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1. Isolation, screening and identification of phytase producing organism

The phytase producing strains were isolated from various sources including soil and water samples from livestock units, poultry farms, agriculture fields, rotten wood logs, etc. The samples were serially diluted in sterile water and plated on to phytase screening agar medium (PSM) plates of different pH (4.5, 5.5 and 6.5). The plates were incubated at varying temperature (30, 50 and 60°C) and positive isolates were selected on the basis of zone of clearance produced by phytic acid degrading organisms. Microbial cultures so obtained were purified by re-streaking the individual colonies on PSM agar plates. Main criteria during the isolation were to look for an isolate, which produces acid-stable and thermostable phytase. A number of positive isolates were obtained from various soil samples on PSM-agar plate and their production pattern was checked at two different temperatures (30 and 50°C) in liquid medium by inoculating loopful of each isolate in various liquid media i.e., minimal media, phytic acid media, Luria broth and starch media of different pH (4.5, 5.5 and 6.5). The process was repeated a number of times to select the most potent phytase producer. Though a number of positive bacterial, actinomycetes and fungal isolates were obtained, one fungal isolate showed highly reproducible phytase activity at pH range 2.0-2.5 and 50-55°C temperature. Under unoptimized conditions, the isolate produced 83.33 nkat/ml phytase units after 13 days of incubation at 30°C. This activity is almost comparable with the already known phytase producing strains. The selected isolate was subjected to various tests as per the identification protocols of Raper and Fennell (1965). The isolate had several morphological characteristics for its placement in *Aspergillus niger* group and was further distinguished from several other taxa classified with *A. niger* group based on conidial morphology and mycelial growth on different growth media (Table 1 and Figure 1). The isolate was further subjected to scanning electron microscopy and compared with several authentic *Aspergillus niger* strains to confirm the novelty of strain (Figure 2). 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, India and deposited under the Accession No. F0101 for patent application.
Table 1: Identification and morphological characterization of the selected isolate as per protocols of Raper, K.A., and D.I., Fennell’s monograph on the Genus *Aspergillus* (1965)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>The isolate had the following characteristics for its placement in <em>Aspergillus niger</em> group:</td>
<td></td>
</tr>
<tr>
<td>a. Sterigmata, the conidia-bearing structures</td>
<td>Biseriate or rarely uniseriate,</td>
</tr>
<tr>
<td>b. Conidial heads</td>
<td>Black with smooth colorless to pigmented conidiophores.</td>
</tr>
<tr>
<td>The fungus was further distinguished from several other taxa placed with <em>Aspergillus niger</em> group based on following features:</td>
<td></td>
</tr>
<tr>
<td>a. Growth on Czapex dox agar</td>
<td>Rapid growth at room temperature (20-25°C), up to 8 cm diameter in a week,</td>
</tr>
<tr>
<td></td>
<td>white mycelia almost submerged;</td>
</tr>
</tbody>
</table>
### Results & Discussion

<table>
<thead>
<tr>
<th>i) Conidial mass</th>
<th>Black, highly crowded, reverse uncolored to slightly yellow, exudates rare and if present as clear, small droplets;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ii) Conidial heads</td>
<td>Brownish-black, globose, usually splitting into several well defined columns, 500 μm-1 mm in diameter;</td>
</tr>
<tr>
<td>iii) Conidiophores</td>
<td>Pigmented in brown shades, mostly 1-1.5 mm long, vesicles globose, 30-60 μm in diameter, fertile over the entire surface, sterigmata in two series,</td>
</tr>
<tr>
<td>iv) Conidia</td>
<td>Globose and irregularly roughened when young, becoming horizontally flattened and longitudinally steriate at maturity, 3.5-4 μm in diameter</td>
</tr>
<tr>
<td>b. Growth on Malt Extract Agar</td>
<td>Colonies grow rapidly, reaching 6-9 cm diameter in a week, with thin hyaline basal mycelium, carbon black; reverse faint yellow, with black conidial heads visible through the mycelium. Conidial heads uniformly split into divergent columns.</td>
</tr>
</tbody>
</table>

The isolated strain was identified as *Aspergillus niger* van Teighem and deposited under the Accession No. F0101 with the MTCC, IMTECH, Chandigarh, India.
Figure 1: Plate morphology of sporulating *Aspergillus niger* strain

The fungal isolate was grown on potato dextrose agar (pH 5.5-5.8) at 30°C and allowed to sporulate for 3-4 days.
Figure 2: Scanning electron micrographs of phytate degrading fungal isolate identified as *Aspergillus niger* van Teighem

The representative areas were photographed under varying magnification: (a) 5.91k X; (b) 4.37k X; (c) 4.94k X; (d) 5.38k X.
Results & Discussion

2. Growth and phytase production by *Aspergillus niger* van Teighem in shake flask

The growth and phytase production by *A. niger* was studied under the similar condition used for the isolation experiments in minimal and starch medium (pH 6.5) at 30°C, 200 rpm. During the course of phytase production over a period of time, the fungal isolate produced 184 nkat/ml phytase units at 55°C and pH 2.5, on 17th day of incubation with the parallel increase in cell mass and extracellular protein (Figure 3). As the growth progressed, sharp decline in pH to 1.8-1.6 was observed after 2 days of incubation (data not shown) after which the pH of the medium got stabilized around 1.4-1.5 throughout the course of phytase production. It was observed that decline in pH significantly correlates with parallel increase in phytase units. After the completion of 17 days, there is a decline in phytase production despite culture growth and sugar utilization. The total sugar content of fermentation broth showed sharp decline from 0.72% on 17th day to 0.35% on 25th day of culture growth. Also, the extracellular protein content increased in parallel with cell mass, reaching maximum to 10.5 µg/ml on 23rd day of culture growth. 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 with a higher specific activity (21367 nkat/mg) in the minimal medium containing starch and glucose (3:1) as carbon sources. A low level of phytase appeared in the early stages of fermentation (13.33 nkat/ml on 7th day) but significant increase in phytase activity was observed after 13th day of incubation with maximum phytase secretion on 17th day of fermentation. When compared with the already reported organisms having phytase activity, it was seen that the present isolate was at par with the recombinant phytase producing strains, possessing very high specific activity (Wyss *et al.*, 1999b).

3. Optimization studies for the growth and phytase production by *Aspergillus niger* van Teighem in shake flask

3.1 Effect of environmental factors

The effect of physico-chemical parameters on the growth and phytase yield, by *Aspergillus niger* van Teighem was studied in shake flask. When the production was carried out at different temperatures viz., 25, 30, 37, 45, 50°C by inoculating $5 \times 10^7$
Figure 3: Growth and production pattern of phytase by *Aspergillus niger* van Teighem

The fungal growth and phytase production were studied in starch medium (pH 5.5) at 30°C on an orbital shaker (200 rpm). Samples were withdrawn at regular time intervals and processed for the estimation of cell mass, protein content, total sugar and phytase activity according to the standard assay protocols.
Results & Discussion

spores/ml in the production medium on a rotary shaker (200 rpm), the organism was found to grow maximally at 37°C above which there is a sharp decline with no growth at 45°C and above (Figure 4). However, maximum phytase production was obtained when the organism was incubated at 30°C. The phytase and extracellular protein production declined very sharply with the increase in growth temperature above 37°C. Similarly, when phytase production was checked at different pH viz., 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, maximum phytase yield was obtained when the initial pH of the medium was adjusted to pH 6.5 (Figure 5). The phytase activity, extra-cellular protein content and cell mass concentration increased as the initial pH of medium moved from acidic towards neutral range but above pH 6.5, significant decline in phytase production was observed. As the growth progressed, sharp decline in pH of the medium was observed after the completion of 48 hours, lowering down to pH 1.8-1.5, irrespective of initial pH of the medium. It was observed that in all the cases maximum phytase yield was obtained when the final pH of the fermentation broth became very acidic. These studies suggested a strong correlation between the lowering of pH of the fermentation broth and phytase production, indicating the acidic conditions might be an inducing parameter for phytase productivity, however, initial pH around 5.5-6.5 is pre-requisite for culture growth.

3.2 Carbon and nitrogen source

Various carbon sources like glucose, lactose, fructose, maltose, galactose, sucrose, glycerol, starch, sorbitol, mannitol were used at final concentration of 4%, besides varying combinations of glucose: starch (3:1, 2:2, 1:3) in the production media which essentially consist of starch medium without any carbon. The growth pattern, phytase activity and extracellular protein production with various carbon sources indicated very little or no enzyme production on simple sugars as shown in Figure 6. Maximum phytase production was obtained when starch was used as carbon source followed by combination of glucose and starch. Although maximum phytase production took place with starch as carbon source, however, practically it is very difficult to design a medium of such high viscosity. Hence, for the subsequent experiments, a combination of glucose and starch (3:1) was used in the medium for the production of phytase. In the presence of simple sugars strong repression in enzyme production was observed. Though the phenomenon of
Figure 4: Effect of temperature on phytase production by *Aspergillus niger* van Teighem. The growth and phytase production were studied at varying temperature (25-50°C) in starch media (pH 5.8-6.0) in shake flask (200 rpm). Samples were withdrawn at regular time intervals and processed for the estimation of various parameters according to standard assay protocols.
Figure 5: Effect of pH on phytase production by *Aspergillus niger* van Teighem

The growth and phytase production were studied at 30°C in starch media of varying initial pH (3.5-5.5) in shake flask (200 rpm). Samples were withdrawn at regular time intervals and processed for the estimation of various parameters.
Figure 6: Effect of various carbon source on phytase production by *Aspergillus niger* van teighem


The growth and phytase production were studied at 30°C (200 rpm) in shake flask using production media with different carbon source. Samples were withdrawn at regular time interval and processed for the estimation of various parameters.
Results & Discussion

glucose repression reported to occur in the yeast *A. adeninivorans*, was overcome by replacing glucose over galactose (Sano *et al.*, 1999), but when phytase production was carried out by the present fungal isolate, absolutely no growth was observed even after 10th day of incubation in the presence of varying concentration of galactose (1-4%) and glucose (4%). Yoon *et al.*, (1996) also observed the repression of phytase synthesis by glucose and other simple sugars in *Enterobacter* sp.4.

Similarly, the effect of organic and inorganic nitrogen sources on phytase production, extracellular protein content and cell mass yield was determined at 0.5% final concentration. As indicated in Figure 7, nitrogen source and content also significantly affect enzyme synthesis. When no nitrogen source was added in the growth medium *i.e.*, culture was grown in the presence of only carbon source, growth and phytase production were greatly affected. Of the various organic nitrogen sources *viz.*, biopeptone, yeast extract, peptone, malt extract, soybean meal and tryptone, maximum phytase production was obtained with 0.5% biopeptone followed by inorganic nitrogen source *i.e.*, ammonium nitrate and ammonium sulphate. In general, organic nitrogen sources, being more complex and rich in nitrogen content, favour cell mass production more than inorganic nitrogen sources. However, for practical purposes involving protein purification, production medium with ammonium nitrate as nitrogen source was used to minimize the extraneous protein content.

3.3 Inducer and surfactants

Phosphorus content was reported to play an important role in phytase production (Howson and Davis, 1983; Ullah and Gibson, 1987; Chelius and Wodzinski, 1994; Wodzinski and Ullah, 1996; Gargova *et al.*, 1997; Kim *et al.*, 1999; Mandviwala and Khire, 2000) by regulating the phosphate supplementation in the growth medium, production of enzyme can be substantially regulated. To see the effect of phosphorous, the production of phytase was carried out at different phosphorus concentration (0.002-0.2%) in the optimized medium at 30°C, 200 rpm (Figure 8). Even at 0.002% concentration, 50% decline in phytase production was observed with absolutely no production at 0.05-0.1% and above concentration of phosphorus, indicating the end product inhibition in phytase biosynthesis. However, cell mass and protein concentration
Figure 7: Effect of various organic and inorganic nitrogen source on phytase production by *Aspergillus niger* van Teighem


The growth and phytase production were studied at 30°C in shake flask (200 rpm) using production media with different nitrogen source (0.5%, w/v). Samples were withdrawn at regular time interval and processed for the estimation of various parameters according to standard protocols.
Figure 8: Effect of phosphorus concentration on phytase production by *Aspergillus niger* van Teighem

The growth and phytase production were studied at varying concentration (0.002-0.2%, w/v) of phosphorus at 30°C in shake flask (200 rpm). Samples were withdrawn at regular time interval and processed for various parameter.
Results & Discussion

were not significantly affected by phosphate concentration in the medium. It was observed that with the increase in phosphorus concentration, the growth became dense, smooth, highly viscous consisting of fragmented mycelia reaching maximum at 0.008% phosphorus content.

Sheih et al., (1969) reported that increased phytase yield (113 nkat/ml in shake flask in 5 days) from Aspergillus ficuum was obtained, if the inorganic phosphorus content was controlled in the range of 0.0001-0.005%, optimum being 0.4 mg phosphorus /100 ml medium. Han and Gallagher (1987) also confirmed that high phosphorus concentration inhibited phytase synthesis by Aspergillus niger NRRL 3135. They noted that 1-5 mg phosphorus/100 ml was needed for maximum phytase production while 8 mg phosphorus /100 ml medium was required for maximum cell growth. Chelius and Wodzinski (1994), during the strain improvement study of Aspergillus niger NRRL 3135 by UV radiation, reported that phyA production by the mutant strain was repressed 60% by inorganic phosphate concentration of 0.006% (w/v) and above whereas phytase production in wild type strain was not influenced significantly by the addition of increasing concentration of phosphorus from 0.006-0.015% (w/v). The phenomenon of phosphate repression on phytase production was observed not only in fungi but in many yeast strains where increased level of phytase was synthesized in phosphate depleted medium (Nakamura et al., 2000).

Similarly, various ionic and non-ionic surfactants were tested for their effect on secretion of phytase into the medium (Figure 9). Surfactants like sodium dodecyl sulphate, Tween-20, Tween-80, Triton-X-100, cetyl trimethyl ammonium bromide (CTAB) were used at 0.5% final concentration in the production medium. The surfactants were found to significantly affect the culture growth and phytase productivity. Even after 4th day of incubation, no growth was observed in SDS containing medium while very homogeneous, smooth, highly fragmented growth was observed with CTAB. However, non-ionic detergents favour growth and enzyme productivity where comparatively more, very thick, homogeneous growth was observed than in control medium (without surfactant). Of the various non-ionic detergents, maximum growth was obtained with Tween-80 where highly viscous, smooth, fragmented mycelial growth was seen as compared to Tween-20, which resulted in thick, fragmented mycelia with very small
Figure 9: Effect of ionic and non-ionic surfactants on the secretion of phytase by Aspergillus niger van Teighem

The growth and phytase production were studied in the presence of surfactants (0.5%, w/v) at 30°C in the shake flask (200 rpm). Samples were withdrawn at regular time interval and processed for the estimation of various parameters by standard assay protocols.
Results & Discussion

fungal pellets, turning smooth, mycelial and homogeneous. In Triton-X-100, fungal growth appeared as very little, tiny pellets. Phytase production was significantly affected as ionic surfactants (cationic and anionic) resulted in complete inhibition of phytase activity while non-ionic surfactants resulted in increased phytase yield and cell mass growth, suggesting that these surfactants alter the cell permeability, resulting in higher release of enzyme. Of the various non-ionic surfactants, Tween-80 resulted in maximum secretion followed by Tween-20 and Triton-X-100. The phytase activity increased 3.9 times after 10th day of fermentation when Tween-80 was used in the medium and even after 14 days, the phytase units were twice than in control. Han and Gallagher (1987) tested the effect of various surfactants in phytase production by *Aspergillus ficuum* in liquid medium and reported that besides the dispersed growth of mycelium, an increase in phytase level of 1.3, 1.7 and 4.8 times resulted, when 0.5% of each Triton-X-100, Tween-80 and Na-oleate was added to the medium, respectively. Similarly, 30% increase in phytase yield was obtained upon using 0.5% Triton-X-100 in the production medium by *Aspergillus niger* NCIM 563 (Mandviwala and Khire, 2000). The effect of surfactants on the reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during solid-state fermentation was studied in detail, suggesting their possible role in enzyme secretion (Al-Asheh and Duvnjak, 1994).

4. Optimization studies for the production of phytase by *Aspergillus niger* van Teighem in laboratory scale fermenter

Despite the fact that filamentous fungi make a substantial contribution to the global economy and are often classified as GRAS (generally recognized as safe) by FDA, a number of process engineering problems are associated with the submerged culture of these organisms because of their filamentous growth (Gibbs *et al.*, 2000). The viscous nature of the fungal fermentation broth resulted in improper mixing of contents and formation of nutrient gradient within the vessel, with oxygen limitation particularly a problem with aerobic fermentation (McNeil and Harvey, 1993). Also the influence of culture pH, agitation rate and ultimately the shearing action of impellers on the morphology and productivity of filamentous fungi has received considerable attention. Besides this, in early fermentation, final product yield and substrate conversion were the
only criteria of performance but as the technology developed, greater attention was paid to time factors: *productivity*, the average rate of product formation, became more significant factor for comparison. Therefore, various aspects of phytase production, its controlling parameters and process development associated problems related to fungal fermentation, besides defined attempts to improve phytase productivity were studied in laboratory scale fermenter. The production pattern of phytase by *Aspergillus niger* van Teighem, under varying operating conditions, was studied in a 7 L laboratory scale fermenter (Chemap AG, Switzerland) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively. The inoculum was prepared by inoculating five 500 ml flasks each containing 100 ml sterile starch medium with $5 \times 10^7$ spores/ml and incubating at 30°C (200 rpm) for 72 hour. Fermenter containing starch medium (4.5 L) was in-situ sterilized and then inoculated aseptically. The fermenter was equipped with different controls such as pH, temperature, dissolved oxygen, agitation and antifoam.

### 4.1 Production studies without pH control

Culture pH is one of the important process parameters that have been reported to affect fungal morphology and ultimately product yield. The effect of initial pH on phytase production was studied at 30°C in 7 L fermenter (Chemap AG, Switzerland) with a working volume of 5 L. Earlier studies on the phytase production by *Aspergillus niger* van Teighem in shake flask showed that the pH of medium played an important role in the phytase productivity (Vats and Banerjee, 2002). In order to confirm the exact inducing parameters 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 with aeration and agitation of 0.5 vvm and 400 rpm, respectively. The pH of medium was adjusted initially to the required range after which it was only monitored and not controlled. Samples were withdrawn aseptically after every 24 hour and the viscosity of culture broth was estimated as described in section 2.4.4. The samples were centrifuged for the estimation of phytase activity, extracellular protein and residual substrate using standard assay protocols (section 2.4). The cell mass yield was estimated as dry weight according to the standard estimation protocol (section 2.4.3).
Results & Discussion

During these studies it was observed (Figure 10) 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 with 120 and 141.33 nkat/ml phytase units on 6th and 10th day of fermentation were 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. When the initial pH of medium was adjusted to 2.5-3.0, maximum of 40 nkat/ml phytase activity was obtained after 6 days of incubation. Also comparatively less cell mass yield at pH 3.0 than at 6.0 indicated that the acidic conditions significantly affect the initial build up of fungal cell mass. At pH 7.0, maximum of 96.66 nkat/ml phytase units were obtained after 10 days of incubation though cell mass yield was comparable to that at pH 6.0. 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. The total sugar content of the fermentation broth reduced to 0.3-0.4% after 10th day of culture growth, irrespective of the initial pH of medium. During these fermentation experiments, though no significant increase in phytase activity was obtained, the productivity level increased three-fold as compared to shake flask. Also shake flask studies indicated that there might be direct correlation between phytase production and pH of the medium (Vats and Banerjee, 2002). However, Howson and Davis (1983) reported that inorganic phosphorus content of medium controlled the synthesis of phytase which was further supported by various studies carried out on phytase production using different types of starch containing phosphorus (Gibson, 1987). From our shake flask studies, it was observed that it is not the phosphorus concentration (reported to be one of the controlling parameters for phytase production) but the acidic enviornment that induced the culture to produce phytase. Therefore, to further study this aspect, various fermenter runs were taken under controlled environment.

4.2 Production studies with pH control

To obtain the better understanding of the effect of pH on phytase production, various experiments in fermenter were carried out under controlled pH environment at 30°C with
Figure 10: Course of phytase production by *A. niger* van Teighem in a stirred tank reactor without pH control

Various batch fermenter runs were taken using starch medium of different initial pH (3-7) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively. The broth pH was allowed to be monitored throughout the fermentation run.
Results & Discussion

aeration and agitation of 0.5 vvm and 400 rpm, respectively. Batch fermentation runs were taken in starch medium containing glucose: starch (3:1; 40 g/L) as carbon source. The initial pH of medium was adjusted to 5.5-6.0 and after inoculation of the fermenter, the pH of medium was allowed to fall to a particular value at which it was controlled using 5N NaOH. When the pH of fermentation broth was controlled at 5.5, maximum of 16.66 nkat/ml phytase units were obtained after 7th day (Figure 11a). But an increase in phytase yield was observed when the pH of fermentation broth was maintained towards acidic range. A significant increase in phytase yield (133.33 nkat/ml) was obtained when the pH was maintained at 2.5 as compared to 109.33 nkat/ml at pH 3.5 (Figure 11b & 11c). But as observed from the production data under uncontrolled initial pH condition, if we adjust the pH of medium initially to 2.5-3.0, negligible phytase production resulted with a proportional decline in cell mass indicating that an initial pH around 5.5-6.0 was required for culture growth. During these fermentation runs, maximum cell mass and sugar utilization was obtained when the pH of fermentation broth was maintained at 3.5.

Once the culture grew and fall in pH was observed, maintaining the pH in acidic range significantly affected the phytase production. This was further confirmed by the fact that there was negligible phytase production when the pH was controlled at 5.5 even after 10 days of fermentation run but when that broth was aseptically transferred to shake flasks where pH was not getting controlled, significant increase in phytase yield with parallel decline in pH was seen, suggesting the strong correlation between decline in pH and phytase production. This further supported the observation that the inducing parameter, in the present study on phytase production, was the acidic conditions and not the phosphorus concentration as reported earlier from phytases (phyA and phyB) from Aspergillus niger NRRL 3135 (Wodzinski and Ullah, 1996).

4.3 Production studies at varying carbon concentration

The effect of substrate concentration on cell mass and phytase yield was studied under uncontrolled pH condition in a 7 L Chemap fermenter with a working volume of 5 L using a medium of varying carbon concentration (10-50 g/L) keeping fixed ratio of glucose : starch (3:1) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively. Samples were withdrawn aseptically after every 24 hour and the viscosity of
Figure 11a: Course of phytase production by *A. niger* van Teighem in a stirred tank reactor with pH control. Batch fermentation run was taken in starch medium (pH 5.8-6.0) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively. The broth pH was allowed to fall to 5.5 and then controlled using NaOH throughout the course of fermentation. Samples were withdrawn at regular time interval and processed for the estimation of various parameters.

Total 5N NaOH consumed to maintain pH at 5.5: 420 ml
Figure 11b: Course of phytase production by *A. niger* van Teighem in a stirred tank reactor with pH control. Batch fermentation run was taken in starch medium (pH 5.8-6.0) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively. The broth pH was allowed to fall to 3.5 and then controlled using NaOH throughout the course of fermentation. Samples were withdrawn aseptically and processed for the estimation of various parameters.

Total 5N NaOH consumed to maintain the pH at 3.5: 340 ml
Figure 11c: Course of phytase production by *A. niger* van Teighem in a stirred tank reactor with pH control. Batch fermentation run was taken in starch medium (pH 5.8-6.0) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm respectively. The broth pH was allowed to fall to 2.5 and then controlled using NaOH throughout the course of fermentation. Samples were withdrawn at regular time interval and processed for the estimation of various parameters.

Total 5N NaOH consumed to maintain pH at 2.5: 4.2 ml
Results & Discussion

culture broth was measured as described in section 2.4.4. The samples were centrifuged at 10,000 x g for 20 minute and processed for the estimation of phytase activity, extracellular protein, cell mass and residual substrate using standard assay protocols. It was observed during the course of these fermentation runs that there was a parallel increase in cell mass yield with the increasing carbon concentration without any significant improvement in phytase units. Initial pH of the medium was adjusted to 6.0 after which it was not controlled and only monitored to compare the pH profile (Figure 12). At 10 g/L substrate concentration, 97.33 nkat/ml phytase units with approximately 1.5 g/L cell mass was obtained after 10 days of incubation. At lower carbon concentration, culture became highly particulate with decline in broth viscosity. But as the carbon concentration in this medium increased, the culture broth became highly viscous with dense, homogenous growth. At substrate level of 50 g/L, 102.33 nkat/ml of phytase units with 11 g/L cell mass were obtained on 10th day of fermentation. During these studies, maximum phytase yield (141.33 nkat/ml) was obtained when production was carried out in starch medium with 40 g-carbon source/L.

4.4 Production studies at varying agitation

The influence of agitation rate, and hence the shearing action of impellers on the morphology and productivity of filamentous fungi understandably has received considerable attention. In order to perform a given bioconversion in a fermenter, mass transfer, gas dispersion and a certain homogenization are required which are normally achieved by agitation with an impeller. In case of fungal fermentation, agitation not only fulfills the above functions, but as a side effect also influences the fungal morphology (Cui et al., 1998). Besides this, high broth viscosity due to increase in filamentous cell concentration and the nature of substrate (starch) decrease the oxygen transfer coefficient, though an increase in agitation speed and aeration rate normally ensure adequate oxygenation (Lee et al., 1995). To study the effect of shearing on the growth and phytase production, batch fermentations were carried out at different agitation rates (200, 300, 400, 500 rpm) at 30°C with aeration of 0.5 vvm and without pH control in a starch medium (40 g/L, pH 5.5). As indicated in Figure 13, growth and phytase production by Aspergillus niger van Teighem were significantly influenced by agitation rate. Maximum
Figure 12: Effect of carbon concentration on the growth and phytase production by *A. niger* van Teighem in a stirred tank reactor

Various batch fermenter runs were taken at varying carbon concentration (10-50 g/L) keeping a fixed ratio of glucose : starch (3:1) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively without pH control. Samples were withdrawn aseptically and processed for the estimation of various parameters.
Figure 13: Effect of agitation rate on the growth and phytase production by *A. niger* van Teighem in a stirred tank reactor

Various batch fermentation runs were taken at different agitation rates (200-500 rpm) in starch medium (pH 5.8-6.0) without pH control at 30°C and 0.5 vvm aeration.
Results & Discussion

of 203.33 nkat/ml phytase units were obtained at 300 rpm as compared to 90.66 nkat/ml at 500 rpm. It was observed that the agitation rate affected both growth and morphology of fungus in submerged culture where cell mass of mycelial organism varied from filamentous to pellet type, thus, ultimately affecting broth rheology and aeration efficiency. The viscosity of culture broth generally decreased with the increase in agitation rate, due to shearing action of impellers, where short, highly fragmented hyphae were produced as compared to lower agitation rate where culture became very viscous with long, highly branched mycelia leading to oxygen transfer problem. At lower agitation rate, the fermentation broth became highly viscous with 90-95 cp at 200 rpm as compared to 30-35 cp at 500 rpm. Thus, the degree of agitation considerably influence the morphology of culture and rheological properties of fermentation broth which became increasingly pseudoplastic due to the development of long, branched mycelial hyphae. However, not much change in extracellular protein secretion, sugar utilization and pH of fermentation broth was observed over range of agitation speed.

4.5 Estimation of volumetric oxygen transfer coefficient ($K_La$)

4.5.1 Estimation of $K_La$ at varying agitation rate

As the phytase productivity and viscosity of the culture broth significantly varied with agitation rate, the volumetric oxygen transfer coefficient was determined using Static Gassing Out method at different agitation rates. This method involved purging the liquid free of oxygen by bubbling nitrogen through it and then switching on the air supply. The value of $K_La$ may be calculated from the equation as described earlier (section 2.6.5.1): 

$$\frac{dC_L}{dt} = K_La \left( C^* - C_L \right),$$

the integration of which result as:

$$\ln \left( \frac{C^* - C_L}{C^*} \right) = - K_La \cdot t$$

Thus, a graph of $\ln \left( 1 - \frac{C_L}{C^*} \right)$ vs time gave a straight line whose slope was $K_La$ (Atkinson and Mavituna, 1991).

It was seen during fermenter operation that $K_La$ and ultimately OTR were affected by the airflow rate and degree of agitation. The airflow rate had relatively small effect on $K_La$ in agitated fermenter and generally, at high flow rates (1.0-1.5 vvm), flooding results leading to the increased foaming without much increase in OTR. Banks (1977) demonstrated that agitation rate had profound effect on oxygen transfer efficiency of a
stirred tank reactor. Thus to determine the aeration efficiency of stirred tank reactor under given conditions, $K_{La}$ at different agitation rates in 7 L Chemap fermenter was determined. The aeration capacity of the 7 L fermenter was found to be maximum at 500 rpm with a $K_{La}$ of 124.56 h$^{-1}$ and significant decline in $K_{La}$ was observed with the decrease in agitation rate where $K_{La}$ of 58.75 h$^{-1}$ at 300 rpm as compared to 38.16 h$^{-1}$ at 200 rpm resulted (Table 2).

4.5.2 Estimation of $K_{La}$ at different phases of fungal growth

Since culture morphology and viscosity changed over a period of time during batch fermentation we used dynamic gassing out method to determine $K_{La}$ and oxygen transfer rate (OTR) at different phases of culture growth in 7 L Chemap fermenter at 30°C. In the Dynamic gassing out method, the air supply in the running fermenter was completely stopped and DO concentration was allowed to fall due to the respiratory action of growing culture. After a time, the air supply was switched on and the oxygen concentration rises again to its initial steady state value (Atkinson and Mavituna, 1991). The value of $K_{La}$ may be calculated from the equation as described in section 2.6.5.2:

$$\frac{dC_L}{dt} = K_{La} (C^* - C_L) - xQO_2$$

Rearranging the equation gives

$$C_L = \frac{-1}{K_{La}} [\frac{dC_L}{dt} + xQO_2] + C^*$$

Thus a graph of $C_L$ vs $[\frac{dC_L}{dt} + xQO_2]$ gave a straight line, whose slope was $-1/K_{La}$ and $C^*$ as intercept.

As the growth progressed and developed mycelial form, the apparent viscosity of broth gradually increased to 20-25 cp after 48 hour leading to pseudoplastic nature of fermentation broth. As the fungal cell mass varied from highly fragmented branched mycelia to unbranched long filaments with initial pellet formation, the oxygen transfer efficiency was significantly affected. When the volumetric oxygen transfer coefficient was estimated after the initial build up of significant cell mass, $K_{La}$ was found to be 97.2 h$^{-1}$ on 2nd day while under similar condition, $K_{La}$ of 121.03 h$^{-1}$ was obtained by Static gassing out method. Further decline in $K_{La}$ to 63.36 h$^{-1}$ was seen on 6th day of fermentation due to the increase in fungal cell mass after which no significant change was observed for the rest of fermentation run (Figure 14).
Table 2: Estimation of volumetric oxygen transfer coefficient ($K_{La}$) by Static Gassing Out method at varying agitation rate

Aeration rate : 0.5 vvm  
Temperature : 30°C

<table>
<thead>
<tr>
<th>Agitation (rpm)</th>
<th>$K_{La}$ (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>38.16</td>
</tr>
<tr>
<td>300</td>
<td>58.75</td>
</tr>
<tr>
<td>400</td>
<td>121.03</td>
</tr>
<tr>
<td>500</td>
<td>124.56</td>
</tr>
</tbody>
</table>
Figure 14: Profile of $K_L a$ at different phases of *A. niger* van Teighem growth in a stirred tank reactor.

The volumetric oxygen transfer coefficient was estimated by *dynamic gassing out* method in a 7L stirred tank reactor using starch medium (pH 5.8-6.0) at 30°C with aeration and agitation of 0.5 vvm and 400 rpm, respectively.
5. Purification of phytase

The phytase was produced in minimal medium comprising starch and glucose (1 and 3%, respectively) as carbon source with 0.5% ammonium nitrate as nitrogen source at 30°C. The 14-day culture broth was then subjected to centrifugation at 10,000 x g for 20 minute and the supernatant was concentrated using membrane ultrafiltration. It was found that almost complete activity was retained in the retentate with negligible activity in permeate when the culture supernatant was subjected to ultrafiltration cut-off with 30 kDa membrane. The enzyme was then purified using ion exchange and gel filtration column chromatography. The concentrated protein fraction was loaded onto Mono-Q HR5/5 and most of inactive material was removed without much loss in phytase activity. Enzyme was eluted at 0.35-0.40 M NaCl (Figure 15). The active fractions were pooled and concentrated using amicon UFMC (10 kDa) and subjected to gel permeation chromatography using Superdex-200, pre-equilibrated with 0.1 M Tris-HCl (pH 6.5) buffer and 150 mM NaCl at a flow rate of 6 ml/hour, where phytase was eluted as a single sharp activity peak (Figure 16). All the active fractions were pooled, desalted and then concentrated using 10 kDa UFMC after which it was analyzed using polyacrylamide gel electrophoresis. The purified enzyme was then characterized to determine its molecular properties. A summary of purification procedure is given in Table 3. The enzyme was purified 1.8 fold over the culture supernatant with 76% yield. The purified enzyme had specific activity of 22592 units/mg and was found to be very stable at room temperature for many months.

Phytases have been purified from several bacterial, yeast and fungal strains using ion exchange and molecular sieving chromatography. It was observed that *Aspergillus niger* NRRL 3135 phytases viz., *phyA*, *phyB* and pH 6.0 optimum acid phosphatase were secreted in relatively higher amounts under phosphate starvation condition in starch medium and only 5-25 fold purification was required to achieve near homogeneity using ion-exchange chromatography and chromatofocussing. The purification profiles of these proteins indicated that approximately 40% of the total secreted proteins are *phyA*, *phyB* and the pH 6-optimum acid phosphatase (Ullah and Gibson, 1987; Ullah and Cummins, 1987b; Ullah and Cummins, 1988). Nagashima *et al.*, (1999) purified phytase from *Aspergillus niger* SK-57 to homogeneity in four steps by using ion-exchange
Figure 15: Purification profile of phytase on Mono-Q HR5/5 column
The concentrated protein sample was loaded onto the column and eluted with 0.02M Tris.HCl, pH 6.5 in a linear salt gradient (0.02-1.5 M NaCl) at a flow rate of 30 ml/h.
The active fractions of Mono-Q purified protein were pooled, concentrated and loaded onto gel filtration column. The protein was eluted using 0.1 M Tris.HCl buffer (pH 6.5) containing 0.15 M NaCl at a flow rate of 6 ml/h.

Figure 16: Elution profile of phytase protein on the Sephacryl S-200
Table 3: Summary of the various steps involved in the purification of phytase from *Aspergillus niger* van Teighem

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (IU/ml)</th>
<th>Protein (µg/ml)</th>
<th>Specific activity (IU/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>106.66</td>
<td>8.5</td>
<td>12548</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>97.33</td>
<td>6.0</td>
<td>16222</td>
<td>91.25</td>
<td>1.29</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>81.33</td>
<td>3.6</td>
<td>22592</td>
<td>76.25</td>
<td>1.80</td>
</tr>
</tbody>
</table>
Results & Discussion

chromatography, gel-filtration chromatography and chromatofocussing. An extracellular phytase from Bacillus subtilis (natto) N-77 was purified 322-fold to homogeneity by ultrafiltration and a combination of Sephadex G-100 and DEAE-Sepharose CL-6B column chromatography. Several phytases from Klebsiella species were purified using ion exchange and gel filtration chromatography (Shah and Parekh, 1990; Tambe et al., 1994; Greiner et al., 1997).

6. Molecular characterization of purified phytase
The molecular characterization of purified phytase was carried out to determine its native and subunit molecular mass, pl, N-terminal sequence and the glycosylation status.

6.1 Estimation of relative molecular weight
6.1.1 Relative molecular weight of native phytase
The molecular mass and homogeneity of the purified native phytase was estimated by gel-filtration chromatography on calibrated Superdex-200 column using Pharmacia SMART® system (Pharmacia Biotech). The column was pre-equilibrated and eluted using 0.1 M Tris-HCl (pH 6.5) buffer with 150 mM NaCl at a flow rate of 6 ml/hour. Various standard gel filtration molecular weight markers (Sigma Chemicals Co., USA) viz., 669 kDa-thyroglobulin; 443 kDa-apoferritin; 200 kDa-β-amylase; 150 kDa-alcohol dehydrogenase; 66 kDa-bovine serum albumin; 29 kDa-carbonic anhydrase; 12.4 kDa-cytochrome c; 6.5 kDa-aprotinin were run to calibrate Superdex-200 column. The void volume (Vo) of column using Blue dextran (2000 kDa) was found to be 0.942 ml under standard operating condition. The elution profile of protein indicated the molecular mass of phytase to be 353 kDa as calculated from the standard plot of log molecular mass vs Ve/Vo (Figure 17). Also on 10% non-denaturing polyacrylamide gel (native PAGE), single protein band of purified phytase indicated the homogeneity and purity of protein. Depending upon the sources of origin (plant, fungal, yeast and bacterial sp.), phytases vary in their molecular mass (35-700 kDa) and were generally microheterogenous due to differential glycosylation (Ullah and Phillippy, 1988).

6.1.2 Relative molecular weight of subunits of phytase
For the estimation of relative molecular weight of subunits of purified phytase, 12% SDS
Figure 17: Estimation of relative molecular weight of purified native phytase

The Superdex 200 column was pre-equilibrated using 0.1M Tris-HCl (pH 6.5) buffer with 0.15 M NaCl at a flow rate of 6 ml/h. The purified protein along with various gel filtration molecular weight (kDa) markers was eluted on standardized S-200 column. The markers used were: 669-thyroglobulin; 443-apoferritin; 200- β-amylase; 150- alcohol dehydrogenase; 66- bovine serum albumin; 29-carbonic anhydrase.
Results & Discussion

-PAGE was run with the known protein standards under denaturing conditions as described earlier. Proteins were visualized by staining with Coomassie brilliant blue R 250. As shown in Figure 18, a broad, diffused band of 66 kDa indicated the monomeric form of purified protein and suggested the present phytase to be oligomeric having native mass of 353 kDa. This was similar to phytase B secreted by Aspergillus niger NRRL 3135 having molecular mass of 68 kDa and reported to be dimeric (Ullah and Phillipy, 1988; Ullah and Sethumadhavan, 1998a). Shimizu (1993) purified extracellular phytase having molecular mass of 60 kDa from Aspergillus oryzae and was reported to be dimeric. Wyss et al., (1999) compared phytases from various sources by gel permeation, SDS-PAGE, mass spectra, amino acid sequence analysis and showed that all of the fungal phytases as well as E. coli phytase are monomeric proteins though phytases of A. terreus, A. oryzae and Schwanniomyces castellii were reported to be homohexameric, heterotetrameric or dimeric proteins (Yamamoto et al., 1972; Seguielha et al., 1992; Shimizu, 1993).

6.2 Estimation of isoelectric point

The two-dimensional gel electrophoresis of purified phytase was carried out to determine the isoelectric point (pI) using standard protocol of Patrick and O’Farrel (1975). The protein bands were visualized by silver staining (Figure 19) and the isoelectric point of phytase was determined by plotting standard curve of pI vs distance of standard isoelectric focusing markers (Sigma Chemicals Co., St. Louis, MO, USA) from anode. The standard proteins (pI in parentheses) used were trypsinogen (9.3); lentil lectin basic protein (8.65); myoglobin basic band (7.2); myoglobin acidic band (6.8); human carbonic anhydrase (6.6); bovine carbonic anhydrase (5.9); β-lactoglobulin A (5.1); soybean trypsin inhibitor (4.6) and amyloglucosidase (3.6). The pI of purified phytase protein was estimated to be 3.8 under given experimental conditions (Figure 20). Generally, the extracellular phytases from various fungal strains displayed acidic pI, below 5, though phytases with neutral pI (5.5-6.5) were also reported from Enterobacter, E. coli, Klebsiella, Bacillus and Pseudomonas sp (Greiner et al., 1993; Tambe et al., 1994; Yoon et al., 1996; Richardson and Hadobas, 1997; Kerovuo et al., 1998; Kim et al., 1999; Wyss et al., 1999; Golovan et al., 2000). The phytases produced by Aspergillus niger
Figure 18: SDS-polyacrylamide gel electrophoresis of phytase
The crude culture supernatant after ultra-filtration (membrane cut off 30 kDa NMWC) and concentration, was purified using gel filtration and ion exchange chromatography. The protein purity was checked by electrophoresing the purified fraction on 12% slab gel at 20 mA and staining the protein band with Coomassie brilliant blue R 250. Lane M: molecular weight standard markers and Lane T: purified protein obtained after chromatographic steps.
Figure 19: Two-dimensional gel electrophoresis of purified phytase

The isoelectric point of purified phytase was estimated by two-dimensional gel electrophoresis. The purified protein along with standard IEF markers was electrophoresed and protein bands were visualized by silver staining. Various visualized protein bands were identified as: a: amylglucosidase (70 kDa/3.6 pI); b: β-lactoglobulin (18.4 kDa/5.1 pI); c: carbonic anhydrase II (29 kDa/5.9 pI); d: carbonic anhydrase I (29 kDa/6.6 pI); P: purified phytase protein. The pI of purified phytase was calculated from the standard curve of pI vs distance of standard IEF markers from anode.
Figure 20: Estimation of isoelectric point (pI) of purified phytase

The pI of phytase was estimated by plotting the standard curve of pI vs distance of standard IEF markers from anode. Various standard markers used were: trypsinogen (9.3), lentil lectin basic protein (8.65), myoglobin basic band (7.2), myoglobin acidic band (6.8), human carbonic anhydrase (6.6), bovine carbonic anhydrase (5.9), β-lactoglobulin A (5.1), amyloglucosidase (3.6).

Estimated pI of purified phytase : 3.8
Results & Discussion

NRRL 3135 viz., *phyA*, *phyB* and pH 6-optimum acid phosphatase were secretory glycoproteins with pI 4.5, 4, 4.9 respectively. The isoelectric point of a novel phytase (*phyC*) from *Bacillus subtilis* VTT E-68013, which showed no HAP motifs, was found to be 6.5. However, the present experiments showed several molecular similarities with reported *phyB* of *Aspergillus niger* NRRL 3135 which displayed the isoelectric point of 4 (Ullah and Cummins, 1987).

6.3 N-terminal amino acid sequencing

The purified phytase was electrophoresed on 12% SDS-PAGE as described earlier. One lane of resolved protein on gel was cut and 66 kDa protein band was excised out after which it was electro-eluted to estimate the phytase activity colorimetrically using standard assay protocol. As the electro-eluted fraction of purified protein showed significant phytase activity (data not shown), the remaining gel was soaked in “transfer buffer” for 30 minute and electro blotted on PVDF membrane. The blotted band (66 kDa) was cut out as a strip, destained completely and air-dried before using it for sequencing. The protein sequence was determined by using an automated protein sequencer and was found to be FYYGAALPQS. The amino acid sequence was then compared to the NCBI protein database by BLAST search and no significant homology was observed with *phyA* phytase (Table 4). However, 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 later referred to as *phyB* phytase (Ehrlich et al., 1993) as shown in Figure 2 in review of literature.

6.4 Periodic acid Schiff (PAS) staining

PAS staining was carried out to identify the glycosylated proteins where oligosaccharides were oxidized with periodic acid and reacted with Schiff’s reagent. The appearance of pink colored bands indicated the glycosylated protein. The purified protein was separated on 12% SDS-PAGE and stained with Schiff’s reagent to determine the glycosylation status of given phytase. The purified phytase was a glycosylated protein as judged by positive PAS staining of the electrophoresed protein (Figure 21). This is in accordance to the several phytases reported from *Aspergillus ficuum* (*phyA* and *phyB*), *S. cerevisiae* acid
Table 4: Comparison of N-terminal amino acid sequence of phytases from various sources.

<table>
<thead>
<tr>
<th>Origin</th>
<th>N-terminal sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus sp. DS11</em></td>
<td>SDPYHFTVNAAXET</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>SE(P)ELKL(E)AVV</td>
</tr>
<tr>
<td><em>Aspergillus ficuum</em> (natuphos)</td>
<td>PASRXQSSCDTV</td>
</tr>
<tr>
<td><em>Aspergillus ficuum</em> (phyB)</td>
<td>FSYGAAIPQSTQEKQF</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> CB</td>
<td>ASRXQSTXDTV</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em> 9A1</td>
<td>SDXNSVDHGTY</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em> CBS</td>
<td>TALGPXGXSDFX</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>SKSXDTVQILGY</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> van Teighem (present study)</td>
<td>FYYGAALPQS</td>
</tr>
</tbody>
</table>

The sequence of purified phytase (marked as dark red) from the present isolate showed remarked homology with *A. ficuum* phytase B (marked as dark blue). The sequence information was obtained from the following references:
(*) : Kim et al., 1998a and without any superscript from: Wyss et al., 1999a.
Figure 21: PAS staining for the identification of glycosylated nature of purified phytase

The purified protein was electrophoresed on 12% SDS-PAGE and stained using Periodic Acid Schiff's method where oligosaccharides were oxidized with periodic acid and stained with Schiff's reagent. The purified phytase was found to be glycosylated as judged by positive PAS staining where pink bands represented the oxidized oligosaccharides.
Results & Discussion

Phosphatase and *A. ficuum* pH 6-optimum acid phosphatase that were found to be heavily glycosylated (Ullah, 1988; Ullah and Cummins, 1988b; Wodzinski and Ullah, 1996). Glycosylation is one of the major naturally occurring modifications in the covalent structure of proteins. As the given purified phytase was found to be stable at high temperature and was not inactivated at room temperature even after several days, thus it can be speculated that the glyco-conjugates present in phytase prevent the protease from degrading the peptide bonds. This observation was supported by several reports where deglycosylation resulted in significant loss of phytase activity and susceptible to proteolytic degradation and denaturation at higher temperature (Ullah and Dischinger, 1993; Han and Lei, 1999).

7. Biochemical characterization of purified phytase

7.1 Temperature and pH dependence studies

The activity of native phytase was estimated over a range of temperature and pH using standard assay protocol as described in section 2.4. The optimum pH for phytase activity under standard assay conditions was found to be in the range of pH 2.0-2.5 (Figure 22), with a sharp decline in activity as the pH moves towards neutral range showing negligible activity at pH 5.0-5.5. At pH 1.5, the purified protein retained 48.61% of the maximum phytase activity (384 nkat/ml). This was similar to that reported for pH 2.5 optimum acid phosphatase, which was later referred to as phytase B (*phyB*) from *A. ficuum* NRRL 3135 showing maximum phosphohydrolase activity at pH 2.5 with negligible catalytic activity at pH 5.5 (Ullah and Cummins, 1987; Ehrlich *et al.*, 1993). The *phyB* lack phytate degrading activity at pH 5.0 while at pH 2.5, it efficiently hydrolyzes phytate with a 628 second⁻¹ turnover number (Ullah and Phillippy, 1994). However, the pH optima of most of the reported fungal phytases range from 2.5-7.0. The cloned and over-expressed *Aspergillus terreus* CBS phytase in *H. polymorpha* and *A. terreus* 9A1 in *A. niger* exhibited maximum phytase activity in the pH range of 5.0-6.0 and at pH 5.5, respectively while *A. fumigatus* had a broad pH optima between 4.0-7.3. In contrast, phytase from *E. nidulans* had a narrow pH optimum of 6.5 for maximum catalytic activity. The commercial *A. niger* phytase (Natuphos) exhibited dual pH optima of 2.5 and 5.5, having 60 and 97% of maximal activity at pH 2.5 and 5.5, respectively (Wyss *et al*.
Figure 22: Effect of pH on the catalytic activity of phytase from *Aspergillus niger* van Teighem

To ascertain the pH optima of the purified enzyme, phytase activity was measured at different pHs using 0.1M buffer (HCl-KCl: 1-2; Glycine-HCl: 2.5-3.5 and Acetate buffer: 4.5-5.5) at 55°C under standard conditions.
Results & Discussion

Generally, the phytases from bacteria and yeast were optimally active in neutral to alkaline pH while in fungi, maximum activity was displayed in 2.5-6.0 pH range and the stability of phytase decreased dramatically above 7.5 and below pH 3.0. This wide range of differences in pH optima could be due to variation in molecular conformation or stereo specificity of the protein from different sources (Shah and Parekh, 1990; Shimizu, 1992; Yoon et al., 1996; Kerovuo et al., 1998).

To determine the optimum temperature for phytate hydrolysis, phytase activity was estimated over a temperature range of 30-70°C (pH 2.5). As viewed from Figure 23a, the activity of purified enzyme increased with increasing temperature and was highest at 55°C, above which the phytase activity declined very sharply with negligible activity at 70°C. Thus, the temperature optimum for phytase activity under given condition was found to be in the range of 52-55°C and was comparable to those reported for several phytases from various *Aspergillus* sp. (Wyss et al., 1999b). In contrary, phytases from yeast sp. were found to possess comparatively higher temperature optima. Sano et al., (1999) reported secretory phytase from *Arxula adeninivorans* that was found to be optimally active at 75°C, pH 4.5-5.0 while Nakamura et al., (2000) reported phytase from several yeast strains viz., *Pichia, Candida, Kluyveromyces, Torulaspora, Schwanniomyces* sp. and were found to possess optimal temperature ranging from 60-80°C. Temperature effect on the hydrolysis of sodium phytate was also investigated and the activation and deactivation energy, as calculated from the Arrhenius plot, were found to be 384 and 1438 cal/mol, respectively (Figure 23b).

### 7.2 pH and thermal stability

In order to check thermal stability, the purified protein was pre-incubated at different temperatures with and without any additive and assayed for phytase activity using the standard protocol at 55°C (pH 2.5). Absolutely no loss of enzyme activity was observed even after 24 hour of incubation at 30, 37 and 45°C in the absence of any additive. Enzyme retained 89% activity in the absence of any stabilizing agent while 93 and 97% activity were retained in the presence of 10 mM CaCl$_2$ and 10 mM glycine, respectively after 24 hours of incubation at 55°C (Figure 24a). At 65°C, half-life of phytase reduced to 30 minute in the absence of any stabilizing agent while 25% phytase activity was retained...
Figure 23: Temperature dependence studies for the estimation of:
(a) optimum temperature; (b) activation and deactivation energy of purified phytase from Arrhenius plot.

The optimum temperature for the catalysis of phytase was estimated in the temperature range of 30-70°C, pH 2.5 using standard assay protocol.
Figure 24: Thermostability profile of purified phytase in the presence of glycine and calcium chloride at: (a) 55°C; (b) 65°C

The purified protein was incubated over a range of temperature (4-65°C, pH 2.5) in the presence of additives. Samples were withdrawn at regular time interval to estimate the residual phytase activity using standard assay protocol.
Results & Discussion

after 4 hour. The half-life of enzyme activity increased significantly by the addition of thermo-protectants like glycine and calcium chloride. The half-life of phytase at 65°C increased to 6 hour in the presence of glycine (10 mM) and to 2 hour in the presence of CaCl₂ (10 mM) suggesting glycine to be more thermoprotectant than calcium (Figure 24b). Also, the enzyme was found to be highly stable at pH 2.5 (4°C) without any significant loss of activity even after 45 day. The enzyme remained active even at room temperature for 20-25 day (data not shown). This observation was supported by the fact that secreted phytase was heavily glycosylated and thus the glyco-conjugates present in phytase prevent the protease from degrading the peptide bonds (Ullah and Dischinger, 1993). Han and Lei (1999) also observed the functional expression of phytase in Pichia pastoris and found that glycosylation was vital to the enzyme thermostability. Various compounds have been reported to enhance the stability of phytase at higher temperature. Calcium was reported to contribute towards heat tolerance of phytases from many microbes (Kim et al., 1998) while phytate enhanced the activity of Arxula adeninivorans phytase (Sano et al., 1999). Chen et al., (2001) reported the stabilizing effect of sorghum liquor waste on phytase where 90% of phytase activity was retained at 70°C. Similarly, phytase stability was tested at various pHs by incubating the enzyme with buffers at room temperature (22-25°C) and withdrawing samples at regular interval to estimate the residual phytase activity under standard assay condition. Almost complete phytase activity was retained over pH range 2-7 even after 12 hour incubation 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 (Figure 25) and was able to refold properly when assayed under its optimum condition.

7.3 Substrate specificity

The biochemical action of purified phytase on several phosphate esters was investigated at 55°C (pH 2.5). The relative rates of hydrolysis are summarized in Table 5. The enzyme was found to possess broad substrate specificity as evidenced by its capability to hydrolyze phosphate groups from a variety of phosphomonoesters. The different substrates tested were p-nitrophenylphosphate, sodium dihydrogen phosphate, glucose-6-phosphate, α-napthyl phosphate and ATP besides phytic acid. Except sodium di-
Figure 25: Stability profile of phytase at varying pH
The purified protein was incubated with 0.1 M buffers of varying pH (2-7) at room temperature and samples were withdrawn at regular time interval to estimate the residual phytase activity at 55°C. Various buffers used were: HCl-KCl (1-2), Glycine-HCl (2.5-3.5), Acetate buffer (4.5-5.5) and Tris-Cl (6.5-7.5).
Table 5: Substrate specificity of purified phytase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative phytase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phytate</td>
<td>100</td>
</tr>
<tr>
<td>p-Nitrophenylphosphate</td>
<td>115</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>6</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>109</td>
</tr>
<tr>
<td>a-Napthyl phosphate</td>
<td>105</td>
</tr>
<tr>
<td>ATP</td>
<td>119</td>
</tr>
</tbody>
</table>

The substrate specificity of phytase was estimated at 55°C (pH 2.5) using series of phosphate compounds (10 mM) besides phytic acid by the standard assay method. Separate blanks with individual substrate were prepared and relative phytase activity was expressed as a percentage of the activity level obtained with sodium phytate as substrate.
hydrogen phosphate, all other substrates showed more activity than phytic acid, which was taken as standard assay substrate. Thus, it can be considered as a special form of acid-phosphatase having broad substrate specificity. Though phytases are fairly specific for phytic acid, the substrate specificity may vary due to differences in molecular characteristics. This purified phytase was similar to phytase B (phyB) and *A. fumigatus* phytase exhibiting broader substrate specificity than substrate specific phyA and several other phytases reported from *A. niger, A. terreus* 9A1, *A. terreus* CBS (Ullah and Cummins, 1988; Wyss *et al.*, 1999a). This was attributed to neutral electrostatic field of phyB site where wide variety of phosphomonoesters can be utilized while the highly positive electrostatic field of phyA’s substrate binding site would accommodate negatively charged phytate (Kostrewa *et al.*, 1999). Also, it was observed that the phytases with broad substrate specificity inherently had low specific activities, but the present phytase besides possessing broader specificity has comparatively high specific activity, thus making it a potential candidate for animal nutrition.

### 7.4 Effect of metal ions on catalytic activity

The effect of metal ions on the phytase activity was determined by incubating the purified enzyme with varying concentrations (1-20 mM) of individual metal ions viz., Cu$^{+2}$, Mg$^{+2}$, Mn$^{+2}$, Ba$^{+2}$, Fe$^{+2}$, Ni$^{+2}$, Hg$^+$, Co$^{+2}$, Zn$^{+2}$, Al$^{+3}$, Ca$^{+2}$, K$^+$ at 25°C for 2 hour. 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 (Table 6). Several authors have shown (Segueilha *et al.*, 1992; Greiner *et al.*, 1993; Yoon *et al.*, 1996; Greiner *et al.*, 1997) the effect of metal ions on phytase, thus suggesting that they play important role in regulating the phytase activity. EDTA, Zn$^{+2}$, Cd$^{+2}$, Ba$^{+2}$, Cu$^{+2}$, Fe$^{+2}$ and Al$^{+3}$ were found to readily inhibit the enzyme activity from *B. subtilis* (natto) N-77 (Shimizu, M., 1992). Yoon *et al.*, (1996) reported severe inhibition of phytase activity from *Enterobacter* sp.4 by Zn$^{+2}$, Ba$^{+2}$, Cu$^{+2}$, Al$^{+3}$ and EDTA. Kerovuo *et al.*, (2000) also studied the metal-ion requirement of *Bacillus subtilis* phytase that showed complete inactivation of enzyme upon removal of metal ions by EDTA and speculated it to be due to conformational change.
Table 6: Effect of metal ions on the catalytic activity of phytase

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Concentration (mM)</th>
<th>Relative phytase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{+2}$</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Mg$^{+2}$</td>
<td>5</td>
<td>106</td>
</tr>
<tr>
<td>Mn$^{+2}$</td>
<td>10</td>
<td>107</td>
</tr>
<tr>
<td>*Ba$^{+2}$</td>
<td>15</td>
<td>153</td>
</tr>
<tr>
<td>*Fe$^{+2}$</td>
<td>20</td>
<td>113</td>
</tr>
<tr>
<td>Ni$^{+2}$</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>Hg$^{+}$</td>
<td>10</td>
<td>113</td>
</tr>
<tr>
<td>Co$^{+2}$</td>
<td>15</td>
<td>135</td>
</tr>
<tr>
<td>Zn$^{+2}$</td>
<td>20</td>
<td>113</td>
</tr>
<tr>
<td>Al$^{+3}$</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Ca$^{+2}$</td>
<td>20</td>
<td>145</td>
</tr>
<tr>
<td>K$^{+}$</td>
<td>20</td>
<td>140</td>
</tr>
</tbody>
</table>

* : interferes with colorimetric assay.

The enzyme was pre-incubated with the individual metal ions at room temperature for 2 hour and the phytase activity was estimated using standard assay protocol. Separate blanks with individual metal ions were prepared. Control was taken as 100% with no metal ions. Relative activity was expressed as a percentage of the activity level in the absence of metal ions.
7.5 Inhibition studies
Various compounds that tend to chelate metal ions viz., EDTA, oxalate, citrate, tartarate and sulfhydryl inhibitors like β-mercaptoethanol and iodoacetate were tested for their effect on phytase activity. Also, serine and cysteine specific modifying agents like PMSF and para-hydroxymercurybenzoic acid (pHMB), phosphomycin were used at varying concentration to investigate their effect on catalytic property. It was noticed that none of them was an inhibitor even up to 2 mM concentration, in contrast they all were slightly activating as evident from Table 7. The fact that inhibitor at 0.1 mM level stimulate the enzyme activity may suggest that proteolytic enzymes were getting inhibited. The effect of metal ions as described above (Table 5) and the fact that EDTA had no effect on phytase activity indicated that the fungal phytases clearly differ from the metal-ion dependent phytase of *Bacillus subtilis* that was readily inhibited by EDTA (Kerovuo *et al.*, 1998). This was further supported by the lack of metal ions in the crystal structure of *A. niger* phytase (Kestrel *et al.*, 1997) and lack of protein sequence homology between fungal and *Bacillus subtilis* phytase. When more specific sulfhydryl inhibitors like β-mercaptoethanol and iodoacetate were tested, no effect was seen even at 25 mM concentration, indicating that either protein has no free and accessible sulfhydryl groups or the missing participation in the active site of enzyme. This was further confirmed 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. The enzyme was insensitive to serine-specific reagent PMSF up to 0.5 mM concentration, above which drastic fall in enzyme activity was seen. 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. Also sodium-azide showed slight activating effect up to 2 mM though even at 20 mM concentration, the purified protein retained 92% phytase activity.

7.6 Effect of organic solvents on enzymatic activity
To investigate the effect of organic solvents on phytase activity, enzyme was incubated with various solvents (10% v/v) viz., hexane, butanol, dimethylsulphoxide (DMSO),
Table 7: Effect of inhibitors on the catalytic activity phytase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>154</td>
<td>152</td>
<td>147</td>
<td>141</td>
</tr>
<tr>
<td>PMSF</td>
<td>163</td>
<td>103</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>L-tartaric acid</td>
<td>134</td>
<td>154</td>
<td>161</td>
<td>-</td>
</tr>
<tr>
<td>Oxalate</td>
<td>152</td>
<td>154</td>
<td>141</td>
<td>121</td>
</tr>
<tr>
<td>Citrate</td>
<td>145</td>
<td>142</td>
<td>136</td>
<td>125</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>137</td>
<td>137</td>
<td>136</td>
<td>132</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>159</td>
<td>152</td>
<td>126</td>
<td>123</td>
</tr>
<tr>
<td>Phosphomycin</td>
<td>137</td>
<td>121</td>
<td>118</td>
<td>114</td>
</tr>
<tr>
<td>p-hydroxymercuric benzoate</td>
<td>122</td>
<td>110</td>
<td>98</td>
<td>92</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>128</td>
<td>98</td>
<td>96</td>
<td>95</td>
</tr>
</tbody>
</table>

The enzyme was pre-incubated with the individual inhibitor at room temperature for 2 hour. The phytase activity was estimated using standard assay protocol. Separate blanks with individual inhibitor were prepared. Control was taken as 100% with no inhibitor. Relative activity was expressed as a percentage of the activity level in the absence of inhibitor.
dimethylfluoride (DMF), benzyl alcohol at room temperature (22-25°C), for 2 hour and assayed at 55°C (pH 2.5) under standard assay condition. As depicted from Table 8, enzyme retained complete activity in the presence of hexane, DMSO, DMF while in the presence of benzyl alcohol and butanol, 83 and 88% activity was retained, respectively. This might suggest that the hydrophobic residues were not involved or played any significant role in the catalytic property of present phytase.

7.7 Effect of detergents and chaotropic agents
Of the various detergents tested (0.1-5%), increasing concentration of 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 (Table 9). Also, chaotropic agents like guanidinium hydrochloride, urea and potassium iodide (0.5-8 M) significantly affected the phytase activity (Table 10). Potassium iodide was found to be comparatively more potent resulting in 60% loss of activity at 1 M with sharp decline in activity at increasing concentration.

7.8 Salt tolerance
The purified enzyme was incubated with varying concentration (0.2-2 M) of sodium chloride and ammonium sulphate and aliquots were withdrawn at regular interval to estimate residual phytase activity at 55°C. As indicated in Figure 26, the purified protein retained 90% phytase activity at 1 M NaCl and 85% at 2 M NaCl after 2 hour incubation at room temperature. Even in the presence of (NH₄)₂SO₄, no significant decline in enzyme activity was observed and 78% phytase activity was retained at 2 M salt concentration after 2 hour. The ability of purified protein to retain almost complete phytase activity at higher salt concentration favors its suitability for practical use in feed industry.

7.9 Estimation of kinetic constants
To study the enzyme-substrate affinity, the kinetic parameters of phytase were estimated over a range of substrate concentration (0.075-2.25 mM) at 55°C, pH 2.5 under standard
Table 8: Effect of organic solvents on the catalytic activity of phytase

<table>
<thead>
<tr>
<th>Organic solvent (10%, v/v)</th>
<th>Relative phytase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Hexane</td>
<td>112</td>
</tr>
<tr>
<td>Dimethylsulphoxide (DMSO)</td>
<td>110</td>
</tr>
<tr>
<td>Dimethylfluoride (DMF)</td>
<td>109</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>83</td>
</tr>
<tr>
<td>Butanol</td>
<td>88</td>
</tr>
</tbody>
</table>

The purified protein was pre-incubated with the individual solvent at room temperature for 2 hour and the phytase activity was estimated using standard assay protocol. Separate blanks with individual solvents were prepared. Relative activity was expressed as a percentage of the activity level in the absence of solvent. Control was taken as 100% with no solvent.
Table 9: Effect of detergents on catalytic activity of phytase

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Concentration (%)</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton-X-100</td>
<td></td>
<td>139</td>
<td>145</td>
<td>167</td>
<td>175</td>
</tr>
<tr>
<td>Tween-20</td>
<td></td>
<td>144</td>
<td>154</td>
<td>180</td>
<td>*</td>
</tr>
<tr>
<td>Tween-80</td>
<td></td>
<td>152</td>
<td>171</td>
<td>177</td>
<td>*</td>
</tr>
<tr>
<td>SDS</td>
<td></td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CTAB</td>
<td></td>
<td>154</td>
<td>162</td>
<td>180</td>
<td>*</td>
</tr>
</tbody>
</table>

*: interferes with colorimetric assay due to the formation of turbidity.

The purified protein was pre-incubated with the individual detergent at room temperature for 2 hour and the phytase activity was estimated using standard assay protocol. Separate blanks with individual detergent were prepared. Control was taken as 100% with no detergent. Relative activity was expressed as a percentage of the activity level in the absence of any detergent.
Table 10: Effect of various chaotropic agents on phytase activity

| Additive          | Concentration (M) | 0.5 | 1   | 2   | 4 | 6 | 8 | *:
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanidinium-HCl</td>
<td></td>
<td>90</td>
<td>68</td>
<td>59</td>
<td>23</td>
<td>8</td>
<td>*</td>
<td>interferes with colorimetric assay due to the formation of turbidity.</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td>90</td>
<td>75</td>
<td>65</td>
<td>59</td>
<td>45</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Potassium iodide</td>
<td></td>
<td>77</td>
<td>40</td>
<td>28</td>
<td>14</td>
<td>5</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

The purified protein was pre-incubated with the individual reagent at room temperature for 2 hour and the phytase activity was estimated using standard assay protocol. Separate blanks with individual chaotropic agent were prepared. Control was taken as 100% with no chaotropic agent. Relative activity was expressed as a percentage of the phytase activity level in the absence of chaotropic agent.
Figure 26: Effect of salt concentration on the catalytic activity of purified phytase
The purified protein was incubated with varying concentration (0.2-2 M) of salt at room temperature for 2 hour and phytase activity was estimated at 55°C, pH 2.5 using the standard assay protocol. Separate blanks corresponding to each salt concentration were prepared and phytase activity was expressed as percentage of activity obtained relative to control (without salt).
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assay condition. The maximal hydrolysis rate ($V_{\text{max}}$) and apparent Michaelis Menten constant ($K_m$) of the phytase for phytic acid were calculated from Lineweaver-Burk plot. As evident from Figure 27, the phytase activity increased linearly with increasing substrate concentration up to 0.6-0.75 mM above which substrate saturation resulted. The kinetic constants were found to be 0.606 mM and 1074 IU/ml as $K_m$ and $V_{\text{max}}$, respectively from Lineweaver-Burk plot. Kinetic linearity of phytase was estimated by incubating the substrate with varying amount of purified protein and estimating the phytase activity under standard condition. During this estimation, the substrate concentration was fixed at 0.75 mM where enzyme was saturated. The phytase activity was observed to be linear from 12-120 ng of protein (Figure 28). The estimated $K_{\text{cat}}$ of the enzyme as computed from the kinetic linearity experiment was $3 \times 10^5$ sec$^{-1}$ with catalytic efficiency of $3.69 \times 10^8$ 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 (Ullah and Sethumadhavan, 1998). The reported phyB was found to possess comparatively high affinity for phytate with $K_m$ of 103 $\mu$M and catalytic turnover of 628 sec$^{-1}$. However, the present phytase, though displayed higher $K_m$ of 606 $\mu$M, was much more efficient in hydrolyzing phytate with significantly high catalytic turnover and approximately 60 times more catalytic efficiency than as reported for phyB ($6.1 \times 10^6$ M$^{-1}$ sec$^{-1}$).

7.10 Effect of myo-inositolhexasulphate (MIHS) on phytase activity

To study the effect of MIHS, a structural analogue of phytic acid, the following experiments were carried out:

7.10.1 Inhibition of phytase activity by MIHS

To determine the inhibition of phytase by MIHS, phytase activity was estimated in the presence of increasing concentration of MIHS (0-800 $\mu$M) at 55°C, pH 2.5 using standard assay protocol (section 2.4.1). The enzyme was severely inhibited by MIHS with 50% inhibition of the phytase activity at 100 $\mu$M concentration (Figure 29). This was contrary to the inhibition concentration reported for phyB from Aspergillus ficuum NRRL 3135 which was much more susceptible to the inhibitory effect of myo-inositolhexasulphate. Most of the phyB activity was inhibited at a concentration of 1 $\mu$M MIHS and at 0.2 $\mu$M,
Figure 27: Estimation of kinetic constants of purified phytase from Lineweaver-Burk plot
Phytase activity was estimated over a range of substrate concentration at 55°C, pH 2.5 under standard assay condition. The graph of phytase units vs phytic acid concentration was plotted in double reciprocal manner to calculate the kinetic constants (Km and Vmax) of purified phytase.

\[ K_m : 0.606 \text{ mM} \]
\[ V_{\text{max}} : 1074 \text{ IU/ml} \]
Figure 28: Estimation of kinetic linearity and catalytic turnover number ($K_{\text{cat}}$) of purified phytase

To determine the kinetic linearity, catalytic turnover number and catalytic efficiency of purified phytase, enzyme activity towards sodium phytate was determined at various concentration of enzyme-protein (12-120 ng) keeping fixed substrate concentration at 55°C, pH 2.5 under standard assay condition.

Apparent $K_{\text{cat}}$ of phytase: $3 \times 10^5 \text{ sec}^{-1}$
Figure 29: Effect of myo-inositolhexasulphate (MIHS); a structural analogue of phytic acid on the catalytic activity of phytase
The purified protein was assayed for phytase activity with sodium phytate as substrate at 55°C, pH 2.5 in the presence of varying concentration of MIHS (0-800 μM) to estimate its concentration for 50% inhibition of phytase activity.
half of the phytase activity was retained (Ullah and Sethumadhavan, 1998b) while the present phytase retained 65% phytase activity even at 50 μM concentration.

7.10.2 Estimation of inhibition constant (Ki) of MIHS

The inhibition constant of MIHS was determined by varying substrate concentration (0.15-1.5 mM) in the presence and absence of MIHS. The inhibitor concentration was fixed at 100 μM where 50% inhibition of phytase activity was obtained. The enzyme showed an interesting inhibition profile and in the absence of inhibitor, a typical sigmoidal curve for catalytic activity vs phytate concentration was obtained. However, in the presence of inhibitor, the enzyme hydrolyzed phytate linearly at a diminished rate (Figure 30). When plotted in double reciprocal Lineweaver-burk fashion, the enzyme showed competitive inhibition by MIHS. 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 (Figure 31). This was similar to the inhibition pattern obtained for the phytases, phyA and phyB, from Aspergillus ficuum NRRL 3135. Despite the fact that both phyA and phyB were structurally different proteins (Kostrewa et al., 1997), the inhibitor MIHS showed very similar affinity for the active site as reflected by similar K_i, indicating that both the phytases assume similar active site structure at pH 2.5 and share identical active site residues (Ullah and Sethumadhavan, 1998b). Thus, the MIHS was confirmed to be potent competitive inhibitor of phytases.

7.11 Effect of inorganic phosphorus on phytase activity

To determine the effect of inorganic phosphorus, phytase activity was estimated by varying substrate concentration (0.15-1.5 mM) in the presence of inorganic phosphorus. The plot of phytase activity (nkat/ml) vs phytic acid concentration (mM) in the absence and presence of varying concentration of inorganic phosphorus clearly show the significant inhibition in phytase activity (Figure 32). At 0.2 mM P_i concentration, approximately 50% inhibition of phytase activity, yielding a typical sigmoidal curve was observed and as the concentration increased to 0.5 mM, a sharp decline in activity resulted indicating the phenomenon of end product inhibition. But at higher
Figure 30: Estimation of inhibition constant of myo-inositolhexa sulphate at varying substrate concentration

The phytase activity of purified protein was determined at varying substrate concentration (0.15-1.5 mM) in the absence and presence of MIHS at 55°C, pH 2.5 under standard condition. The inhibitor concentration was fixed at a value where 50% inhibition in phytase activity resulted.
Figure 31: Lineweaver Burk plot indicating the inhibition pattern of phytase by myo-inositolhexasulphate
The graph of phytase units vs phytic acid concentration in the absence and presence of 100 µM myo-inositolhexasulphate (MIHS) was plotted in double reciprocal manner to calculate the inhibition constant besides the nature of inhibition exerted by MIHS on the purified phytase.

\[ K_m : 0.625 \text{ mM} ; \quad V_{max} : 1117.44 \quad \text{and} \quad K_m : 1.898 \text{ mM} ; \quad V_{max} : 1125.87 \]

Estimated \( K_I : 0.05 \text{ mM} \)
Figure 32: Effect of inorganic phosphorus on the catalytic activity of purified phytase

The purified protein was assayed for phytase activity at varying substrate concentration (0.15-1.5 mM) in the presence of inorganic phosphorus at 55°C, pH 2.5 using standard protocol to determine the inhibition profile.
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collection, the inhibitory effect of Pi was so strong that no significant variation in phytase activity was observed even at higher substrate concentration.

To further determine the nature of inhibition exerted by Pi on phytase, \( K_m \) study by varying substrate concentration corresponding to each inorganic phosphorus concentration viz., 0, 0.2 and 0.5 mM was performed. By plotting the data in double reciprocal fashion, it was found that the given phytase was inhibited in uncompetitive manner by Pi (Figure 33). The inhibition constant of the purified protein was found to be 0.16 mM in the presence of 0.2 mM Pi.

7.12 Effect of DEPC on phytase activity

As most of the phytases reported from fungal origin are histidine acid phosphatases, the effect of DEPC, histidine specific modifying agent, was studied to view the role of His residues in enzyme catalysis. DEPC was reported to be reasonably specific for histidyl residues in the pH range 5.5-7.5 and at moderate excess, it results in the substitution of one of the nitrogen positions on imidazole ring. The dialyzed, suitably diluted purified enzyme was incubated with the varying concentration of DEPC (2.5-25 mM) and aliquots were withdrawn after every 15 minute to estimate the residual phytase activity at 55°C, pH 2.5 using standard assay protocol. Though phytase activity decreased with increasing concentration of diethylpyrocarbonate but the enzyme was able to retain significant phytase activity even at higher DEPC concentration. 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 (Figure 34). This was contrary to the previously reported phytases from *Aspergillus niger* NRRL 3135 (*phyA* and *phyB*), pH 2.5 optimum acid phosphatase in *E. coli*, pho3 and pho5 in yeast, where a sensitive histidine at the active site played important role in catalysis (Wodzinski and Ullah, 1996).

8. Enhancement of phosphorus bioavailability of livestock feed using phytase

The in-vitro system used to mimic the condition in digestive tract consisted of autoclaved feed, buffer and crude phytase supernatant. The autoclaved feed was suspended in 20 ml sterile 0.1M Glycine-HCl buffer (pH 2.5) in a 100 ml conical flask and supplemented
Figure 33: Lineweaver Burk plot indicating the inhibition pattern of phytase by inorganic phosphorus

The graph of phytase units vs phytic acid concentration in the absence and presence of 0.2 mM inorganic phosphorus was plotted in double reciprocal manner to determine the nature of inhibition and to calculate the inhibition constant.

$K_m : 0.606 \text{ mM} ; V_{\text{max}} : 1074.99 \quad \text{and} \quad K_m : 0.282 \text{ mM} ; V_{\text{max}} : 461.91$

Estimated $K_i : 0.16 \text{ mM}$
Figure 34: Effect of diethylpyrocarbonate (DEPC) on the catalytic activity of phytase

The dialyzed and purified protein was incubated with varying concentration of DEPC (2.5-25 mM) at room temperature. The samples were withdrawn at regular time interval to estimate the residual phytase activity at 55°C, pH 2.5 using standard assay protocol. Separate blanks corresponding to individual inhibitor concentration and control (no inhibitor) were also prepared.
Results & Discussion

with known phytase units. The autoclaved feed suspension without any enzyme acts as control. The flasks were then incubated at 37°C (200 rpm) and samples were withdrawn at regular time interval for the quantification of the released phosphorus from the enzyme treated feed.

8.1 Phosphate liberation from various commercial livestock feed

The ability of present phytase to hydrolyze phytate content in various commercial livestock feed besides its ability to retain enzyme activity over a period of time in the presence of feed constituents and additives was studied. Four commercial feeds of different composition were investigated for their effect on the catalytic activity of present phytase. The feeds were autoclaved and aliquots were suspended in sterile buffer (0.1M Glycine-HCl, pH 2.5) and supplemented with crude phytase. The final reaction mixture contained 140 nkat/ml phytase unit. As viewed from Figure 35a, the enzyme was able to effectively hydrolyze phytate content in all the feeds tested. The maximum phosphorus release was observed in Feed 2 where 31.33 nmoles $P_i$/ml were liberated after 48 hour at 37°C as compared to 48 nmoles $P_i$/ml at 55°C. When compared to untreated control samples, an increase of 30 nmoles $P_i$/ml resulted in phytase supplemented samples at 55°C, indicating the ability of the given phytase to hydrolyze phosphate moieties present in commercial feed. In all the feeds tested, the present phytase was much more efficient when used at higher temperature (55°C) than at 37°C. This can be speculated from the earlier studies that the present phytase was optimally active at 55°C, thus resulting in the better performance. Han et al., (1999) expressed phyA gene in S. cerevisiae and reported the effectiveness of recombinant phytase in hydrolyzing phytate phosphorus invitro from corn and soybean meal. When the stability of phytase was investigated, the present phytase was able to retain complete phytase activity in Feed 1 while 90-95% enzyme activity was retained in Feed 2-4 at 55°C after 72 hour of incubation (Figure 35b). Thus the present phytase was effective in hydrolyzing phytate phosphorus with negligible loss in catalytic activity in the presence of phosphates, antibiotics and other feed constituents.

8.2 Feed hydrolysis at varying feed: enzyme ratio

To estimate the ability of given quantity of phytase to hydrolyze phytate present in feed-sample (Feed 2), hydrolysis pattern was studied at varying concentration of feed (5, 8, 10,
Figure 35a: *Invitro* phosphate liberation studies from various livestock feed using phytase from *A. niger* van Teighem.

The autoclaved feed samples were suspended in 0.1 M Glycine-HCl buffer (pH 2.5) and supplemented with crude phytase. Both test and control samples were incubated at 37 and 55°C, separately. The solid lines indicate phytase supplemented samples while "dotted lines" symbolize their corresponding controls.
Figure 35b: Enzyme deactivation profile of phytase in the presence of various livestock feed
Different livestock feed were incubated with crude phytase at 55°C, pH 2.5 on rotary shaker (200 rpm) and samples were withdrawn at regular time interval to estimate the residual phytase activity.
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12, 15 g / 20 ml 0.1 M Glycine-HCl buffer, pH 2.5) keeping fixed phytase units (141.33 nkat/ml) at 37 and 55°C on a rotary shaker (200 rpm). Samples were withdrawn at regular interval from test and control flasks to quantitate the released phosphorus. An increase in phosphorus release was observed with increase in substrate concentration at 37 and 55°C (Figure 36). The enzyme showed better performance in feed hydrolysis at 55°C as compared to 37°C. After 24 hour of incubation at 37°C, increased release of 12.66 nmoles P_i/ml was observed in phytase supplemented 5 g sample over control as compared to 13.66 nmoles P_i/ml and 17.33 nmoles P_i/ml in 10 and 15 g phytase treated samples, respectively. A significant increase in hydrolysis pattern was observed at 55°C where 23.33 nmoles P_i/ml was released after 24 hour in 5 g sample as compared to 26.66 nmoles P_i/ml at 10 g concentration. As with the increase in feed concentration, thick slurry resulted, therefore, in all the subsequent experiments, an optimum feed concentration of 5 g supplemented in 20 ml buffer with 5 ml enzyme extract was used. When the enzyme deactivation pattern was observed, the enzyme extract retained 90-94% phytase activity at all the concentration tested even after 72 hour of incubation at 55°C. Similarly, the hydrolysis pattern was studied at varying phytase units keeping fixed feed concentration (5 g) at 37 and 55°C on a rotary shaker (200 rpm). As viewed from Figure 37, the enzyme was significantly more effective at 55°C than 37°C in hydrolyzing phytate content of the feed. An increased release of 25.33 nmoles P_i/ml in phytase-treated samples over control was observed when 5 ml enzyme preparation was added as compared to 41.33 nmoles P_i/ml with 10 ml supplemented phytase supernatant after 24 hour incubation at 55°C. This was further increased over a period of time and after 48 hour incubation at 55°C, an increased release of 38 nmoles P_i/ml and 50 nmoles P_i/ml in 5 ml and 10 ml phytase treated samples over control was observed, respectively. When the enzyme deactivation pattern was studied, 90-93% phytase activity was retained even after 72 hour at 55°C, thus indicating the suitability of present phytase in animal feed.

8.3 Feed hydrolysis at different temperature

The phosphate liberation kinetics from the commercial livestock feed using phytase was further investigated at different temperatures (30, 37, 45 and 55 °C) in shake flask by supplementing the aliquots (5 g) of autoclaved with enzyme preparation, (133.33 nkat/ml
Figure 36: *Invitro* phosphate liberation at varying concentration of livestock feed

Varying concentration (5-15 g) of autoclaved feed were suspended in 0.1 M Glycine-HCl buffer (pH 2.5) and supplemented with fixed phytase unit. The test samples and their corresponding controls were incubated for a varying time period at 37 and 55°C. Data showed the liberated inorganic phosphorus in phytase treated sample over control.
Figure 37: *In vitro* phosphate liberation from livestock feed at varying concentration of phytase
The autoclaved feed was suspended in 0.1 M Glycine-HCl buffer (pH 2.5) and supplemented with varying concentration of phytase unit. The test and the control samples were incubated for varying time period at 37 and 55°C. Data showed the liberated inorganic phosphorus in phytase treated sample over control.
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Phytase activity in the final reaction mixture). Separate controls and phytase supplemented test flasks were then incubated at different temperatures (200 rpm) and samples were withdrawn at regular time interval to quantitate the released phosphorus. The present phytase extract was able to release significant amount of phosphorus from commercial livestock feed over untreated control samples (Figure 38a). At 30°C, 25.66 nmoles P$_i$/ml was released in phytase-treated sample as compared to 11.2 nmoles P$_i$/ml in untreated control after 48 hour incubation. With the increase in incubation temperature, parallel increase in the hydrolysis of livestock feed was observed using phytase. At 37°C, 42.66 nmoles P$_i$/ml was liberated in phytase treated samples while 49.33 and 56.33 nmoles P$_i$/ml were liberated at 45 and 55°C, respectively after 48 hour. A significant release of 40 nmoles P$_i$/ml in phytase-treated over control sample was observed at 55°C after 48 hour.

When the phytase stability was investigated over temperature range (30-55°C) in the presence of feed constituents, 91% phytase activity was retained even after 48 hour incubation at 55°C (Figure 38b). Thus, the ability of present phytase to retain activity over a period of time in the presence of feed constituents besides broad substrate specificity indicated its suitability for application in the livestock feed. Wyss et al., (1999b) studied invitro phosphate liberation kinetics by using feed suspensions supplemented with either A. fumigatus or A. niger phytase (Natuphos) at 37°C, pH 5.0 and suggested that the phytases with broad substrate specificity were better suited for animal nutrition purposes than phytases with narrow specificity as even when the initial phytase activities were same, the phytases with broader substrate specificity readily liberated all five equatorial phosphate groups of phytic acid. However, the phytases with broader substrate specificity had generally low specific activities. Since the present wild type phytase had significantly high specific activity besides the ability to hydrolyze a variety of phosphomonoesters, thus it can be considered as a potential candidate in animal nutrition.

8.4 Feed hydrolysis at varying pH

The ability of crude phytase to hydrolyze phytate in the feed was tested at varying pH condition in shake flask. The autoclaved feed was suspended in sterile buffer of desired
Figure 38a: *Invitro* phosphate liberation from livestock feed using crude phytase at different temperature. Autoclaved feed samples were suspended in 0.1 M Glycine-HCl buffer (pH 2.5) and incubated for varying periods of time at different temperatures. The solid lines indicate the phytase supplemented samples while "dotted lines" symbolize their corresponding controls i.e., not supplemented with phytase.
Figure 38b: Enzyme deactivation profile of phytase at different temperature

Aliquots of commercial livestock feed were incubated with crude phytase at different temperature (30-55°C, pH 2.5) on rotary shaker (200 rpm) and samples were withdrawn at regular time interval to estimate the residual phytase activity.
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pH and inoculated with 5 ml of concentrated enzyme supernatant, (133.33 nkat/ml). Separate controls and phytase-supplemented test samples were incubated at 37°C (200 rpm). Samples were withdrawn at regular time interval to quantitate the released phosphorus. As viewed from Figure 39a, significant release of phosphorus was observed in phytase-treated sample as compared to control over a period of time. Though significant amount of phosphorus was liberated by phytase over a range of pH (1.5-5.5), maximum hydrolysis was observed at pH 2.5 where 34.66 nmoles P_i/ml was released after 24 hour of incubation compared to 11.66 nmoles P_i/ml in untreated control sample. The enzyme preparation was able to hydrolyze phosphate moieties increasingly over a period of time and after 48 hour, 40.66 nmoles P_i/ml was released over 8.66 nmoles P_i/ml in control sample. At pH 5.5, decline in phosphorus release was observed where only 17.33 nmoles P_i/ml was liberated in phytase-treated sample as compared to 8 nmoles P_i/ml in untreated control even after 32 hour. This can be speculated from the earlier observation that the given enzyme was optimally active at pH 2.5 with comparatively little activity at pH 5.5. As the gastric pH of simple stomached animals is around 2.5-3.5, this ability of present phytase to hydrolyze the phosphate moieties efficiently in acidic pH range suggested its effectiveness in releasing phytate phosphorus from digesta. This is further supported by the ability of present phytase to hydrolyze not only chemically pure soluble sodium phytate but also its effectiveness in releasing phytate phosphorus from various commercial feeds.

Besides this, the enzyme deactivation pattern was studied at varying pH over a period of time (Figure 39b). The present phytase was able to retain almost complete phytase activity in the presence of feed constituents even after 48 hour over various pH tested. The enzyme preparation retained 95 and 89% phytase activity at pH 2.5 and 5.5, respectively after 48 hour of incubation, indicating the stability of phytase preparation.

9. Identification of various hydrolytic and dephosphorylated products of myo-inositolhexakisphosphate formed by enzymatic hydrolysis

Attempts have been made to separate and identify the various dephosphorylated isomers of myo-inositolhexakisphosphate formed by enzymatic degradation using various chromatographic and spectroscopic techniques.
Figure 39a: *In vitro* phosphate liberation from livestock feed using crude phytase at varying pH

Autoclaved feed samples were suspended in 0.1 M buffers of varying pH (1.5-5.5) and incubated at 37°C, 200 rpm. The solid lines indicate the phytase supplemented samples while "dotted lines" symbolize their corresponding controls *i.e.*, not supplemented with phytase.
Figure 39b: Enzyme deactivation profile of phytase at different pH
Aliquots of commercial livestock feed were suspended in 0.1M buffer of varying pH (1.5-5.5) and incubated with crude phytase at 37°C, 200 rpm. Samples were withdrawn at regular time interval and phytase activity was estimated by standard assay protocol in the presence of feed constituent.
Results & Discussion

9.1 Separation and identification of lower InsP isomers using liquid chromatography-mass spectroscopy (LCMS)

Besides the adverse effect of phytate and other highly phosphorylated inositol phosphates on mineral bioavailability, some novel metabolic effects of some of its degradation products have been recognized. Certain myo-inositol phosphates were found to have positive effect on heart disease by controlling hypercholesterolemia and arteriosclerosis (Jariwalla et al., 1990; Potter, 1995), prevention of diabetes complication (Ruf et al., 1991; Carrington et al., 1993), anti-inflammatory effects (Claxon et al., 1990). The most extensively studied positive aspect of myo-inositol phosphates is their potential for reducing the risk of colon cancer (Ullah and Shamsuddin, 1990; Graf and Eaton, 1993; Vucenik et al., 1993; Yang and Shamsuddin, 1995; Shamsuddin et al., 1997). Furthermore, some of lower myo-inositol phosphates, in particular D-myo-inositol (1,4,5)-triphosphate and D-myo-inositol (1,3,4,5)-tetraphosphate were found to play important role as intracellular secondary messengers in signal transmission (Streb et al., 1983). The position of phosphate groups on inositol ring is, thereby, of great significance for their physiological function. To investigate the physiological effects of defined myo-inositol phosphate isomers, these compounds have to be available in pure form and sufficient quantity. Attempts have been made to separate and identify the various dephosphorylated isomers of myo-inositolhexakisphosphate formed by enzymatic degradation. The kinetic studies of the purified enzyme had shown the enzyme to be optimally active at 55°C, over a pH range of 2.0-2.5. Also the enzyme was found to be stable at this temperature retaining approximately 90% activity in the absence of any stabilizing agent even after 24 hour, suggesting its applicability for hydrolyzing phytate over a varied time period. The reaction was started by the addition of purified phytase to the incubation mixture consisting of glycine-HCl buffer (0.1M, pH 2.5) containing 75 μl of 10 mM sodium phytate as substrate and incubating at 55°C for varying time period. As the lower esters are produced in nanogram quantities, therefore, to produce these compounds for the efficient detection using liquid chromatography, approximately ten tubes, each containing 1ml reaction mixture were set up for a particular time period. The samples were withdrawn at different time interval and the reaction was quenched by heating at 95°C for 10 minute, after which the contents of particular time interval were
pooled and concentrated in Speed-Vac concentrator under vacuum to powder form. Various test samples (5 minute-8 hour, 16-24 hour) were then chromatographed on C\textsubscript{18}-HPLC column (4.6 x 50 mm; 5μ pore size) using gradient solvent system and identified using Electron Spray Ionization-Mass Spectrometric detectors (Finnigan Mat, USA) in positive ion mode. 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. Preliminary studies involved the optimization of various operating conditions on LCMS, besides mode of detection (+p ESI and APCI) and efficient eluant system for the resolution of various lower InsPs. It was observed that when samples were reconstituted in sterile, 0.22 μ filtered water instead of mobile phase and chromatographed on C\textsubscript{18}-column using methanol: water (30:70) as eluant, in +p ESI mode, significant resolution of various InsPs was achieved. The LCMS scans showed that the phosphate moieties were removed from InsP\textsubscript{6} in a sequential manner and within 10 minute of enzymatic reaction (Figure 40-41), InsP\textsubscript{6} was enzymatically cleaved to various lower InsPs (InsP\textsubscript{5}, InsP\textsubscript{4}, InsP\textsubscript{3}, InsP\textsubscript{2}). Though, no major base peak was identified after 10 minutes but as the reaction proceeded over a period of time, most of the substrate got converted to InsP\textsubscript{4} (RT 3.52-4.24) within 30 minute constituting 80-100% relative abundance of the total reaction pool and 33 % relative abundance of InsP\textsubscript{2} (2Na.InsP\textsubscript{2}, base peak m/z 383, RT 4.4-4.54). Within 30 minute of reaction, relative abundance of substrate-InsP\textsubscript{6} reduced to 5%, which along with InsP\textsubscript{5} constituted a total of 10-15% of reaction mixture (Figure 42). But as the enzymatic hydrolysis of InsP\textsubscript{6} with purified phytase proceeded over a period of time, InsP\textsubscript{4} got converted to InsP\textsubscript{3} with increasing abundance of InsP\textsubscript{2} from 33% (after 30 minute) to 82% after 1 hour of enzymatic reaction (Figure 43), and after 2 hour of hydrolytic reaction, whole of the substrate got converted to InsP\textsubscript{2} (RT 4.46, m/z 382.9) with 10-15% relative abundance of mixture of InsP\textsubscript{5,3}. As viewed from (Figure 44), InsP\textsubscript{2} (RT 4.44-4.48) produced by enzymatic hydrolysis of InsP\textsubscript{6} as a major product after 2 hour reaction, appeared as prominent isomer of inositol phosphate as the hydrolytic reaction with phytase proceeded over a period of time (Figure 45). Almost complete conversion of substrate to InsP\textsubscript{2} was observed with < 5 % abundance of higher phosphates (InsP\textsubscript{5,3}) after 8 hour of enzymatic reaction (Figure 46a). As the scanning range was 300-1000 m/z, to further confirm that if any lower InsP was
Figure 40: The liquid chromatography-mass spectrometer analysis of sodium phytate (12 Na.InsP₆)
The elution of standard substrate (12 Na.InsP₆) was carried out on C₁₅⁺ HPLC column using methanol:water (3:7) as mobile phase and detected using mass-spectrometer.
Figure 41: LCMS profile of enzymatically dephosphorylated-InsP₆ over a period of 10 minute

The substrate (InsP₆) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 10 min and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C₁₈-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
Figure 42: LCMS profile of enzymatically dephosphorylated-InsP₆ over a period of 30 minute

The substrate (InsP₆) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 30 min and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C₁₈-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
Figure 43: LCMS profile of enzymatically dephosphorylated-InsP$_6$ over a period of 1 hour

The substrate (InsP$_6$) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 1h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C$_{18}$-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
Figure 44: LCMS profile of enzymatically dephosphorylated-InsP₆ over a period of 2 hours

The substrate (InsP₆) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 2 h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C₁₈-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
Figure 45: LCMS profile of enzymatically dephosphorylated-InsP₆ over a period of 4 hour

The substrate (InsP₆) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 4 h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C₁₈-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
Figure 46a: LCMS profile of enzymatically dephosphorylated-InsP₆ over a period of 8 hours

The substrate (InsP₆) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 8 h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C₁₈-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
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generated, MS scan was carried out in 150-500 m/z, which showed InsP$_2$ as base peak with no lower inositol phosphate isomers (Figure 46b). Further, incubation of substrate (12Na. InsP$_6$) with phytase showed no significant change in the hydrolysis pattern over the period of time (14-24 hour), indicating that InsP$_6$ got converted to InsP$_2$ in a sequential manner, after which the enrichment of isomer resulted as the reaction proceeded over a period of 24 hour (Figure 47). As indicated from the figure, a clean base peak of 383.0 m/z corresponding to the molecular mass of 2Na.InsP$_2$ appeared with traces of higher inositol phosphates. Besides this no base peak corresponding to inositol monophosphate and inositol was found, thus suggesting inositol diphosphate to be the end product of enzymatic dephosphorylation of present purified phytase. To further confirm that if the substrate was getting hydrolyzed at 55°C over a period of time without any enzymatic reaction, controls were set up and assayed for the release of any inorganic phosphorus liberated. Even after 24 hour of incubation, no phosphorus was liberated from 12 Na. InsP$_6$, thus confirming that the liberated phosphate was only due to enzymatic dephosphorylation of substrate. When the enzyme deactivation pattern was studied, purified protein showed negligible loss of phytase activity after 24 hour at 55°C. Thus, the purified phytase from present isolate, Aspergillus niger van Teighem, hydrolyzes InsP$_6$ in sequential manner (InsP$_6$→InsP$_5$→InsP$_4$→InsP$_3$→InsP$_2$), with InsP$_2$ as possible end product of phytate hydrolysis. As the phytases belong to special group of phosphatases capable of sequentially hydrolyzing phytate and are widely distributed in nature, thus using phytases of different origin may lead to production of different myo-inositolphosphate isomers. Besides this reduction in the number of phosphate groups bonded to the inositol ring resulted in the increased solubility and decreased ability to form complexes. Spiers et al., (1996) evaluated the hydrolysis products of InsP$_6$ for their ability to depress OH$^-$ formation and reported that Ins (1,2)P$_2$ potentiated the OH$^-$ production. Greiner et al., (2000) established the stereo specificity of InsP$_6$ dephosphorylation by the P2 phytase of E. coli in a sequential manner (6/1/3/4/5) using combination of high performance ion chromatography along with post column derivatization and reported Ins(2)P or Ins(5)P as end product of enzymatic dephosphorylation. Nakano et al., (2000) deduced the hydrolysis pathway of InsP$_6$ by phytase from wheat bran of Triticum aestivum L. cv. Nourin #61 using gradient
Figure 46b: LCMS profile of enzymatically dephosphorylated-InsP₆ over a period of 8 hour in 150-500 m/z range

In order to confirm the formation of any lower isomer of InsP₂, MS-scan of enzymatically dephosphorylated-8 h sample was taken in 150-500 range.
The substrate (InsP₆) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 24 h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then chromatographed on C₁₈-HPLC column using methanol:water (3:7) as eluant and detected using mass-spectrometer.
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Chromatography on Q-Sepharose and reported myo-inositol as the final product. The identification of intermediates was carried out by a variety of nuclear magnetic resonance techniques and gas chromatography. Vander Kaay and Haastert (1995) reported the dephosphorylation pattern of inositolhexaphosphate ($^{32}$P-labelled InsP$_6$) using Paramecium phytase and found that after prolonged incubation of InsP$_6$ with phytase, mainly Ins (1,2,3) P$_3$ and small amounts of Ins (2,3) P$_2$ were produced. The study showed that InsP$_6$ was dephosphorylated in sequential manner (6/5/4/1), thus allowing the enzyme to produce specific inositol isomers, notably Ins-1, 2,3,4,5-P, Ins-1, 2,3,4-P and Ins-1, 2,3-P with Ins (2,3) P$_2$ as possible end product.

9.2 Thin layer chromatography

The product purity was further confirmed by thin layer chromatography on pre-derivatized silica gel-60 and RP-HPTLC silica plates. The enzymatically-produced sample (24 hour) as described above along with known standards viz., Ins-1, 4-diphosphate and Ins, were chromatographed by spotting different dilutions (1-2 µl) on silica plates. Preliminary experiments involved the development of solvent system for the resolution of inositol and InsPs, besides the detection system. As shown in Figure 48, when the inositol was mixed with 24 hour test sample and chromatographed along with standard Ins-1,4-diphosphate using standardized solvent system consisting of methanol: water (90:10), effective separation of components was achieved. The resolved InsPs were detected using KMnO$_4$ reagent prepared by mixing 1% KMnO$_4$ in 2% NaHCO$_3$ and drying with heat gun at 220°C, which resulted in the appearance of pale spots against colored background. 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. As no other impurity was visualized from the chromatogram, this indicated that the InsP$_2$ was produced in the purified form, with further no hydrolysis to lower InsP and inositol. This observation was also confirmed by the 24 hour LCMS-spectra where a single, neat base peak (383 m/z) corresponding to 2Na. InsP$_2$ was seen. Thus, the given purified phytase was able to cleave InsP$_6$ in a sequential manner over a period of time to InsP$_2$, which appeared to be the end product of enzymatic degradation.
Figure 48: Thin Layer Chromatography of enzymatically produced InsP$_2$ on Silica 60 plate

The enzymatically-hydrolyzed (24 h) sample along with known standards viz., Ins and Ins-1, 4-diphosphate, were dissolved in buffer (0.1M Glycine-HCl, pH 2.5) and chromatographed using methanol : water (9:1) as mobile phase. The resolved InsPs were visualized using KMnO$_4$ reagent prepared by mixing 1% KMnO$_4$ in 2% NaHCO$_3$. 
as seen by mass-spectrometric and TLC detection. To further confirm the isomer of InsP₂, ¹H and ³¹P-NMR (Avance DPX 300 MHz, Bruker, Switzerland) were carried out.

9.3 Nuclear Magnetic Resonance (¹H and ³¹P-NMR) spectroscopy

To further confirm the identity of enzymatically produced 2Na. InsP₂, the reaction was set up using purified phytase and sodium phytate as described above at 55°C, pH 2.5 for 24 hour. The reaction was stopped by heat treatment at 100°C for 5 minute and concentrated to remove the traces of water in Speed Vac after which it was dissolved in deuterated water for the identification by ¹H and ³¹P-NMR (Avance DPX 300 MHz, Bruker, Switzerland). For ³¹P-NMR, H₃PO₄ was used as internal standard. As the required quantities of standard Ins-1, 4-diphosphate and Ins-2,4-diphosphate were not in sufficient amount for NMR, thus the spectra of test sample (Figure 49-50) could not be compared. The ¹H and ³¹P-NMR of Ins-1, 2,3,4,5,6-P and Ins were also taken (App VIII-IX).
Figure 49: $^1$H-NMR of enzymatically produced InsP$_2$ over a period of 24 hour

The substrate (InsP$_4$) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 24 h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then lyophilized and reconstituted in deuterated water which acts as internal standard. The spectra was recorded on 300 MHz (Avance DPX, Bruker) spectrometer.
Figure 50: $^{31}$P-NMR of enzymatically produced InsP$_2$ over a period of 24 hour

The substrate ($\text{InsP}_5$) was hydrolyzed using purified phytase at 55°C, pH 2.5 for 24 h and reaction was stopped by heat treatment (100°C, 5 min). Samples were then lyophilized to remove traces of water and the spectra was recorded on 300 MHz (Avance DPX, Bruker) spectrometer. H$_3$PO$_4$ was used as internal standard.