), 18S rRNA forward (F: 5' -AGA GTT TGA TCC TGG CTC AG -3') and reverse (R: 5' -GGT TAC CTT GTT ACG ACT T-3') primers, and fungal DNA template. PCR was performed according to the recommended thermal cycling conditions, where a cycle of initial denaturation / enzyme activation was conducted at 95°C for 10 min. Then, 35 cycles of annealing were conducted at 65°C for 1.0 min, followed by an extension cycle at 72 °C for 10 min. At the end of the reaction, the PCR products were separated on agarose gel electrophoresis and purified using GeneJET™ PCR purification kit (Thermo Fisher Scientific, USA). Finally, the purified PCR products were sequenced on an ABI 3730XL™ Sequencer. The NCBI BLAST® program searched the DNA databases to find the sequence similarities related to the sequence of this isolate. Then MEGA 5.0 software was used to align the identified sequences to construct the phylogenetic tree.

### Phytase production from *Aspergillus niger* SF58

A disk from 7 days old culture of *Aspergillus niger* SF58 on PDA medium was used to inoculate each 250 ml Erlenmeyer conical flasks containing 25 mL of the medium consists of (g/L): D-glucose, 15; Sodium phytate, 3.0; Ammonium nitrate, 5.0; Potassium chloride, 0.5; Magnesium sulphate, 0.5; Manganese sulphate, 0.01; Ferrous sulphate, 0.01; and Calcium chloride, 1.0. The culture flasks were incubated at 30 °C and pH 6.0 for 8 days under stationary culture conditions. The medium was filtered using Whatman No.1 filter paper and the filtrate was used as the source of crude enzyme.

### Purification of *Aspergillus niger* SF58 phytase enzyme

The obtained crude phytase from *A. niger* SF58 was concentrated using a dialysis bag (Medicell International Ltd, UK), where the crude extract was kept in sucrose at 4°C overnight. The retained fraction was cleared by centrifugation and prepared for the next step of purification using Sephadex G-100 column chromatography. The column (50 ×1.0

cm) was previously equilibrated with acetate buffer (0.2 M, pH 5.5) and enzyme elution was done with the same buffer at a flow rate of 30 ml/h. Collected fractions (2.0 ml) were analyzed for protein concentration and phytase activity determination. The fractions with the greatest specific activity were pooled and collected for subsequent analysis and characterization.

### Phytase enzyme assay

The activity of phytase enzyme was measured by the method described by Boyce *et al.*,<sup>17</sup> where phytase activity was determined by incubation of 0.5 ml of phytic acid sodium salt hydrate 0.1 % (w/v) dissolved in 200 mM sodium acetate buffer (pH 5.5), with 0.5 ml of diluted phytase enzyme for 15 min at 50 °C. The reaction was terminated by addition of 1.0 ml of 0.5 M TCA solution. The assay was undertaken in duplicate, along with an appropriate blank. The blank is conveniently prepared by separate incubation of substrate and enzyme individually with the subsequent addition of TCA to the enzyme, followed by the substrate. Color development due to the release of inorganic phosphorus was achieved by adding 1.0 ml of a freshly prepared FeSO<sub>4</sub>·7H<sub>2</sub>O/ammonium molybdate/H<sub>2</sub>SO<sub>4</sub> reagent. The solution was incubated for 5 min at room temperature and absorbance values were recorded at 660 nm. FeSO<sub>4</sub>·7H<sub>2</sub>O/ammonium molybdate/H<sub>2</sub>SO<sub>4</sub> reagent used was prepared by dissolving 5.0 g of FeSO<sub>4</sub>·7H<sub>2</sub>O in 90 mL of distilled water and adding 10.0 mL of 8.0% (w/v) ammonium molybdate. The 8.0% ammonium molybdate solution was made by dissolving 8.0 g of ammonium molybdate in 50 mL of distilled H<sub>2</sub>O, then 27 mL of 10 M H<sub>2</sub>SO<sub>4</sub> were added and the solution was brought to 100 ml with distilled water. The absorbency at 660 nm was determined after standing for 5 min at room temperature. One phytase unit was defined as the activity that released 1.0 μmol of inorganic phosphorous from sodium phytate within one min. Soluble protein was determined according to the method described by Lowry *et al.*<sup>18</sup>

### Molecular mass determination of *Aspergillus niger* SF58 purified phytase

The purified enzyme was subjected to SDS-PAGE in order to check the purity and determine its molecular mass according to the method of Laemmli.<sup>19</sup> The gel system consisted of 7.5% acrylamide stacking gel and 15% acrylamide separating gel. SANTA CRUZ broad range marker SC-2361 was used as the molecular weight standard (20 - 200 kDa). Electrophoresis was performed with a discontinuous buffer system in a BIO-RAD mini protein tetra-vertical cell protein system. Then, the gels were stained overnight, and de-stained later for 24 h. Gels were scanned and analyzed using (Gel analyzer2010a) to determine and compare the molecular weights of proteins.

### Characterization of the purified *Aspergillus niger* SF58 phytase

Effect of reaction time on the activity of the purified phytase was determined by incubating the reaction mixture for different time periods (5, 10, 15, 20, 25, 30, and 35 min) at 55°C and pH 5.5. Impact of reaction temperature on phytase activity was determined via estimation of phytase relative activities at different reaction temperatures which ranged between 20 and 100°C. Also, the effect of different pH values on phytase activity was detected through phytase activity determination at various pH values in the range of 3.0 and 8.0. In this regard, citrate buffer was used in pH 3.0 - 4.5 range, acetate buffer was used in pH 5.0 - 6.5 range, and Tris-HCl buffer was used in pH 7.0 - 8.0 range. Equal volumes of buffers at different pH containing 0.1 % of the substrate and 0.1 ml of purified phytase were incubated at 60°C for 15 min. Thermal stability of the purified enzyme was estimated by initial incubation of similar aliquots of the purified phytase, separately at different temperature (50, 60, 70 and 80°C) for different incubation periods (5, 10, 15, 20 and 30 min). Then, phytase relative activities were determined by incubating each preheated enzyme aliquot with the other components of the standard reaction mixture (sodium acetate buffer containing 0.1% of the substrate at pH 5.5). The effects of EDTA as a chelating agent and metal ions on the activity of the purified phytase were tested. Mineral salts of various cations Mg<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Fe<sup>2+</sup>, Al<sup>3+</sup> or Zn<sup>2+</sup> were examined at a concentration of 5.0 mM in the reaction mixtures. The

control was prepared without any metallic salts. Different substrates were used to determine the enzyme specificity toward the different substrates. Soy bean, wheat bran, corn flower and wheat flower were added to sodium acetate buffer pH 5.5 instead of sodium phytate, and then the specificity of the purified phytase toward each substrate was assayed. The purified phytase was stored at 4°C for 4 months, and samples were taken periodically to assess its residual activity.

#### Dephytinization of food ingredients

Ten grams of soybean and wheat bran were suspended separately in a 30 mL of Na-acetate buffer (0.2 M, pH 5.5) and incubated under shaking conditions at 100 rpm with the purified *A. niger* SF58 phytase (10.0 U) at 60°C for 3 days. Samples were withdrawn at regular intervals for soluble protein and inorganic phosphate analysis.<sup>20</sup>

#### Statistical analysis

All experiments were done in triplicates, and the mean value was represented  $\pm$  standard deviation.

## Results and Discussion

#### Qualitative screening of phytase producing fungi

Ten fungal isolates were selected from 99 fungal isolates to be screened qualitatively for phytase production using phytase screening medium. These isolates exerted the highest capability to hydrolyze phytate as a phosphorus source in screening experiments, as an indication for phytase production. Based on the highest clear zone of phytate hydrolysis (Table 1), Isolate 62 was defined to be the best phytase producing fungus which was then used for subsequent investigations (Figure 1).

#### Identification of the most active phytase producer isolate

The fungal Isolate 62, the best tested phytase producer isolate, was identified via both microscopic and molecular aspects. Microscopic observation revealed that the fungus has septated mycelia, non-septated conidiophores, radial conidia, and black colour on the cultivation medium. These descriptions indicated that the isolate belongs to the genus of *Aspergillus*. For molecular identification, the nuclear small subunit ribosomal DNA (18S rDNA) was amplified via PCR. This region was targeted for fungal identification due to the availability of well-established universal fungal primers that were based on the conserved regions of 18S rDNA which allows possible comparison of nucleotide sequences. Furthermore, the 18S rDNA sequences are available in large numbers in the GenBank which make similarity searches more convenient. Several researchers have also reported that identification of fungi was based on the 18S rRNA gene sequence analysis.<sup>21</sup> Sequencing of the PCR product showed a 701 bp fragment which was submitted to the NCBI GenBank database. Based on sequence homology, the alignment of *Aspergillus niger* 5.8S ribosomal RNA gene sequences revealed a high identity with the published *Aspergillus niger* ITS in the GenBank library. Therefore, the sequence was submitted to the NCBI GenBank with an accession number of MT119452. A tree dendrogram of phenology and genetic correlation between different *A. niger* strains and Isolate 62 of *A. niger* SF58 obtained in this study was presented in Figure 2.

#### Purification and molecular weight determination of *Aspergillus niger* SF58 phytase

The results of phytase purification (Table 2) highlighted that a considerable purification fold (2.303) and high percentage recovery values were obtained. Figure 3 illustrates the profile of *A. niger* SF58 phytase purification using Sephadex G-100 chromatographic column. From the electrophoresis results (Figure 4), the molecular weight of phytase enzyme was about 22 kDa and it was represented by one band. This observation agrees with that obtained by Patki *et al.*,<sup>22</sup> where they found that the molecular weight of phytase enzyme

produced by bacteria isolated from mangrove soil was in the range of 16-22 kDa. Also, Jatuwong *et al.*<sup>2</sup> reported that phytases produced by fungi are considered as monomeric proteins and possess molecular weights in the range between 14 and 353 kDa. On the other hand, Zhang *et al.*<sup>23</sup> reported that fungal phytase which was extracted from fruiting bodies of the shiitake mushroom (*Lentinus edodes*), had a monomeric structure with a molecular mass of 14 kDa. The molecular weight of *A. niger* CFR 335 phytase was around 66 kDa.<sup>11,24</sup> Neira-Vielma *et al.*<sup>11</sup> found that the molecular weight of phytase produced by *A. niger* was 89 kDa as demonstrated by SDS-PAGE.

#### Effect of reaction time on the activity of the purified *A. niger* SF58 phytase

The impact of incubation time on the purified phytase activity was investigated and the results indicated that the optimum reaction time for the purified enzyme was 15 min (Figure 5). The decrease or increase of reaction time led to a decrease in the relative activity of the enzymatic reaction which was 72.23% after 35 min of incubation.

#### Effect of reaction temperature on the activity of the purified *A. niger* SF58 phytase

The effect of reaction temperature on the activity of the purified *A. niger* SF58 phytase was studied. The results were presented in Figure 6, which indicated that 60°C was the optimum temperature for phytase activity. The relative activity was decreased at lower or higher degrees of temperature. The gradual increase in activity by the increase in reaction temperature up to the optimal temperature (60°C) could be due to the increase of interaction between the enzyme and its substrate. This increase in the activity is due to improving in the kinetic energy between the reactant molecules. After the optimum temperature limit, the denaturation of the enzyme caused the activity to drop at higher reaction temperatures.<sup>25</sup> The current observation is in agreement with that previously reported by Hong *et al.*<sup>26</sup> where the purified phytase produced by *B. subtilis* CF92 showed an optimal activity at 60°C. On the other hand, Gunashree and Venkateswaran<sup>24</sup> reported that the optimum temperature of *A. niger* CFR 335 phytase was 30°C. Also, Sanni *et al.*<sup>27</sup> reported that *Aspergillus fumigatus* phytase had an optimum temperature of 40°C.

#### Thermal stability behaviour of the purified *A. niger* SF58 phytase

Thermal stability is considered as one of the important criteria for enzymes evaluation with vast biotechnological and industrial applications.<sup>28</sup> Thermal stability behaviour of the purified *A. niger* SF58 phytase was illustrated in Figure 7. The results showed that the purified phytase had good thermal stability at 50 and 60°C with 100% relative activity after 30 min. By increasing the incubation temperature, the relative activity was decreased to be 90% and 67.10% at 70 and 80°C, respectively. The obtained results are in accordance with the study of Bandyopadhyay *et al.*,<sup>29</sup> where they stated that *Amycolatopsis vancoresmycina* S-12 phytase thermal stability was at 60 °C and the stability decreases with increasing temperature. On the other hand, Hong *et al.*,<sup>26</sup> reported that *B. subtilis* CF92 purified phytase retained 40% of its initial activity after 30 min of incubation at 80°C. Additionally, Zhang *et al.*<sup>23</sup> reported a residual activity of more than 60 and 40% at 70 and 95°C, respectively.

#### Effect of pH values on the activity of the purified *A. niger* SF58 phytase

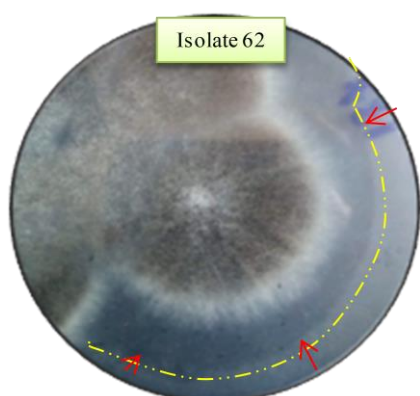
The results presented in Figure 8 showed that the optimum pH value of the purified phytase activity was recorded at pH 5.5 and the enzymatic activity was gradually decreased before or beyond this pH value to give a relative activity of 25.53% at pH 8.0. Previous studies on this aspect showed variable results with different strains, where the optima phytase activity of *Amycolatopsis vancoresmycina* S-12, *Aspergillus niger* CFR 335, and *Aspergillus fumigatus* were reported at pH values of 5.4,<sup>29</sup> pH 4.5,<sup>24</sup> and pH 6.0,<sup>27</sup> respectively.

**Table 1:** Qualitative screening of phytase producing fungal isolates

Isolate	Incubation time (days)					
	3		5		7	
	Clear zone (mm)	Mycelial growth (mm)	Clear zone (mm)	Mycelial growth (mm)	Clear zone (mm)	Mycelial growth (mm)
10	4 ± 0.35	12	6 ± 0.70	25	18 ± 0.21	30
24	8 ± 0.14	5	15 ± 0.35	20	23 ± 0.07	20
28	4 ± 0.07	7	10 ± 0.07	7	16 ± 0.14	10
30	4 ± 0.14	5	7 ± 0.14	10	15 ± 0.21	10
37	5 ± 0.21	15	8 ± 0.08	30	15 ± 0.07	30
38	4 ± 0.35	5	10 ± 0.21	10	17 ± 0.35	14
41	4 ± 0.21	9	11 ± 0.35	15	17 ± 0.07	18
58	1 ± 0.07	11	1 ± 0.21	40	5 ± 0.28	40
62	2 ± 0.07	7	15 ± 0.14	15	24 ± 0.21	23
96	5 ± 0.28	7	15 ± 0.07	15	20 ± 0.14	20

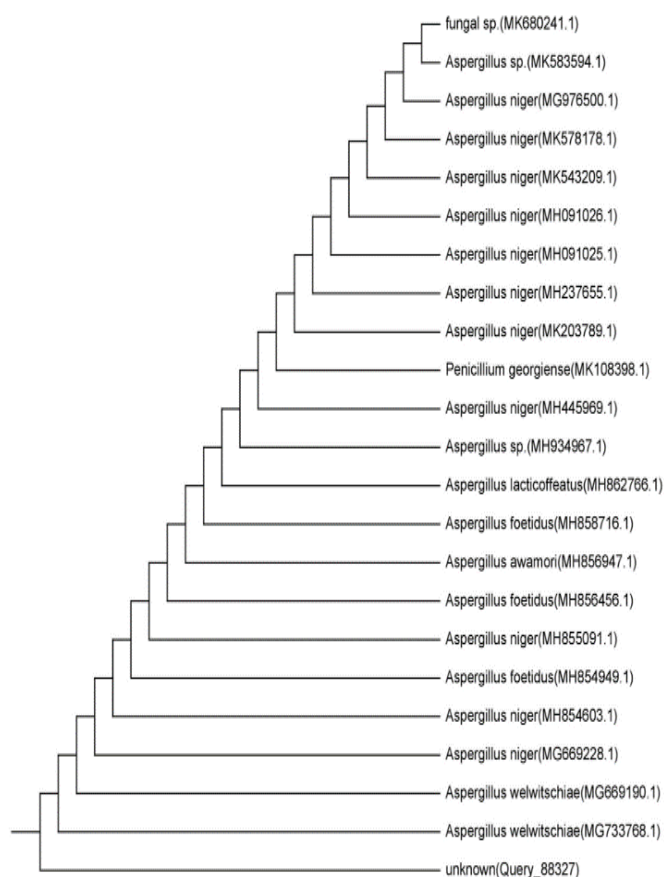
**Table 2:** Purification steps of phytase enzyme extracted from *Aspergillus niger* SF58

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	402.25	76.95	5.227	100	1.00
Dialysis bag	387.73	38.85	9.980	96.39	1.909
Sephadex G 100	292.62	24.3	12.041	72.74	2.303

**Figure 1:** A qualitative phytase screening of fungal Isolate 62 using phytase screening agar medium

#### Effect of EDTA and metal ions on the activity of the purified *A. niger* SF58 phytase

The effects of EDTA and different metallic ions on the activity of the purified *A. niger* SF58 phytase at a concentration of 5.0 mM were illustrated in Figure 9. Results obtained showed that  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$  and EDTA acted as activators for the purified phytase activity. On the other hand,  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Al}^{3+}$  had an inhibitory role on the enzyme activity. The present findings are in agreement with that illustrated by Hong *et al.*,<sup>26</sup> where they found that EDTA had an induction effect on phytase enzyme. Also, the present study is in line with what was previously described by Sanni *et al.*,<sup>27</sup> where they stated that *Aspergillus fumigatus* phytase was activated by  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$ , but inhibited by  $\text{Al}^{3+}$  and urea. On the contrary, Zhang *et al.*<sup>23</sup> found that  $\text{Al}^{3+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$  acted as inhibitors to *Lentinus edodes* phytase enzyme, while  $\text{Ca}^{2+}$  played an activator role to the enzyme at 5.0 mM concentration.

**Figure 2:** A tree dendrogram showing phenology and genetic correlation between different *Aspergillus niger* strains and Isolate 62 of *A. niger* in the present study.

*Effect of substrate specificity on the activity of the purified A. niger SF58 phytase*

Soy-bean, corn-flower, wheat bran and wheat flour were applied separately to investigate the substrate specificity of purified phytase. The effect of different substrates on the purified phytase activity was highlighted in Table 3. The specificity of the enzyme toward the different tested substrates was in a descending order of wheat bran > wheat flour > soy bean > corn flower. The previous investigations on this aspect showed various responses on the basis of specific substrate and enzyme source. Marlida *et al.*<sup>3</sup> reported that the substrate specificity of *Fusarium verticillioides* phytase was in the following order: rice bran > soybean meal > corn > coconut cake. Also, Gunashree and Venkateswaran<sup>24</sup> found that *Aspergillus niger* CFR 335 phytase has a high specificity toward sodium phytate which in agree and support our findings.

*Effect of storage time on the activity of the purified A. niger SF58 phytase*

The effect of storage time on the purified phytase activity at 4°C for 4 months (Figure 10) revealed a gradual decrease in the phytase relative activity by time to be 46.93% of its initial activity after 4 months. Bandyopadhyay *et al.*<sup>29</sup> reported that 80 % of the *Amycolatopsis vancoresmycina* S-12 initial phytase activity was retained after storing the enzyme for 6 months at 4°C.

*Dephytinization of soybean and wheat bran using the purified A. niger SF58 phytase*

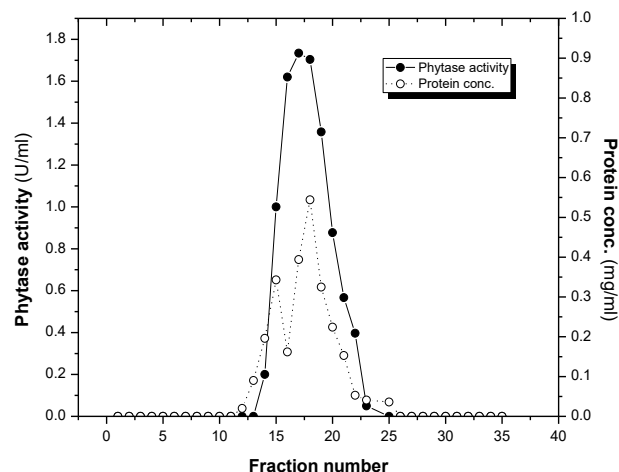
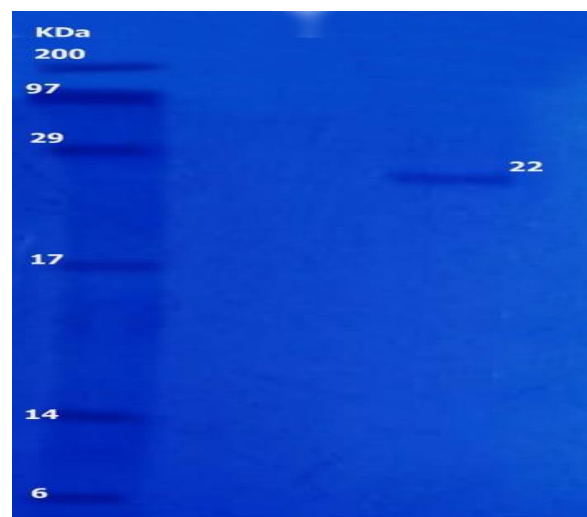
The dephytinization ability of *A. niger* SF58 phytase enzyme was investigated with soybean and wheat bran (Table 4). The phytase enzyme caused a dephytinization of both soybean and wheat bran, which accompanied by an increase in the amount of liberated inorganic phosphate. By increasing the dephytinization reaction time, an increase in the liberated inorganic phosphate and soluble proteins were noticed (Table 4). Bala *et al.*,<sup>20</sup> reported the dephytinization of wheat flour and gram flour by *H. nigrescens* phytase enzyme. Also, Sapna and Singh,<sup>30</sup> also reported that *A. oryzae* phytase caused the dephytinization of wheat bran which indicated by inorganic phosphate liberation.

**Table 3:** Substrate specificity of the purified *Aspergillus niger* SF58 phytase

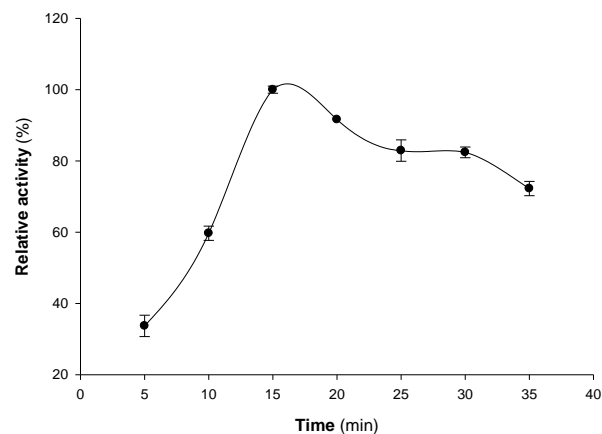
Substrate	Relative activity (%)
Sodium phytate	100.00
Wheat bran	43.75
Soybean	38.39
Wheat flour	38.61
Corn-flower	24.55

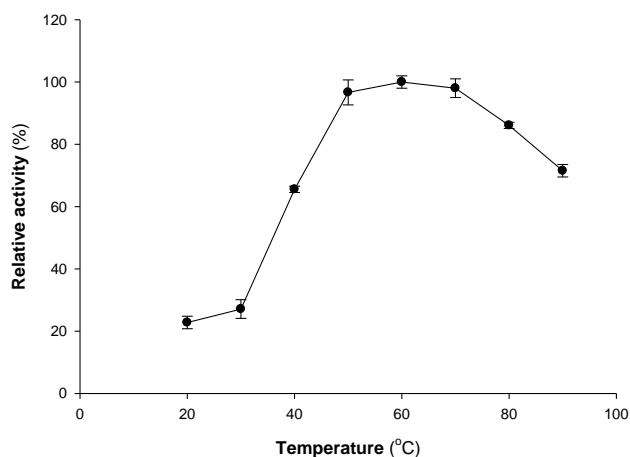
**Table 4:** Dephytinization of soybean and wheat bran by *Aspergillus niger* SF58 phytase

Cereal	Time of reaction (day)	Liberated inorganic phosphate (µg/g substrate)	Soluble protein (µg/g substrate)
Soybean	1	84.80	40.50
	2	276.8	130.5
	3	345.6	276.0
Wheat bran	1	70.40	222.0
	2	168.0	370.0
	3	289.6	400.5

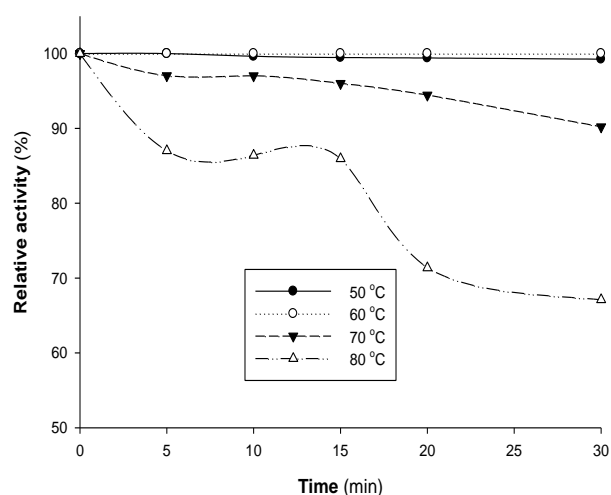
**Figure 3:** Purification of *Aspergillus niger* SF58 phytase enzyme by means of Sephadex G 100 chromatographic column.**Figure 4:** SDS-PAGE of purified *Aspergillus niger* SF58 phytase.

M: Standard molecular weight protein marker; Lane 1: purified enzyme from Sephadex G-100 column chromatography.

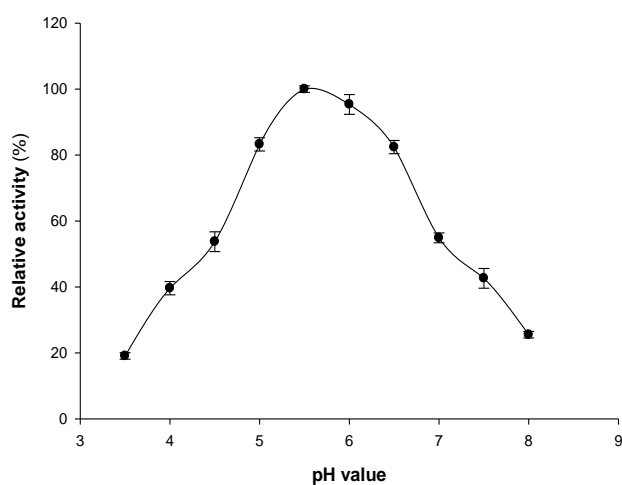
**Figure 5:** Reaction progress with time (time curve) of the purified *Aspergillus niger* SF58 phytase



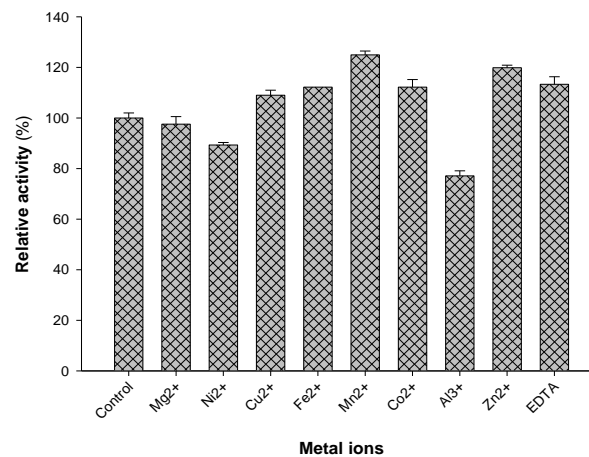
**Figure 6:** Effect of reaction temperature on the activity of purified *Aspergillus niger* SF58 purified phytase.



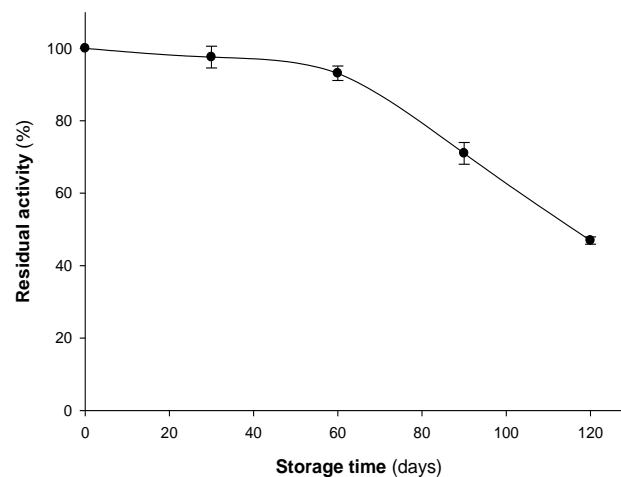
**Figure 7:** Thermal stability behavior of purified *Aspergillus niger* SF58 purified phytase



**Figure 8:** Effect of different pH values on the activity of purified *Aspergillus niger* SF58 purified phytase



**Figure 9:** Effect of different metal ions (5 mM) on the activity of purified *Aspergillus niger* SF58 purified phytase



**Figure 10:** Effect of storage time on the activity of the purified phytase produced by *Aspergillus niger* SF58

## Conclusion

Phytase from *Aspergillus niger* SF58 local isolate was purified to show one protein band corresponding to phytase activity at 22 kDa on SDS-PAGE. The optimum conditions for phytase activity were found to be 15 min of reaction at 60 °C and pH 5.5. Co<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup> metal ions and EDTA acted as activators for the purified phytase, whereas Mg<sup>2+</sup>, Ni<sup>2+</sup> and Al<sup>3+</sup> acted as enzyme inhibitors. The enzyme retained 46.93% of its initial activity after 4 months of storage at 4°C and showed high substrate specificity toward wheat bran, wheat flour, soy bean, and corn flour. In addition, the phytase enzyme was able to cause a dephytination of wheat bran and soybean, indicating the possibility of enzyme application to increase the nutritional values of feed and food additives.

## 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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