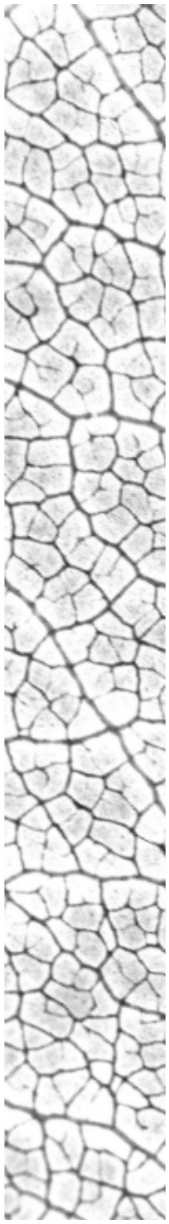

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Biochemical characterization of phytase purified from *Aspergillus niger* S2

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Abstract

Background: Phytic acid is the large reservoir of phosphorous of plant origin feed of monogastric animals. These animals are incapable of metabolizing phytic acid due to lack of phytase enzyme production. Phytases are a group of enzymes which are involved in hydrolyzing phytic acid and releasing inorganic phosphate Microorganisms are the main sources of phytases especially purified from filamentous fungi *Aspergillus niger*S2.

Materials and Methods: In the present study, a three step purification study of phytase from *Aspergillus niger* S2 was done by using Sephadex G-50. The active fractions pooled after chromatography step was used for molecular mass determination through sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDSPAGE). Phytase activity was determined at different pH between 3.0 and 8.0. The effect of temperature on enzyme activity was studied of temperature range of 30-60 ºC. The action of the enzyme on several phosphorylated substrates was tested at concentrations of 3mM. The phytase enzyme function was tested on digestion of plant material.

Results: The molecular mass of *A. niger* phytase S2was also found to be 50kDa. The enzyme showed optimal pH at 5.0. The enzyme showed maximum activity at 40^0C. The enzyme showed high substrate affinity towards sodium phytate. The purified enzyme activity on digestion of grass and hay showed positive results with increased protein content, reducing sugar and phosphate.

Conclusion: The purified enzyme of the present study with these specifications can able to play a vital role in feed industry as a feed supplement in order to metabolize phytic acid and hence reduce soil pollution and eutrophication.

Keywords: *Aspergillus niger* S2, phytase enzyme, purification and Characterization, SDS PAGE, sodium phytate

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INTRODUCTION

Phosphorous is one of the key elements necessary for the growth and development of all living organisms. Phytic acid is the principal storage form of phosphorus and inositol. It represents ~60-80% of the total phosphorus content in cereals, legumes, and oilseeds (Selle et al. 2000). It has high collaborative ability to chelate with metal ions such Ca, Mg, Fe, Zn and Mn because of having rich source of phosphate groups in its structure. Seeds and grains are the main ingredients of the feed supplements of non-ruminant mono gastric animals. Majority of these animals lack enzymes such as phytases to degrade phytate. The undigested phytate enters into the environment via manure results in elevated levels of phosphorous and become a pollutant to the environment. Conversion of phytic acid into an assimilatable form of phosphorus has been an object of biotechnological interest for human as well as animal nutrition.

Phytases are the class of enzymes which are mainly involved in sequential hydrolysis of phytic acid. These phytases play a vital role in the animal feed industry as a feed supplement and also in preparation of myo inositol phosphate, in the pulp, paper industry and in agriculture (Soni 2009). Although phytase is reported in various groups of microbes, plants and animals, microbial sources are promising among them. Among microorganisms the better sources in terms of commercial production of phytase is especially purified from filamentous fungi *Aspergillus niger.* Higher thermal and pH stability makes fungal phytase a suitable choice as feed additive (Bhavsar et al. 2012, Gaind et al. 2015). The phytases of fungal origin are high molecular weight proteins ranging from 35 to 500 KDa. They are optimally active with in pH and temperature ranges between 4.5 to 6.0, and 45 to 70 °C respectively. The main theme of the present study focuses on the purification and characterization of phytase produced by *Aspergillus*

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niger S2 isolated from agricultural fields with special interest in its application to enhance phosphorous levels and also as an enzyme source in animal feed at industry level.

MATERIALS AND METHODS

The strain *Aspergillus niger* S2 was obtained by routine microbiological isolation procedures from soil sample collected from rice fields. The strain was grown on PDA (Potato Dextrose Agar) medium slants and maintained at 4^0C on same medium until further use.

Enzyme Purification

Isolated strains were cultured in phytase production media containing sodium phytate as substrate and kept in a shaker incubator at 200 rpm at 37°C for 3-5 days. After incubation, the fermented broth was subjected to centrifugation at 10000 rpm for 10 minutes at 4°C. The cell free supernatant was collected and used as crude enzyme for purification. All purification steps were carried out at 4 °C unless otherwise stated. The supernatant obtained after centrifugation was fractionated by step- wise precipitation with Ammonium sulphate at different concentrations up to saturation. The protein pellet from 80% $(NH4)_2SO_4$ saturation was suspended in 50 mM acetate buffer, pH 5.0 and desalted by dialysis. Dialysis was carried out in a cellulose dialysis membrane until complete removal of $(NH4)_2SO_4$. Removal of $(NH4)_2SO_4$ was determined by Nessler's reagent.The desalted enzyme preparation was subjected to gel filtration chromatography on SEPHADEX – G-50 (20-80 µm) pre-equilibrated with 50 mM acetate buffer, at pH 5.0 and eluted with the same buffer at a flow rate of 12 ml h-1. Fractions of 1.5 ml were collected and analyzed for protein and phytase activity. The active fractions eluted as a single peak were pooled stored at 4°C and used further for molecular mass determination.

Assay of Phytase

Phytase enzyme assay was performed according to Heinonen and Lahti (1981) method. Samples were diluted accordingly before the analysis. 0.5 ml diluted samples and 25 mM sodium phytate in 0.2 M phosphate buffer (pH7.0) were incubated separately at 55°C for 10 min. Then, 0.5 ml of the substrate was added to the sample and the mixture was incubated for another 10 min. Thereafter, 2 ml of 10 mM Ammonium Molybdate: 5N Sulphuric acid: acetone (1:1:2) was added. The reaction was allowed to proceed for 30s. The reaction was halted by adding 0.1 ml of 1 M citric acid. The color of the reaction of the Pi (inorganic phosphate) Mo complex was read at A380. A reference standard (Potassium Dihydrogen phosphate, 0.1-0.4 µM) was simultaneously assayed with the samples. A unit (U) of phytase activity was defined as the amount of enzyme required to release 1 µM of inorganic phosphate per minute under assay conditions.

Characterization of Purified Enzyme

Protein estimation

Quantitative estimation of protein was done by Lowry et al. (1959) method using Bovine serum albumin as standard.

Molecular mass determination

The enzyme fractions at various steps of purification were separated by SDS-PAGE using the method of Laemmli (1970). The molecular weight was determined by using high molecular weight markers (97, 66, 46, 29, 20.1, 14.3 kDa).

Effect of pH and temperature

The purified enzyme was characterized for its temperature and pH optima. The effect of temperature on purified enzyme was performed by incubating the reaction mixture at a range of 25-60^0C at an interval of 5^0C. The optimum PH of the purified enzyme was determined by using a pH range of 3-8.

Effect of various substrates

The effect of substrates on purified enzyme activity was studied by using various phosphate containing compounds substrates such as sodium phytate, Sodium dihydrogen phosphate, Adenosine monophosphate, adenosine diphosphate, P-nitro phenyl phosphate, Glucose 1 phosphate at a concentration of 3mM. The phytase enzyme activity was done as described above.

Digestion of Grass and Hay Using Enzyme Extract

The digestion of grass and hay was done by mixing 1 gm of grass or hay in a reaction mixture containing 18 ml of 0.05 M acetate buffer, pH 5.5, and 2 ml of crude enzyme and purified enzyme. The reaction was incubated at 37°C for 1 hour (Jin Young Cha et al. 2010). The amount of reducing sugar, protein and the available phosphate for animal absorption were determined.

DNS assay for reducing sugar concentration

The reducing sugar concentration was estimated by DNS assay method of Miller (1959).

Phosphorus assay

This was done following the method for phytase assay of Englen et al. (1994) with slight changes in the volume of the assay mixture.

RESULTS AND DISCUSSION

Purification Studies

The extracellular phytase produced by *Aspergillus niger* S2strain was purified by various stages of purification and the results are summarized in **Table 1**. The phytase purified after three step purification exhibited 51U/ml activity at 26.02 fold with a yield of 8.12%.

Table 1. Purification scheme for the phytase enzyme from *Aspergillus niger* S2

Step	Protein (mg)	Total unit of phytase activity(U/ml)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	84.36	196	2.33	1.00	100
Ammoniumsulphate fractionation	38.21	164	4.31	1.84	83.67
SephadexG-50column chromatography	7.54	51	7.28	8.12	26.02

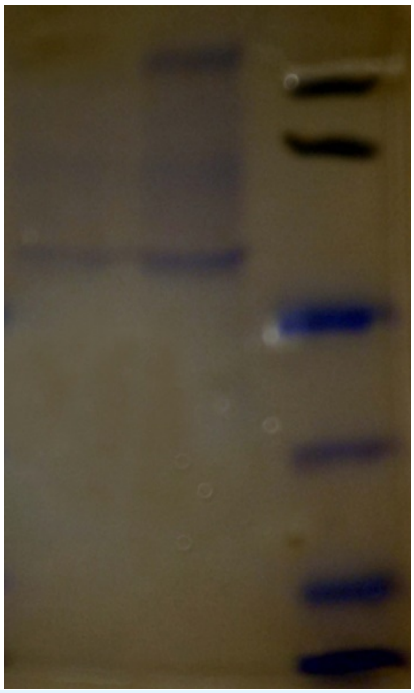

Fig. 1. SDS-PAGE: Lane:1 purified enzyme, Lane 2: Partially Purified enzyme, Lane 3:Standard protein marker (97, 66, 46, 29, 20.1, 14.3 Kda)

Molecular Weight Determination

The purified phytase showed one single band on SDS PAGE electrophoresis as shown in **Fig. 1**. The single band on SDS-PAGE suggested that enzyme was homogenous. The molecular mass of *A. niger* phytase was also found to be 54 KDa in the present study coinciding with Gunasree et al., (2015) report. Molecular mass of *Aspergillus niger* phytase within the range of 40–100 KDa, is one of the characteristics of phytases from filamentous fungi. (Wodzinski and Ullah 1996; Judith et al. 2006).

Biochemical Characterization of Purified Phytase Enzyme

Phytase enzyme from various origins has different optimal pH and temperature. In the present study the enzyme activity of purified phytase at different temperatures were shown in **Fig. 2**. Extracellular enzymes produced by mesophilic fungi generally display temperature optima below 60 °C although a few of them display higher temperature optima (Manzanares et al. 1997, Chien et al. 2002).This is also evident in the present study. The optimum temperature of the phytase enzyme was 40 ^{0}C. Several studies have investigated the optimum temperature for reaction of the phytase and phytase reported with optimal activity between 40˚C and

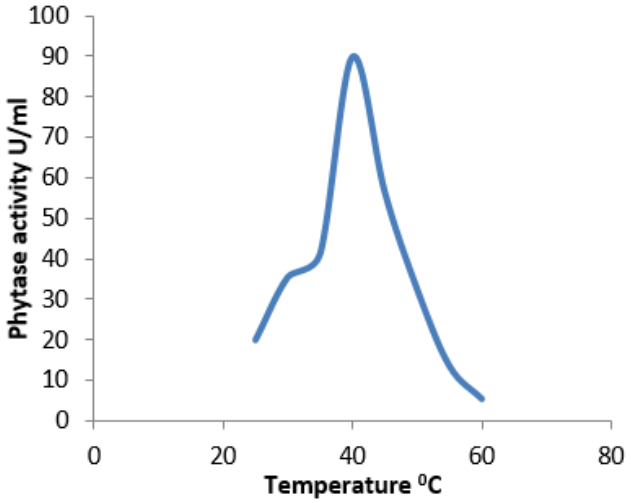

Fig. 2. Effect of Temperature on purified phytase activity from *A.niger* S2

60˚C, such as those obtained for the fungi *Aspergillus niger* phytase reported by Nagasimha et al. (1999) was found to be 50°C, recombinant *Aspergillus fumigatus* phytase displayed maximal activity at 60°C and above (Judith et al. 2006). The decrease in the enzyme activity with the increase in temperature may lead to the denaturation of enzyme by destructing the three dimensional structure of protein that causes a change in the active site which leads to inactivation of the enzyme at high temperatures (Khalaf 2012). Temperature may effect on the protein structure by breaking the bonds that stabilizes secondary and tertiary structure of protein which results in denaturation (Chesworth et al. 1998).

The purified enzyme's activity at different pH was shown at **Fig. 3**. The maximum activity was shown at pH 5.0, similar to various reports. These results are in agreement with Vohra and Satyanarayana (2003) that described the initial pH from 5.0 to 6.0 as characteristic for production of most microbial phytases. The purified phytase from *A.niger* quoted by Alberto et al. (2018) was most active at pH 5.3. Gunasree et al. (2008) reported that the enzyme purified from *Aspergillus niger* displayed maximum activity at P^H 4.5. Judith et al. (2006) also stated that the recombinant *A.fumigatus* phytase exhibited highest activity in a range of P^H 4.0 to 7.0. The enzymes activity with this pH range is one of the required criteria in animal feed digestion why because the pH range of 3.5-5.0, is the characteristic P^H of stomach after initial ingestion of feed (Boyce and Walsh 2007). Outside the range of pH and temperature the activity is reduced.

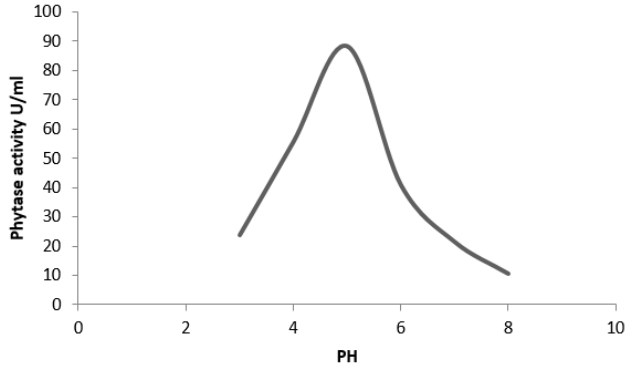

Fig. 3. Effect of pH on phytase activity purified from *A.niger* S2

Table 2. Effect of various substrates on *A.niger* purified phytase enzyme activity

Substrate	Specific activity (U/mg)
Sodium phytate	52.8
Sodium dihydrogen phosphate	29.6
Adenosine monophosphate	19.2
Adenosine diphosphate	16.4
p-nitrophhenyl phosphate	13.5
Glucose 1 phosphate	9.8

The ability of purified phytase enzyme to dephosphorylate various substrates such as sodium phyate, calcium phytate was shown in **Table 2**. The enzyme showed higher rate of dephosphorization in case of sodium phytate which was proven to be the best substrate similar to various authors quoted (Gaind et al. 2015, Monteiro 2015, Shimizu 2014, Walsh 2018). According to Monteiro et al. (2015), Casey et al. (2003), phytases from *Aspergillus* were able to hydrolyze calcium phytate, phenyl phosphate, and glucose-1-phosphate. However, Gaind et al. (2015) reported that phytases with a very small margin of substrate specificity, like *Aspergillus flavus* ITCC 6720, which displays specificity only for sodium phytate and p-nitrophenylphosphate. Substrate specificity studies showed that the purified enzyme accepts phytate as a good substrate. All other compounds tested were only marginally hydrolysed by the purified enzyme. These results are broadly similar to values reported for phytases purified from other *A. niger* strains (Casey et al. 2003, Dvorakova et al. 1997, Sariyska 2005, Skowronski 1978).

Efficiency of Enzyme Extract in Digestion of Grass and Hay

The present study was focused to check the efficiency of enzyme extract in digestion of plant material in order to derive the potential use as animal feed supplement. After digesting of fresh grass, the protein content was increased by 15 folds; i.e. from 5.7 µg/gm to 85.5.3 µg/gm; the reducing sugar increased by 6 folds; i.e. from 150 µg/gm to 900 µg/gm; and the free absorbable phosphate was increased by 19 folds; i.e. from 0.6 mg/gm to 17.1 mg/gm. In the digestion of 1 gm of dried hay the amount of absorbable reducing sugar increased markedly from 21.5 µg/gm to 193.5 µg/gm; the protein content was increased from 21.2 µg/gm to 167.4 µg/gm; and the free absorbable phosphate was increased from 1.6 mg/gm to 7.4 mg/gm. This revealed the efficacy of purified enzyme in increasing the nutrient consumption and hence to use it as an additive in animal feed industry (Jin Young Cha et al. 2010).

CONCLUSION

In this study a phytase enzyme from locally isolated *Aspergillus niger* was purified and characterized biochemically. The molecular mass of the enzyme was found to be 50kDa.The results demonstrated that the phytase showed optimal activity at acidic pH 5.0, temperature at 40^0C. The enzyme shows higher affinity towards sodium phytate and has broad substrate specificity. Additionally the enzyme showed its efficacy in digestion of grass and hay. The overall properties of phytase enzyme concluded that it is one of the best enzyme sources in feed and food industries for hydrolysis of phytates. Application of phytase in food industry seems to be a gifted approach nutritionally and economically.

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