Phytases (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) belong to the family of histidine acid phosphatases, a subclass of phosphatases, which hydrolyses phytic acid in a stepwise manner to lower inositol phosphates, *myo*-inositol and inorganic phosphate, besides increasing the phosphorus bioavailability (Mitchell *et al.,* 1997). Thus the phytases become potential candidate for the production of special isomers of different lower phosphate esters of *myo*-inositol, some of which are pharmacoactive and play important intracellular secondary messengers (Greiner and Konietzny, 1996).

Although there are reports regarding phytases from several microbial sources, but in order to have better and alternative source *viz.,* thermostable and acid stable phytase with broad substrate specificity and high specific activity, we extensively screened several microbial cultures isolated from various soil and water samples collected from diverse Indian niches with the ultimate aim to produce this enzyme to cost effective level and establish the suitability for its industrial application. Of the various isolates obtained, one fungal isolate was selected on the basis of reproducible high phytase titer at 50-55°C and 2.0-2.5 pH range. Under unoptimized conditions the isolate produced 83.33 nkat/ml phytase units in the minimal medium after 13 days of incubation at 30°C. The selected isolate was identified as ***Aspergillus niger* van Teighem** on the basis of morphological characteristics and scanning electron microscopy studies. The identification was done in association with Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and deposited under the Accession No. F0101.

To study the effect of environmental factors and media composition on the growth and phytase production by *Aspergillus niger* van Teighem, experiments were carried out in the shake flask. The optimum growth temperature of *Aspergillus niger* van Teighem was found to be 37°C while maximum phytase production was obtained at 30°C. Maximum phytase production took place when the initial pH of the medium was adjusted at pH 6.0-6.5. Though there are reports regarding the production of phytase from *Aspergillus* sp. using cornstarch and semi-synthetic media by submerged fermentation (Wodzinski and Ullah, 1996), the present strain produced a highly active phytase (184 nkat/ml) with a higher specific activity (21367 nkat/mg) in the minimal medium of initial pH 5.8-6.0, containing starch and glucose (3:1) as carbon sources along with KCl, $MgSO_4.7H_2O$,

$FeSO_4.7H_2O$ as metal supplements. Of the various organic nitrogen sources used, maximum phytase production was obtained with 0.5% biopeptone followed by ammonium nitrate where a significant increase in specific activity of phytase was obtained. Also, for all the practical purposes involving protein purification, production medium with ammonium nitrate as nitrogen source (to minimize the extraneous protein content) was used. The phosphorus content was reported to play an important role in phytase production (Wodzinski and Ullah, 1996; Gargova *et al.,* 1997; Kim *et al.,* 1999; Mandviwala and Khire, 2000) and by regulating the phosphate supplementation in the growth medium, production of enzyme can be substantially regulated. However, a sharp decline in phytase production by the present isolate was observed even at 0.002% phosphorus where 50% phytase yield resulted with absolutely no production at 0.05-0.1% phosphorus, indicating the end product inhibition in phytase biosynthesis. Also, the surfactants were found to significantly affect the culture growth and phytase production. Of the various ionic and non-ionic detergents, Tween-80 resulted in maximum secretion followed by Tween-20 and Triton-X-100. The phytase activity increased 3.9 times over control after 10^{th} day of fermentation when Tween-80 was used in the medium.

A number of process engineering problems are associated with the submerged culture of the filamentous fungi. The growth and production pattern of phytase by filamentous fungus, *Aspergillus niger* van Teighem, was studied in a 7 L laboratory scale fermenter (Chemap AG, Switzerland) at 30°C with aeration (0.5 vvm) at varying agitation rates, controlled and uncontrolled pH conditions. As the culture pH was reported to significantly affect fungal morphology and ultimately product yield, therefore, to confirm the exact inducing parameter resulting in the extracellular release of phytase, various fermenter runs were taken in starch medium of varying initial pH *viz.,* 3.0, 4.0, 5.0, 6.0 and 7.0 at 30°C. During these studies it was observed that as the growth progressed, the pH of culture broth drastically declined to 1.5-1.8 within 24-48 hour irrespective of the initial pH of medium, after which it got stabilized for the rest of fermentation run. Maximum phytase yield (120 and 141.33 nkat/ml phytase units on 6^{th} and 10^{th} day of fermentation, respectively) was obtained when the initial pH of production medium was adjusted to 6.0. As the initial pH of medium was adjusted towards acidic range, a decline in phytase productivity and cell mass yield was observed with maximum of 40 nkat/ml

phytase activity at pH 2.5-3.0. These studies suggested that the initial pH in the neutral range was pre-requisite for the culture growth and as the growth progressed, decline in pH with subsequent increase in phytase yield was observed. To further study this aspect, various fermenter runs were taken under controlled pH environment. When the pH of fermentation broth was controlled at 5.5, maximum of 16.66 nkat/ml phytase units were obtained after 7^{th} day. But an increase in phytase yield was observed when the pH of fermentation broth was maintained towards acidic range and a significant increase in phytase activity (133.33 nkat/ml) was obtained when the pH was maintained at 2.5, thus further confirmed that neutral pH was required for optimum growth while maximum phytase production was obtained by maintaining the pH in acidic range (2.0-2.5).

Various fermentation runs were taken at different carbon concentration (10-50 g/L), and a parallel increase in cell mass yield with increasing carbon content without any significant improvement in phytase yield was observed. Besides this, the agitation rate significantly affected the fungal morphology and rheological properties of fermentation broth. The viscosity of fermentation broth was found to decrease with the increase in agitation rate, showing 30-35 cp at 500 rpm as compared to 90-95 cp at 200 rpm. The volumetric oxygen transfer coefficient was determined using *static gassing out* method at different agitation rates. The aeration capacity of fermenter (K_La) was found to be 124.56 h^{-1} at 500 rpm as compared to 38.16 h^{-1} at 200 rpm. Since fungal morphology changed over a period of time during batch fermentation, OTR was also determined at different phases of culture growth using *dynamic gassing out* method where a gradual decline of K_La from 97.2 h^{-1} on 2^{nd} day to 62.28 h^{-1} on 6^{th} day of fermentation was observed. During these fermentation runs, phytase productivity increased three-fold as compared to shake flask.

To study various molecular and biochemical properties of present phytase, the enzyme was purified to near homogeneity using ion exchange and gel-filtration chromatography. The enzyme was purified 1.8 fold over the culture supernatant with 76% yield and had specific activity of 22592 units/mg. The elution profile of purified protein on calibrated Superdex-200 column indicated the molecular mass of native phytase as 353 kDa. The subunit molecular mass was found to be 66 kDa by denaturing polyacrylamide gel electrophoresis, thus suggesting the present phytase to be oligomeric. The pI of purified phytase protein was estimated by two-dimensional gel electrophoresis and was found to

be 3.8 under given experimental conditions. The N-terminal sequence of purified phytase was determined to be FYYGAALPQS and was then compared to the NCBI protein database by BLAST search. Though no significant homology was observed with *phyA* phytase, the given amino acid sequence showed strong homology with the N-terminal sequence of *Aspergillus ficuum* pH 2.5 optimum acid phosphatase (Ullah and Cummins, 1987), which was later referred to as *phyB* phytase (Ehrlich *et al.*, 1993). The purified phytase was a glycosylated protein as judged by positive PAS staining where oligosaccharides were oxidized with periodic acid and reacted with Schiff's reagent, thus resulting in the appearance of pink colored bands of the electrophoresed protein.

The biochemical characterization of purified phytase was carried out to determine various physico-chemical and kinetic parameters. The purified enzyme showed maximum phytase activity in the temperature range of 50-55°C and pH 2.0-2.5. Temperature effect on the hydrolysis of sodium phytate was also investigated and the activation and deactivation energy were found to be 384 and 1438 cal/mol, respectively. Also, the enzyme was found to be very much thermostable and retained 89% phytase activity in the absence of any stabilizing agent even after 24 hour at 55°C. The half-life of phytase at 65°C was increased from 30 minute in the absence of any stabilizing agent to 6 hour in the presence of glycine (10 mM) and to 2 hour in the presence of $CaCl_2$ (10 mM). Also, the enzyme was highly stable at pH 2.5 (4°C) for months together without any significant loss of activity even after 45 days at room temperature. This observation was supported by the fact that the secreted phytase was heavily glycosylated and thus the glyco-conjugates present in phytase prevent the protease from degrading the peptide bonds. Besides this, almost complete phytase activity was retained over pH range 2.0-7.0 even after 12 hour incubation at room temperature and no significant loss in phytase activity was observed even after 24 hour, indicating that purified protein was not getting denatured under the varying pH condition and was able to refold properly when assayed under its optimum conditions. Among the various metal ions tested, the phytase activity was severely inhibited by Al^{+3} ions and at 0.5 mM, 50% inhibition was observed, above which the activity declined very drastically. In general, all the metal ions tested above 1 mM were found to be inhibitory except Ca^{+2} and K^{+}, where 90% phytase activity was retained even at 15-20 mM.

The purified enzyme remained active in the presence of most of chelating agents and sulfhydryl inhibitors suggesting the missing participation or absence of free, accessible sulfhydryl groups in the active site of the enzyme. This interpretation was further supported by the observation that para-hydroxymercurybenzoic acid, a specific modifying agent of cysteine residue, did not affect phytase activity even up to 10 mM concentration indicating the absence of free or missing participation of cysteine residues in the active moiety. Phosphomycin, a competitive inhibitor of pH 6-optimum acid phosphatase from *A. ficuum* (Ullah and Cummins, 1987) did not affect the enzyme activity, rather it was slightly activating in its action. The purified protein retained 83 and 82 % phytase activity even after 2 hour incubation at 20 and 25 mM DEPC respectively, suggesting the missing participation of histidine residues in the catalysis of given phytase. The purified protein did not show any loss in phytase activity in the presence of various organic solvents and retained 90% activity after 2 hour incubation in the presence of 2 M sodium chloride and ammonium sulphate each separately. Of the various detergents tested, non-ionic detergents like Tween-20, Tween-80, Triton-X-100 and cationic detergents like CTAB stabilize the enzyme while anionic detergent (SDS) even at 0.1% concentration severely inhibited the enzyme resulting in the loss of 92% phytase activity. Also, chaotropic agents like guanidinium hydrochloride, urea and potassium iodide significantly affected the phytase activity. Potassium iodide was found to be comparatively more potent resulting in 60% loss of activity at 1 M with sharp decline in activity at higher concentration.

When examined for substrate specificity, the enzyme showed considerable activity with a broad range of phosphate compounds including p-nitrophenylphosphate, glucose-6-phosphate, α-napthyl phosphate and ATP besides phytic acid. Since it was observed that the phytases with broad substrate specificity inherently had low specific activities, the present phytase can be considered as special form of acid-phosphatase that possesses broader specificity with comparatively high specific activity, thus making it a potential candidate for animal nutrition.

The maximal hydrolysis rate (V_{max}) and apparent Michaelis Menten constant (K_m) of the phytase for phytic acid were found to be 0.606 mM and 1074 IU/ml. The catalytic turnover number of purified phytase was found to be 3×10^5 sec^{-1} with catalytic

efficiency of 3.69 x 10^8 M^{-1} sec^{-1}. Despite the fact that the present phytase showed similar pH optimum like *phyB*, it showed marked differences in catalytic constants from already reported *phyB* that had a K_m of 103 μM and catalytic turnover of 628 sec^{-1}. The present phytase, though displayed higher K_m of 606 μM, was much more efficient in hydrolyzing phytate with significantly high catalytic turnover and approximately 60 times increased catalytic efficiency than as reported for *phyB* (6.1 x 10^6 M^{-1} sec^{-1}). When the kinetic studies were carried out using *myo*-inositolhexasulphate (MIHS), a structural analogue of phytic acid, the enzyme was found to be competitively inhibited by MIHS with 50% inhibition of the phytase activity at 100 μM concentration. The K_m of the phytase increased from 0.625 mM to 1.898 mM in the presence of structural analog, with apparent K_i of 0.05 mM. Also, the enzyme was significantly inhibited by inorganic phosphorus in uncompetitive manner with 50% inhibition of phytase activity at 0.2 mM P_i concentration.

It was observed that the enzyme was able to release significant amount of phosphorus from different commercial livestock feed over untreated control sample and was not inactivated by various formulations and antibiotics present. The enzyme retained 90-95% phytase activity at 55°C after 72 hour of incubation in all the commercial feeds tested, thus indicating its suitability in feed application. The phosphate liberation kinetics from the commercial livestock feed using phytase was investigated at different temperatures (30, 37, 45 and 55 °C) and pH (1.5-5.5) in shake flask. The hydrolysis pattern increased with the increase in temperature and a significant release of 40 nmoles P_i/ml in phytase-treated over control sample was observed at 55°C after 48 hour. Besides this, the enzyme was maximally effective when used under acidic condition, releasing 20.66 and 25.33 nmoles P_i/ml at pH 1.5-2.5, respectively. As the pH shifted towards 5.5, significant decline in phosphorus release was observed. However, the enzyme was able to retain almost complete phytase activity in the presence of feed constituent even after 48 hour over various pH tested. Thus it can be a potential candidate in animal nutrition where the ability of present phytase to retain activity over period of time in the presence of feed constituent is desired.

During course of this study, attempts have been made to separate and identify the various dephosphorylated isomers of *myo*-inositolhexakisphosphate formed by enzymatic

degradation using high performance liquid chromatography and mass spectroscopy. Since these compounds do not absorb UV or visible light, nor can be identified using specific colorimetric reagents, the efficient analysis of inositol phosphates (InsPs) is difficult. It was observed that when various test samples (5 min-8 h, 16h-24h) were chromatographed on C_{18}-column using methanol: water (30:70) as eluant and identified using Electron Spray Ionization-Mass Spectrometric detectors in positive ion mode, significant resolution of various InsPs was achieved. The LCMS scans showed that the phosphate moieties were removed from $InsP_6$ in a sequential manner and within 10 minute of enzymatic reaction $InsP_6$ was enzymatically cleaved to various lower InsPs ($InsP_5$, $InsP_4$, $InsP_3$, $InsP_2$). As the reaction proceeded over a period of time, whole of the substrate got converted to $InsP_2$ (2Na.$InsP_2$, RT 4.4-4.54, base peak m/z 382.9) with 10-15% relative abundance of mixture of $InsP_{5\text{-}3}$ after 2 hour of hydrolytic reaction. Thus, the purified phytase from present isolate, *Aspergillus niger* van Teighem, hydrolyzes $InsP_6$ in sequential manner ($InsP_6 \rightarrow InsP_5 \rightarrow InsP_4 \rightarrow InsP_3 \rightarrow InsP_2$), with $InsP_2$ as possible end product of phytate hydrolysis with further no hydrolysis to lower inositol phosphate isomers (MS scans; 150-500 m/z). The product purity was further confirmed by thin layer chromatography on pre-derivatized silica gel-60 and RP-HPTLC silica plates. A single spot, moving below inositol and parallel to standard Ins-1,4-diphosphate, in 24 hour test sample confirmed the presence of $InsP_2$ produced by enzymatic reaction in the purified form, with further no hydrolysis to lower InsP and inositol. To further confirm the identity of $InsP_2$, $^1H_1$ and ^{31}P-NMR of various standard Ins-1, 2,3,4,5,6-P and Ins along with 24 h test sample were recorded.

To conclude, the present isolate *Aspergillus niger* van Teighem produced thermostable and acid stable phytase with broad substrate specificity and high specific activity. The enzyme was able to hydrolyze phosphate moieties from various commercial live stock feeds without any significant loss of phytase activity, thus indicating its suitability in feed applications. Besides this, purified phytase hydrolyzes $InsP_6$ in sequential manner ($InsP_6 \rightarrow InsP_5 \rightarrow InsP_4 \rightarrow InsP_3 \rightarrow InsP_2$), with $InsP_2$ as possible end product of phytate hydrolysis.