PURIFICATION AND CHARACTERIZATION OF PHYTASE PRODUCED FROM *ASPERGILLUS NIGER* USING SOLID STATE FERMENTATION

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The current research work was carried out to purify and characterize phytase. The enzyme was purified through precipitation of ammonium sulfate and gel filtration chromatography. The results revealed that, after all the purification steps, the maximum specific activity of 697 U/mg, 15.2 purification fold with 43% phytase yield was obtained. The result showed the appearance of only one band of purified phytase in SDS-PAGE, which indicates high purification of the enzyme and efficiency of the process used for purification. Phytase, after purification, showed a molecular weight of 45.7 kDa. After that, the characterization of the purified phytase was also conducted. The results exhibited that the enzyme was active optimally at pH 5 and 50 °C temperature for 10 min of the incubation period. Enzyme kinetics revealed that purified phytase exhibited V_{max} and K_m values of 1207.0 IU/mL and 0.5738 mg/mL, respectively, using Na-phytate as substrate. Phytase showed 98% and 86% thermostability at 50 °C and 60 °C, respectively, for 1 h of pre-incubation temperature treatment. The pH stability studies revealed that the enzyme retained 100% and 98% relative activity at pH 5 and 6, respectively. Ca^{2+} and Mg^{2+} showed positive effects, whereas Mn^{2+}, Na^{+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} exhibited negative effects on phytase activity.

Keywords: *Aspergillus niger*, phytase, purification, FPLC, SDS-PAGE, characterization

INTRODUCTION

Phytic acid, also known as *myo*-inositol 1,2,3,4,5,6-hexakisphosphate, is a phosphate-containing organic compound that acts as a primary reservoir of phosphorus, inositol, monovalent, and divalent cations and deposited in plants seeds. Cereal grains, legume seeds, oilseeds, nuts, and pollen are the main constituents of animal feed and contain about 60-90% of phosphorus as phytic acid.[1-3] Phytic acid is considered an antinutrient factor due to its strong binding affinity with proteins and minerals, *e.g*., zinc, iron, magnesium, and calcium.[2,4,5]

Instead of being a rich source of organic phosphorus found in animals' feed, the non-ruminant animals, like poultry and fish, cannot properly absorb and assimilate phytic acid in their bodies because the digestive tracts of these animals have very low levels or insufficient amounts of phytase, which is an essential enzyme for the breakdown of phytic acid.[1,2,6] Thus, most of the phytic acid is not digested by the non-ruminant animals and is ultimately excreted as manure, which causes environmental pollution or eutrophication.[1,7,8]

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase), EC 3.1.3.8, is a hydrolytic enzyme that speeds up the splitting of phytate and phytic acid and produces inorganic phosphate, lower *myo*-inositol phosphates, bound minerals, and free *myo*-inositol in stepwise manners.[3,5,6] In addition to animal feed additives, phytase has been used in many industries, *e.g*., the paper

industry, food and feed industry, pharmaceutical industry, preparation of *myo*-inositol phosphate, detoxified agents, elimination of environmental pollution, and soil improvement.[9-11]

The aim of the present study was the purification of phytase obtained through *Aspergillus niger* and the optimization of various assay conditions, *i.e*., temperature, pH, and incubation period for the highest enzyme activity. K_m and V_{max} values of the enzyme were also calculated after enzyme purification. Thus, purified enzyme may then be used as an animal feed additive and in many other industries.

EXPERIMENTAL

Microorganism procurement and production of phytase

Aspergillus niger was collected and used in the experiments and belongs to the collection of the Microbiology Laboratory of PCSIR (Pakistan Council of Scientific and Industrial Research) laboratories complex in Lahore, Pakistan. The fungal culture was revived on slants containing PDA (potato dextrose agar) incubated at 37 °C for 5 days, and stored in a refrigerator at 4 °C. Solid state fermentation (SSF) process was employed to produce phytase from *Aspergillus niger*, using agro-industrial waste, *i.e*., rice polish as substrate and different inorganic salts ($FeSO_4.7H_2O$, $MgSO_4.7H_2O$, KCl 0.1% each and 0.5% NH_4NO_3) were used as medium components.

Purification of phytase

During the current research work, phytase obtained from *Aspergillus niger* using SSF was purified. Then, SDS-PAGE was conducted to confirm purity and calculate the molecular weight of enzyme. Different steps involved in the purification of phytase are described below.

Ammonium sulphate precipitation

$(NH_4)_2SO_4$ salt was added at various saturation levels, *i.e.* 40-80% in crude enzyme, and stirring was done at 4 °C for 1 h, and then placed for 24 h at 4 °C to get enzyme precipitation. Centrifugation was done at a speed of 12,000 rpm for 30 min at 4 °C in a centrifuge machine. The pellets, thus obtained, were dissolved in the appropriate amount of citrate buffer 0.2 M with pH 5.5. To remove ammonium sulphate ions from precipitated phytase, dialysis was conducted against the same buffer for 24 h at 4 °C, to remove ammonium sulphate ions.

Gel filtration chromatography

Dialyzed enzyme was eluted with 0.2 M citrate buffer having pH 5.5 in a Sephadex G-200 column (2 cm × 30 cm) of the FPLC system. Buffers (2 mL) after elution were collected in a fraction collector (Model 2110, Bio-Rad). The sample's absorbance was monitored at 280 nm continuously at a flow rate of 30 mL/h. Different fractions were analyzed for phytase activity and stored at -20 °C for further studies.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

12% SDS-PAGE was conducted using the procedure of Laemmli[12] under denaturation conditions. Determination of the molecular weight of purified phytase was carried out by drawing a graph between relative mobility (R_f values) vs relative molecular weights. Standard molecular weight markers were used.

Phytase characterization

Different characteristics of purified phytase, *i.e*., the effect of incubation period, incubation temperature, pH, substrate concentration, and metal ions on the activity of phytase, were determined. The relative activities of phytase were determined using the following formula:

$$\text{Relative activity (\%)} = \frac{\text{Actual enzyme activity}}{\text{Maximum enzyme activity}} \times 100 \quad (1)$$

The highest enzyme activity was considered 100% relative activity in all experiments.

Effect of incubation period on phytase activity

Phytase was incubated with the substrate for different time intervals, *i.e*., 5-50 min, to determine the optimum incubation period for the best enzyme activity. 10 min was reported as the optimum incubation period for maximum phytase activity.

Effect of temperature on phytase activity and stability

Enzyme-substrate mixtures were incubated for 10 min at different temperatures (20-90 °C) in a water bath (Eyela, Japan), and the influence of temperature on phytase activity was studied. 50 °C was found as the optimum temperature for the highest enzyme activity.

To determine thermal stability, pre-incubation of enzyme samples was done for various incubation times, *i.e.* 1, 4, 8 and 24 h at different temperatures (30-80 °C). Afterwards, enzyme activities were measured per assay procedure for incubation of 10 min at 50 °C.

Effect of pH on phytase activity and stability

Determination of phytase activities was done at different pH values, *e.g*., 4, 5, 6, 7, 8, and 9, using various buffer solutions at optimum temperature (50 °C) for 10 min of incubation, and the optimum pH value was determined.

Phytase enzyme was pre-incubated for different durations (1, 4, 8, 24 h) at pH 4, 5, 6, 7, 8, 9 to determine its pH stability. After pre-incubation at different pH values, enzyme-substrate mixtures were

incubated at optimized assay conditions (*i.e.* 50 °C for 10 min at pH 5), and relative enzyme activities were calculated.

Effect of different metal ions

Different metal ions, *i.e.*, K^+, Na^+, Mn^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, and Mg^{2+}, were mixed in the enzyme-substrate mixtures, and their effects on enzyme activity were determined using standard assay conditions.

Kinetics of purified phytase

The kinetic parameters, *i.e.*, V_{max} (maximum velocity) and K_m (Michaelis-Menten constant) of phytase hydrolysis of Na-phytate as substrate with different concentrations (0.25-2% w/v), were studied. The Lineweaver-Burk plot can be obtained by plotting against V_{max} and K_m, which was employed to calculate V_{max} and K_m values at optimal assay conditions.

Determination of phytase activity and total protein content

A slightly modified procedure of McKie and McCleary[13] was used to determine phytase activity. 0.2 mL phytic acid 1% (w/v), having pH 5.5, and 0.2 mL enzyme sample were incubated in a test tube kept in a water bath for 15 min at 37 °C. Afterward, 0.4 mL of 15% trichloroacetic acid (TCA) was added to stop the reaction. 0.2 mL of the above mixture with 1.8 mL double distilled water was taken in a test tube, a color reagent was added, and then the mixture was incubated at 50 °C for 15 min. The blank contained 0.2 mL of 0.2 M citrate buffer, instead of enzyme extract. The absorbance of the reaction mixture was measured at 655 nm against a blank using a spectrophotometer. One unit of phytase was defined as the enzyme amount required to liberate one micromole of inorganic phosphorous using a standard assay procedure. Lowry *et al.*[14] method was followed for the estimation of the total protein.

Statistical analysis

A completely randomized design (CRD) was employed with triplicate experiments. The COSTAT (Cohart software, 2003, Monterey, California) computer package was used, and ANOVA (analysis of variance) was computed for all parameters.

RESULTS AND DISCUSSION

Purification of phytase

Precipitation of phytase by $(NH_4)_2SO_4$

For purification of the enzyme, precipitation of the enzyme is carried out by a suitable salt; it removes a large amount of water from the enzyme solution. It increases the degree of purity of the enzyme.[15] During the current study, crude phytase was concentrated by adding various quantities (40-80%) of ammonium sulfate salt. Then, the concentrated enzyme suspensions were dialyzed for 24 h at 4 °C. After dialysis, enzyme activities were determined. Results exhibited that maximal phytase activity (374 IU/g) was obtained at 60-70% of $(NH_4)_2SO_4$ saturation level (Table 1).

Dikbas *et al.*[3] used ammonium sulfate salt (0-80%) to partially purify phytase produced by *Lactobacillus brevis* NM-34 strain, and the highest enzyme activity (243.80 U/mL) and maximum protein concentration (0.989 mg/mL) were obtained at 40-60% of saturation level.[3] Crude phytase obtained from oakbug Milkcap (*Lactarius quietus*) was precipitated with 70% ammonium sulfate.[16] Phytase extracted from a local mushroom (*Pleorotus ostreatus*) was precipitated at 70% saturation level,[9] phytase produced by *Bacillus subtilis* KT004404 was precipitated with 70% ammonium sulfate[7] and *Bacillus sp*. with 60% saturation of ammonium sulfate compared to other saturation levels and the highest phytase activities were obtained.[17]

Table 1 shows that during the ammonium sulfate precipitation (saturation level 60-70%), total proteins were decreased to 1.9 mg/g in relation to the initial protein content of 12 mg/g, whereas the specific activity is increased to 197 U/mg compared to a specific activity of crude enzyme, *i.e.*, 46 U/mg. Also, the phytase enzyme was purified to almost 4.3-fold with a 68% yield after the completion of this purification step.

Gel filtration chromatography

In the next step, the enzyme was eluted on the Sephadex G-200 column using the FPLC system for further purification. It was confirmed using standard enzyme assay procedure that the best enzyme activity was found in the fractions obtained from the highest peak of elution profile (Fig. 1). This last extraction and purification step showed 43% yield, 15.2 purification fold, and 697 U/mg specific activity, 237 IU/g enzyme activity and 0.34 mg/g total proteins for purified phytase in comparison to crude enzyme (Table 1).

Among all chromatographic techniques, gel filtration chromatography is the simplest one and isolates the different proteins based on their various molecular size.[6] Studies conducted by Zhang *et al.*[18] and Hassan and Al-Jobory[9] for purification of phytase using gel filtration chromatography by Superdex-75 revealed the highest specific activity of 3.11 and 7.5 U/mg, the purification fold of 31.6 and 19.74, the enzyme yield of 27.1 mg and 42.61 mg, respectively.

Javaid *et al*.[7] conducted research work on the purification of phytase. They used Sephadex-50 column chromatography to purify phytase synthesized by *Bacillus subtilis* KT004404. The results indicated 153.07 U/mg specific activity, 7.2 purification fold, and 46.27% phytase yield.

Table 1
Stepwise purification of phytase produced by *Aspergillus niger*

Purification steps	Total activity (IU)	Total proteins (mg/g)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude phytase	548	12	46	1	100
$(NH_4)_2SO_4$ precipitation (60-70%)	438	3	146	3.2	80
Dialysis	374	1.9	197	4.3	68
Gel filtration (Sephadex G-200)	237	0.34	697	15.2	43

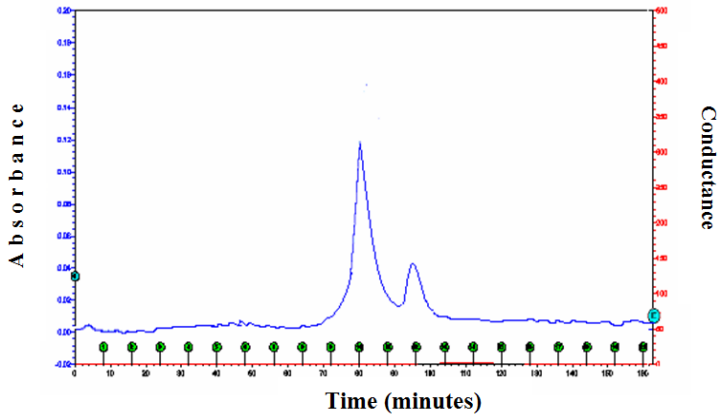

Figure 1: Elution profile of phytase

Purity of phytase by SDS-PAGE technique

In the present research, a purified sample of phytase from gel filtration chromatography was examined on 12% SDS-PAGE under denaturing conditions to confirm the purity of the phytase. Figure 2 shows a single band for purified phytase, indicating the high purity of the enzyme and also showing the success of this purification process.

Estimation of molecular weight of purified phytase

Figure 3 shows the relationship between the Log Mw of standard proteins and their Rf (relative mobility). The molecular weight of the phytase band was calculated using SDS-PAGE and found to be about 44.7 kDa, as shown in Figures 2 and 3.

Separation of enzyme bands by SDS-PAGE depends upon the molecular mass and charges on enzyme molecules. Phytase obtained from different sources showed variation in molecular weight, *e.g*., the molecular weight of phytase extracted from filamentous fungi was 49-85 kDa,[19] whereas the molecular weight of phytase obtained from some mushrooms ranged from 14-45 kDa.[18] The molecular weight of purified phytase extracted from *P. ostreatus* 11 L was determined as 25.12 kDa,[9] that of phytase from *Bacillus subtilis* KT004404 was calculated as 30 kDa,[7] and from *Bacillus subtilis* P6 was estimated as 40 kDa.[4]

Characterization of phytase

The effect of different factors on the activity of purified phytase produced by *Aspergillus niger* was determined as follows.

Effect of incubation time

During the current study, the activity of phytase was checked at different incubation periods (5-50 min). The highest enzyme activity was obtained at 10 min of incubation, and the lowest activity was obtained at 50 min of incubation (Fig. 4). Research conducted by Hassan and Al-Jobory[9] showed that phytase exhibits maximum activity when the reaction mixture is incubated for 15 min.

Effect of temperature on activity and stability of phytase

The influence of various temperatures (20-90 °C) was studied on purified phytase activity. The results showed the highest enzyme activity at 50 °C and lowest at 90 °C. Dikbas *et al.*[3] reported that 50 °C was the temperature at which phytase extracted from *L. brevis* NM-34 exhibited the best activity (115.87 U/mL). Optimum phytase activity was shown by enzyme obtained from *Aspergillus niger* NCIM at 55 °C,[20] from *R. microspores* at 55 °C,[21] from *B. licheniformis* PFBL-03 at 55 °C,[22] from *Bacillus subtilis* at 40 °C[7] and *Bacillus subtilis* p6 at 40 °C.[4]

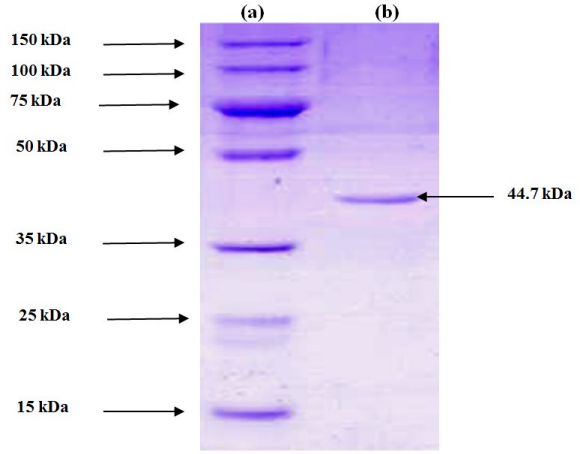

Figure 2: SDS-PAGE, (a) Molecular mass markers, (b) Purified phytase

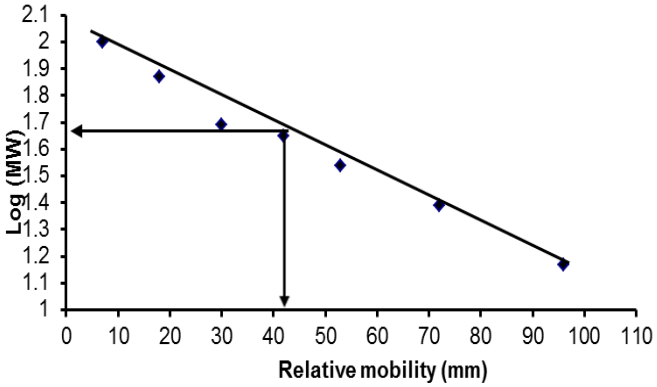

Figure 3: Molecular weight (Mw) determination

The thermostability of phytase was investigated by pre-incubating purified enzyme at different temperatures (30 °C-80 °C) for 1, 4, 8, and 24 h. The enzyme showed maximum stability at low temperatures, with 98% relative activity at 50 °C for 1 h, which decreased to 86% after 24 h, as presented in Figure 6.

Effect of pH on activity and stability of phytase

Phytase activity was determined at different pH values ranging from 4 to 9, incubated for 10 min at 50 °C. It was observed that the enzyme showed maximum activity at pH 5, whereas phytase showed lower activities by increasing or decreasing pH from the optimum value (Fig. 7). Dikbas *et al.*[3] performed experiments at different pH values (2-11) for phytase produced from *L. brevis* NM-34. The results indicated that the highest phytase activity (67.1 U/mL) was obtained at pH 5. Hassan and Al-Jobory[9] reported maximum phytase activity at pH 6. Phytase also showed the highest activity at pH 5, 4.5, 5, and 6 when phytase was extracted from *Aspergillus niger* NCIM 563,[23] *Aspergillus niger* CFR 335,[24] *Bacillus sp.*[17] and *Bacillus subtilis* p6[4], respectively. During the present study, the pH stability of phytase was determined at different pH values (4-9) for various durations (1, 4, 8, 24 h).

The enzyme maintained its relative activity of 100% at pH 5 for 1 h and decreased to 98% after a pre-incubation period of 24 h, as indicated by the results (Fig. 8). The phytase was fully stable at 20 °C-30 °C at pH 5 and 6, for 1 h of the incubation period, as reported by Hassan and Al-Jobory.[9] Another study performed by Alves *et al.*[25] reported that phytase showed maximum stability at pH 5 for 1 h of incubation period at 25 °C.

Effect of different metal ions

Experiments relating to the effect of different metal ions on phytase activity revealed that Ca^{2+}, Mg^{2+}, and K^{+} ions had stimulatory effects, whereas Na^{+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+} showed inhibitory effects (Fig. 9). Hassan and Al-Jobory[9] showed that $MgSO_4$ had a positive effect, whereas $CuSO_4$ and $ZnCl_2$ had a negative impact on phytase activity. In a different work conducted by Sardar *et al.*,[17] the results showed that Ca^{2+} and Mg^{2+} ions remarkably enhanced phytase activity, showing a stimulatory effect, whereas Zn^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+} and Fe^{2+} ions indicated an inhibitory effect on enzyme activity.

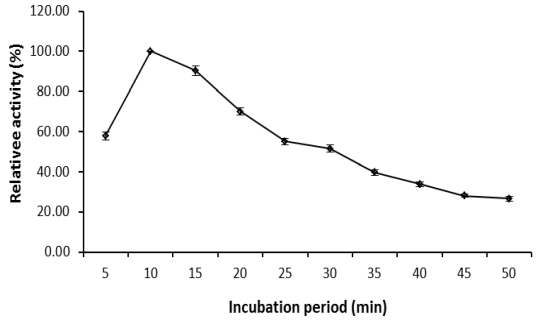

Figure 4: Effect of different incubation periods on phytase activity (bars represent ± S.E.)

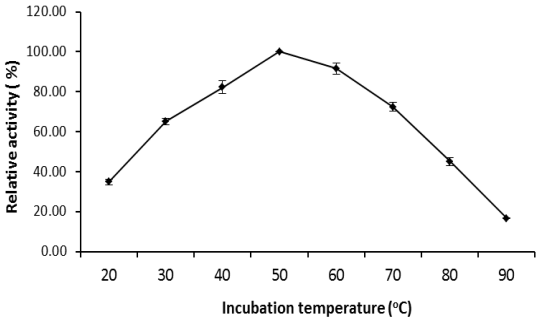

Figure 5: Interaction between various temperatures and phytase activity (bars represent ± S.E.)

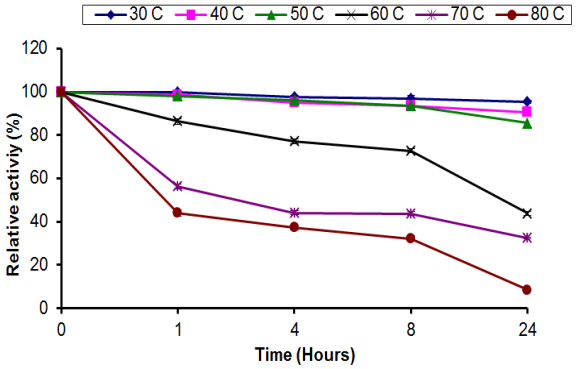

Figure 6: Thermal stability of phytase produced (bars represent ± S.E.)

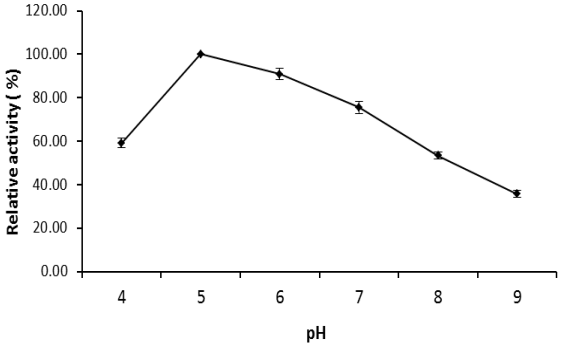

Figure 7: Impact of different pH on phytase activity (bars represent ± S.E.)

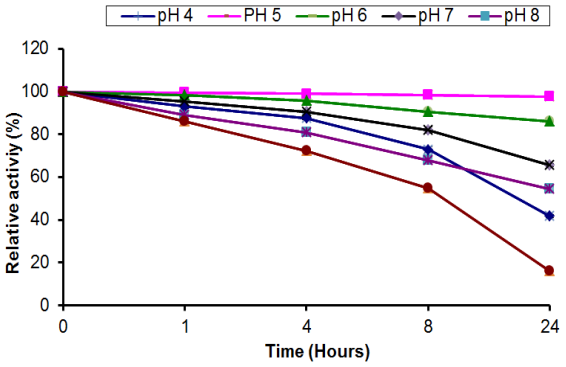

Figure 8: Effect of various pH on stability of phytase (bars represent ± S.E.)

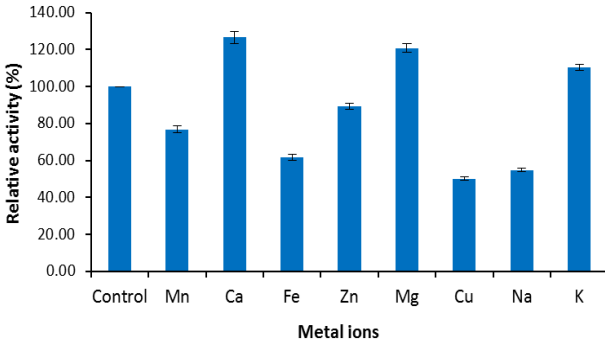

Figure 9: Impact of different metal ions on phytase activity (bars represent ± S.E.)

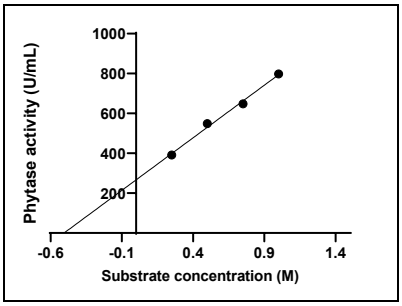

Figure 10: Lineweaver-Burk plot for calculation of V_{max} and K_m values for purified phytase

Determination of kinetic parameters

During the current studies, optimum reaction velocity (V_{max}) and Michaelis-Menten constant (K_m) of phytase on Na-phytate were estimated using the graphic method of the Lineweaver-Burk plot. It was indicated by the results that purified phytase had a V_{max} value of 1207.0 IU/mL and K_m value of 0.5738 mg/mL (Fig. 10). Dikbas *et al*.[3] revealed that phytase obtained from *L. brevis* NM-34 expressed 0.0146 mM of K_m value and 1.6 µmol/min of V_{max} value, when tested against Na-phytate salt. Georgiev *et al*.[26] reported that V_{max} was 19.9 µm/mL and K_m was 0.21 mM for phytase enzyme produced from *Candida melibiosica* 2491. Phytase produced by *Bacillus subtilis* KT004404 exhibited 250 IU/mL V_{max} and 0.175 mM Km value.[7]

CONCLUSION

Phytase has several commercial applications in different industries, but the use of phytase as a poultry feed additive is one of the most important applications to improve the nutritive value of plant-based feed and the growth performance of broiler chicken. During the current research project, phytase extracted by *Aspergillus niger* using solid-state fermentation was successfully purified and characterized. The purified phytase was recovered, with a final yield of 43% with 15.2-fold purification and 697 U/mg specific activity using different purification techniques. Phytase showed the best activity at 50 °C, pH 5, using a 1% (w/v) concentration of phytic acid as substrate and an incubation period of 10 min. Due to the maximum stability of phytase in a slightly acidic condition of pH 5-6 at 50-60 °C, it appears to have a potential application in the poultry feed industry for the improvement of the nutritional value of poultry feed and the growth performance of broilers.

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