This chapter discusses the application of single step aqueous two-phase extraction (ATPE) for the downstream processing of phytase from *Aspergillus niger* NCIM 563, produced under solid state fermentation and compares it with the traditional multi-step procedure involving salt precipitation and column chromatography. High phytase recovery (98.5%) within a short time (3hrs) and improved thermostability was attained by ATPE in comparison to 20% recovery in 96hrs by chromatography process. The ATPE method, therefore, seems to be an interesting alternative for simultaneous partitioning and purification of phytase. The influence of system parameters; such as, phase forming salts, polymer molecular weight and system pH on the partitioning behavior of phytase was evaluated. The ATPE system consisting of combination of polyethylene glycol (PEG) 6000 and 8000 (10.5%) and sodium citrate (20.5%) resulted in one-sided partitioning of phytase in bottom phase with a purification factor of 2.5. This is the first report on phytase purification using liquid-liquid extraction and the results are likely to be beneficial in the poultry feed industry.

Part of the work presented in this chapter is published:

1. Introduction

The cycling of P, a biocritical element in short supply, in nature is an important but slow biogeochemical process. P is a vital mineral important for bone and tissue growth in poultry. The massive growth of livestock production has made it the third most expensive nutrient in poultry production after energy and protein. Plants store P in the form of phytate (inositol 6-phosphate) carrying 6 phosphate groups. But this P present in seed grain as phytate is not readily available to mono-gastric animals, as they lack phytase activity. Phytate also acts as an antinutrient by chelating metal ions and reducing energy uptake [41]. To meet the P requirement, animal diets are generally supplemented with excess of commercial synthetic fertilizers. Human influences on the phosphate cycle come mainly from the introduction and use of these fertilizers. Strict norms for the excretion of large quantities of P effluents, human interference, decomposition of underutilized phytate cause phosphate pollution and price hike in synthetic fertilizers have currently led to the use of microbial phytase in animal feed [18].

Phytase (EC3.1.3.8) is an enzyme that acts by cleaving off 6 phosphate groups from inositol 6-phosphate and hence when added in feed has multiple benefits, mainly in increasing mineral, phosphorous and energy uptake. This decreases the necessity to fortify the fodder with above substances. It is estimated that 10kg dicalcium phosphate can be replaced by just 0.25kg of phytase and even then the worldwide demand for phytase in cattle feed is expected to be approximately 4000 tonnes/annum. In addition, they produce different myo-inositol phosphates that have novel metabolic effects, such as amelioration of heart disease by controlling hypercholesterolemia and atherosclerosis, prevention of renal stone formation, and reduced risk of colon cancer [15].

Hence efforts are needed to produce cost effective phytase with fast and economic downstream processing. The intricacies in phytase production technology can be understood through modeling, kinetics of growth of microbes, control of parameters, optimization, scale-up
and commercialization of the process. We have reported high level phytase production along with up-scaling studies under SSF by using response surface methodology [6]. Likewise production, downstream processing also is an integral part of any product development as the final cost of the product largely depends on the cost incurred in extraction and purification. Conventional procedures, including precipitation or chromatography, are currently employed for phytase purification. They have several limitations, such as, dilute concentration of enzyme, extensive downstream procedures and treatment of generated effluents. The process is also expensive, time consuming and difficult to scale-up. These traditional approaches are currently employed due to lack of alternative methods [27,32,38].

Thus there is a clear need for efficient, scalable and economical process for phytase bioseparation. One such purification method that meets all these criteria is liquid-liquid extraction [24, 37]. ATPE systems are an ideal technology where clarification, concentration, and partial purification can be integrated in just one-step. ATPE is composed of two different polymers or one polymer and one salt mixed at certain concentrations in an aqueous solution. The solution separates into two immiscible phases, with each dissolved component predominating in one or the other phase with water as a solvent in both phases. Due to higher water content, the ATPE systems have several advantages as compared to the commonly used separation and purification techniques, e.g., low interfacial tension, non-toxicity, non-flammability and biocompatibility [29, 5].

Polyethylene glycol (PEG)-salts systems and PEG-dextran systems have been introduced for large-scale protein separation. However, PEG-salt leads to faster separation than PEG-dextran because of the larger droplet size, greater difference in density between the phases, low viscosity and low cost. The industrial application of PEG-salt system is improved by the availability of commercial separators, which allows faster continuous protein separations [33]. PEG–potassium phosphate and PEG–magnesium sulfate are among the other frequently used polymer–salt systems.
These salts, however, lead to high phosphate or sulfate concentration in effluent streams, and are of environmental concern.

One way to reduce the amount of salt discharged into the wastewater is to substitute these inorganic salts by citrate, which is biodegradable and non-toxic and hence can be discharged directly into a wastewater treatment plant [39]. PEG-citrate systems have been applied to the recovery of few biomolecules [42], namely, for the recovery of α-amylase [43], penicillin acylase [1,22,23], hexokinase [26], insulin [3], plasmid DNA [11,13,30] and monoclonal antibodies [4]. For the above reasons, we chose to study the suitability of ATPE system for phytase purification and to find the appropriate conditions concerning PEG molecular mass and citrate concentration.

The present work reports the use of ATPE for separation and purification of phytase and compares it with the conventional chromatography process. To our knowledge; this is the first attempt to evaluate purification methods for downstream processing of phytase from A. niger NCIM 563.

2. Materials and Methods

2.1. Materials

PEG of molecular weights 6000 and 8000 were procured from Himedia. All other chemicals were of analytical grade and Millipore water was used in all experiments. Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA.

2.2. Phytase production and extraction

The SSF medium contained 10g of wheat bran in 250ml Erlenmeyer flask plus glucose 3g, dextrin 1.25g, sodium nitrate 0.2g, magnesium sulphate 0.3g moistened with 20ml distilled water and sterilized by autoclaving at 121°C for 30min. On cooling fermentation medium was inoculated
with 1% spore suspension of *A. niger* NCIM 563 and incubated for 4 days at 30°C. Enzyme production was expressed as enzyme activity IU/g DMB. Phytase extraction from koji was done as mentioned [21] and the specific activity of crude enzyme was approximately up to 5 U/mg of protein. The crude extract was stored at 4°C and used as and when required for the experiments.

2.3. Analytical methods

Phytase measurements were carried out at 50°C. The reaction mixture consisted of 3mM sodium phytate buffered with 100mM acetate buffer (pH 5.5). Enzymatic reactions were started by the addition of 50ml of enzyme solution. After 30min at 50°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [14]. A freshly prepared solution of acetone: 5 N H₂SO₄: 10mM ammonium molybdate (2:1:1 v/v/v) and 400µl 1M citric acid was added to the assay mixture. Absorbance was measured at 370nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1µm P/min under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed. Concentration of protein was determined using Lowry method [19] using bovine serum albumin as standard.

2.4. Purification of phytase by ATPE

Predetermined and weighed quantities of polymer (PEG), salt (sodium citrate) and citric acid were used for obtaining the binodal curves estimated using cloud point method as described by Albertsson [2]. ATPE system is prepared in 15ml centrifuge tubes by adding the appropriate amount of PEG, salt and crude phytase enzyme. Distilled water was added to obtain 5g of the final weight. The contents were mixed thoroughly for 1h using magnetic stirrer for equilibration and was allowed to stand for phase separation. After clear phase separation, the top and bottom phases were collected and analyzed for protein concentration and enzyme activity according to the procedures.
reported in the following sections. The experiments were conducted on micro-scale (5g in centrifuge tube) and macro-scale (100g in separatory funnel). All the experiments were repeated three times and average values are reported.

The partition coefficient, purification factor, activity recovery and phase volume ratios were calculated as follows:

**Partition Coefficient**

The partition coefficient in the aqueous two phase systems is defined as

\[
\text{Partition coefficient } (K_e) = \frac{A_b}{A_i} \quad (1)
\]

where, \(A_b\) and \(A_i\) are the activities of phytase (IU/ml) in bottom phase and crude extract, respectively. Proteins were not partitioned in top phase but precipitated at the interface. The \(K_e\) is therefore calculated using the activities of protein and crude extract as employed in [28].

**Purification Factor**

The purification factor of phytase is defined as the ratio of specific activity of phytase in bottom phase to that of crude extract and calculated by the following equation:

\[
\text{Purification factor } (PF) = \frac{A_b}{A_i} \times \frac{P_i}{P_b} \quad (2)
\]

where, \(P_i\) and \(P_b\) is the protein concentration in the crude extract and bottom phase, respectively and \(A_i\) and \(A_b\) are the activities of phytase (IU/ml) in crude extract and bottom phase, respectively [25].
Percentage Enzyme Activity Recovery

The enzyme activity recovery was calculated for the bottom phase because enzyme
was preferentially partitioned to this phase.

\[
\text{Activity recovery (\%)} = \frac{A_b V_b}{A_i V_i} \times 100
\]  
(3)

where, \(A_i\) and \(A_b\) are the phytase activities (U/ml) in crude extract and bottom
phase, respectively. \(V_i\) and \(V_b\) are the corresponding volumes [25].

Phase volume ratio

The phase volume ratio is defined as the ratio of volume of the top and bottom phases

\[
\text{Phase volume ratio (V_r)} = \frac{V_t}{V_b}
\]  
(4)

where, \(V_t\) and \(V_b\) are the volumes of top and bottom phases, respectively [25].

2.5. Purification of phytase by chromatographic method

The purification of phytase by chromatography protocol was carried out in five steps:

Step 1- In the first step, 50ml of 2% aqueous solution of CaCl₂·2H₂O was added to fermented
koji in flask and kept at 200 rpm for 2h at room temperature for extraction of enzyme. The
suspension was squeezed through a double layer of muslin cloth and centrifuged at 5000xg for 20
min at 4°C. The clear supernatant was designated as the crude enzyme preparation.

Step 2- Crude enzyme obtained was subjected to ammonium sulphate precipitation (95% saturation) with constant stirring. The precipitate was collected by centrifugation (5000xg, 20min) and dissolved in minimum volume of 50mM sodium acetate buffer, pH 5.5.
Step 3- The enzyme was desalted by passing it through Sephadex G-25 column and fractions were estimated for phytase activity and dialyzed overnight at 4°C against the same buffer.

Step 4- The dialyzed solution was subjected to hydrophobic column chromatography using Phenyl-Sepharose CL-4B (30ml bed volume), previously equilibrated with 30% ammonium sulphate in 20mM acetate buffer, pH 5.5. The column was washed thoroughly with 20 bed volumes of the above buffer and eluted with a 120ml linear decreasing gradient of ammonium sulphate (30–0%) with a flow rate of 20ml/h and approximately 3ml fractions were collected. Fractions showing activity at pH 5.5 were pooled and estimated for phytase activity.

Step 5- The fractions were concentrated by rotavapor and loaded on a Sephacryl S-200 gel filtration column with a flow rate of 12 ml/h and 2ml fractions were collected. All the purification procedures were carried out at 4°C.

2.6. SDS-PAGE

The purified samples obtained from ATPE and chromatography separation were analyzed by SDS-PAGE. The samples were boiled for 5min with the presence of 1% SDS, 80mM 2-mercaptoethanol, 100mM Tris–HCl buffer (pH 6.8) and 15% glycerol and loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, the gels were stained with silver staining protocol [8].

2.7. Thermal stability of chromatography phytase versus ATPE phytase

The thermal stability was studied up to 60-80°C and the residual enzyme activity was determined using standard assay conditions and compared with the control without incubation.

3. Results and Discussion

Phytase partitioning in a PEG-citrate ATPE system was studied systematically by varying one variable at a time (OVAT). Since solid state phytase has a pH optimum at around 5.6 and is stable
up to 40°C; all experiments were carried out at pH 5.6 and at room temperature (25°C). Using this approach, the effects of variables, viz., concentration of citrate, molecular weight and concentration of PEG, and the influence of process parameters such as pH, protein loading on phytase partitioning behavior was studied. Multistage extraction and polymer recycling experiments were also studied for the purification of phytase. The results obtained are discussed below.

3.1. Effect of sodium citrate concentration on phytase partitioning

In order to identify the citrate concentration suitable for purification of phytase, ATPE experiments were carried out by adding predetermined weighed quantity of PEG 6000 and different concentrations of citrate salt to crude phytase extract (1ml) making the total weight of the system 5g in 15ml centrifuge tube. The results are shown in Table 1. Phytase is selectively partitioned to bottom phase and the activity recovery was 86.8% with a $PF$ and $V_r$ value of 1.63 and 1.0, respectively. Due to the high molecular weight of PEG and molecular exclusion mechanism, phytase is preferentially driven to the salt phase while the other proteins precipitate at the interface because the solubility in PEG-rich phase is low.

<table>
<thead>
<tr>
<th>Citrate concentration (%)</th>
<th>Partition coefficient ($K_e$)</th>
<th>Specific activity (U/mg)</th>
<th>Activity recovery (%)</th>
<th>Purification factor ($PF$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.76</td>
<td>16.0</td>
<td>76.0</td>
<td>1.52</td>
</tr>
<tr>
<td>20.5</td>
<td>0.87</td>
<td>16.3</td>
<td>86.8</td>
<td>1.63</td>
</tr>
<tr>
<td>18</td>
<td>0.71</td>
<td>14.3</td>
<td>71.0</td>
<td>1.35</td>
</tr>
<tr>
<td>15.2</td>
<td>0.62</td>
<td>13</td>
<td>61.9</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Systems comprising high concentrations of polymer and salt have been reported to partition biomolecules at the interface due to the combined influence of both volume exclusion and salting out effects. Such effects have been observed and discussed for whey proteins [31] and alcohol dehydrogenase [20].

3.2. Effect of PEG molecular weight on phytase partitioning

It has been demonstrated that the molecular weight of PEG affects its distribution in the two phases and polymer–protein interactions [9]. In order to make selection of suitable molecular weight of PEG for purification of phytase, ATPE was performed with different molecular weights of PEG (MW 6000, 8000 and a combination of 6000 and 8000). Other parameters such as citrate, phase volume ratio, temperature and pH of system were kept constant as in Section 3.1. The partitioning coefficient, activity recovery, specific activity, and purification factor for phytase in bottom phase of ATPE with different PEG are presented in Table 2.

Combination of PEG 6000 and 8000 exhibits a better partition coefficient ($K_e$) of 0.96 with the highest activity recovery of 96% (Table 2). However PEG 6000 and 8000 at 10.5% exhibits lower activity recovery 90.5% and 86.8%, respectively.
### Table 2 Effect of polymer molecular weight on phytase partitioning

<table>
<thead>
<tr>
<th>Run number</th>
<th>PEG molecular mass</th>
<th>PEG Concentration (%)</th>
<th>Partition coefficient ($K_e$)</th>
<th>Activity Recovery (%)</th>
<th>Purification Factor ($PF$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6000</td>
<td>9.5</td>
<td>0.42</td>
<td>41.8</td>
<td>0.68</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>90.5</td>
<td>0.90</td>
<td>0.68</td>
<td>2.05</td>
</tr>
<tr>
<td>3</td>
<td>11.5</td>
<td>39.9</td>
<td>0.39</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6000+ 8000</td>
<td>10.5</td>
<td>0.96</td>
<td>96.0</td>
<td>2.30</td>
</tr>
</tbody>
</table>

Phase system: PEG-citrate (20.5%), pH 5.6 at 25±2°C.

Lowering the average molecular weight of PEG is a strategy often employed to increase partition coefficient for the protein of interest and presumably occurs by lowering the hydrophobicity of PEG-rich phase. The molecular weight distribution of PEG can also be easily manipulated by mixing fractions of different average molecular weight PEG and was therefore studied. The mixture of PEG of different molecular weight and its distribution in ATPE influences the phase diagram [16] and results in reduction of free volume and low solubility for other proteins that now precipitate at the interface. Advantageously, phytase which has a high affinity for citrate is selectively partitioned to the bottom phase with high recovery of 96% and $PF$ value of 2.3.
3.3. *Effect of protein load on phytase partitioning*

The protein content of crude extract loaded in ATPE can alter the partition behavior of target protein, i.e., phytase. Therefore, ATPE experiments were carried out by varying the protein loads up to 2.8mg protein/5gm ATPE (PEG 6000+8000) in centrifuge tube. Figure 1 illustrates the effect of protein load on phytase purification factor. Based on the results, crude protein load of 1.68mg gave the highest $PF$ value of 2.4. Further, increase in protein content of crude extract results in a decrease of the ATPE performance as observed by the decrease in $PF$ to a value of 2.2.

**Fig 1** Effect of protein load on phytase partitioning

Purification factor (---■---)

Phase system: PEG (6000+8000)-citrate [10.5/20.5%] at 25±2°C. ATPE system was comprised of crude protein load ranging from 0.1 to 3.0 mg/ml.

This behavior can be explained by the increasing accumulation of precipitate at the interface that affect protein and phytase partitioning and gives low $PF$ value for phytase. The process studied on micro-scale (5g) was successfully scaled-up to 100g on macro-scale in a separatory funnel. It is
clear that a maximum of 33mg crude enzyme in 100g ATPE gives the maximum recovery of phytase and this result is therefore encouraging from the industrial point of view.

### 3.4. Effect of pH on partitioning of phytase

The pH could affect the partition either by changing the charge of the solute or by altering the ratio of the charged species present. To study the influence of pH on phytase partitioning for the selected phase system (PEG 6000+8000-citrate), experiments were performed in the pH range 5.0-6.5 and the results are presented in Table 3. These pH values were chosen so that the aqueous phase is neither too acidic nor basic because the solutions cannot be discharged to the environment without further treatment. The pH of ATPE was controlled by adjusting the ratio of sodium citrate to citric acid and thus regulating the ratio of trivalent to divalent citrate ions.

**Table 3** Effect of pH on phytase partitioning

<table>
<thead>
<tr>
<th>pH</th>
<th>Specific activity (IU/mg)</th>
<th>Activity recovery (%)</th>
<th>Purification factor (PF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>9.2</td>
<td>50.0</td>
<td>1.75</td>
</tr>
<tr>
<td>5.6</td>
<td>14</td>
<td>98.5</td>
<td>2.50</td>
</tr>
<tr>
<td>6.0</td>
<td>6.12</td>
<td>92.6</td>
<td>1.20</td>
</tr>
<tr>
<td>6.5</td>
<td>6.0</td>
<td>90.6</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Phase system: PEG 6000+8000-citrate, 10.5 / 20.5 (%) at 25±2°C

The iso-electric point (pI) of phytase obtained from solid state fermentation is unknown. But all the fungal, bacterial and plant phytase enzymes investigated so far have acidic pI values in the range of 3.65-5.2 for *Aspergillus* spp [40]. The PEG-citrate system are known to have almost no ability for resolving proteins on the basis of surface charge differences because no relation is found
between $K_\alpha$ and charge density. Instead the trivalent to divalent ratio of citrate ions in PEG-citrate system play a major role in separation [10]. Therefore it may be concluded that the enhanced affinity for the bottom phase is due to decrease of the salting-out effect in the lower phase and less hydrophobic interaction in upper PEG-rich phase rather than the effects of surface charge on the protein. At pH 6.5 and 5, the activity recovery is 90% and 50%; respectively, with an optimum pH 5.6 for the efficient separation and purification of phytase as seen in Table 3.

Thus, the optimal ATPE system for phytase purification consisted of 10.5% of PEG 6000+8000 and 20.5% of citrate at pH 5.6 as seen in Table 3. The same system also provided the best activity recovery (98.5%) in the bottom phase with a $PF$ value of 2.5. The addition of different inorganic salts, such as NaCl and KCl was studied as these salts are often used to improve and direct selectivity partitioning between the phases. These salts help in increasing the hydrophobicity and promote the partitioning of hydrophobic proteins in polymer-rich phase [34, 36]. But no further improvement in phytase partitioning is observed upon addition of neutral salts in optimized ATPE indicating that the composition of the PEG-citrate is not influenced by them.

3.5 Multistage extraction and recycling of PEG for the purification of phytase

Multistage extractions were carried out to increase the purity of phytase where beginning from the optimized ATPE system (Table 3, pH 5.6) repeated extractions were performed. In the second extraction, 10.5% PEG (6000+8000) was added to the bottom phase of the first extraction. The concentration of PEG was chosen to obtain a $V_r$ of 1.
The aliquots of the separated phases after the second extraction were analyzed for $PF$ of phytase. In the third extraction, the same procedure was followed using the bottom phase of the second extraction. It was found that in the third extraction, the $PF$ increased to 3.6 from 2.5 (i.e., by a factor of 1.4) with 80% enzyme activity recovery (Table 4). Further extraction attempts resulted in decreased activity recovery due to lower concentrations of citrate salt affecting the pH.

Recycling the components of both phases is important to assure the low-cost of this process and provides an environment-friendly way to avoid unnecessary disposal of chemicals. As phytase is preferentially extracted into the salt phase, the PEG-rich phase may be reused. To study the effect of polymer recycling, used polymer (PEG 6000+8000) was subjected to reuse for subsequent extractions, i.e., up to 8 cycles. It was observed that in the latter cycles, i.e., 5–8, partitioning of phytase to the salt-rich phase was affected with low activity recovery. However, limited recycling of the PEG-rich top phase, i.e., up to four cycles, has a minimum effect on the enzyme activity recovery (98.5%). The result shows that recycling of PEG is possible and this feature can help in process economics.
3.6. Chromatography protocol

The enzyme was extracted from the fermented koji and subjected to ammonium sulphate precipitation and desalting by Sephadex G-25 procedure which resulted in 69% enzyme recovery with purification factor of 2.5 and specific activity of 49.83 IU/mg of protein. The enzyme was further purified by adsorption and elution ion-exchange chromatography on Phenyl-Sepharose CL-4B, followed by gel filtration on Sephacryl S-200. A purification factor of 24.89 was possible although with a low phytase yield of 20%.

3.7. Comparison of ATPE and chromatographic phytase: Separation and purification

It was found that the obtained purification parameter for phytase is higher in ATPE protocol (Table 5). The chromatographic separation involves four major steps (ammonium sulphate precipitation, desalting, hydrophobic ion chromatography and gel filtration), two steps of dialysis and one step of conditioning for obtaining the required concentration.
Table 5 Comparison of downstream processing process of phytase by chromatography and ATPE protocol

<table>
<thead>
<tr>
<th>Property</th>
<th>Chromatography protocol</th>
<th>ATPE protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purification process</strong></td>
<td>Commonly employed</td>
<td>First report</td>
</tr>
<tr>
<td>Ionic interactions and</td>
<td></td>
<td><strong>Enzyme:</strong> Size, shape, charge, pl, MW, hydrophobicity.</td>
</tr>
<tr>
<td>molecular weight</td>
<td></td>
<td><strong>Phase system:</strong> Concentration</td>
</tr>
<tr>
<td><strong>Basis of enzyme</strong></td>
<td></td>
<td>and MW of polymer/salt, pH, protein load, temperature, addition of salt/specific ligand</td>
</tr>
<tr>
<td>separation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pre-treatment of crude enzyme</strong></td>
<td>Repeated centrifugation due to presence of solid particles</td>
<td>Not required</td>
</tr>
<tr>
<td><strong>Capacity</strong></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td><strong>Operation Temp</strong></td>
<td>Low</td>
<td>Room temperature</td>
</tr>
<tr>
<td><strong>Operation Time (hrs)</strong></td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td><strong>Phytase recovery (%)</strong></td>
<td>20</td>
<td>98.5</td>
</tr>
<tr>
<td></td>
<td>Cleaning in place (CIP), Storage and sanitization is mandatory</td>
<td></td>
</tr>
<tr>
<td><strong>Maintenance</strong></td>
<td></td>
<td>Less</td>
</tr>
<tr>
<td><strong>Unit operations</strong></td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td><strong>Phytase enzyme</strong></td>
<td>Thermo-labile</td>
<td>Thermo-stable</td>
</tr>
</tbody>
</table>

The multi-step chromatography process with long processing times (96hrs) however appears to less suitable for phytase purification. Besides it has several other shortcomings such as enzyme
pretreatment, low yield (20%), complex scale-up, high material cost, low capacity and high labor cost.

In contrast, the ATPE system involves a single step operation and has several advantages such as no pretreatment, high yield (98.5%), easy scale-up, low material cost, high capacity and low labor cost. Furthermore, volume reduction, good reproducibility, shorter process time (3hrs), polymer recycling and scope for continuous operation are additional features. Thus, ATPE has considerable potential for the separation and purification of phytase as compared to chromatography process.

The purity of phytase in ATPE process was also comparable to that obtained from the chromatographic separation. Figure 2 shows the SDS-PAGE analysis of phytase obtained by ATPE and column chromatography. The purified enzyme from ATPE in bottom phase and chromatography methods appears as a single band on silver stained SDS-PAGE, corresponding to a molecular mass of 85kDa. The reduction in number of bands compared to the crude extract indicates the purification of phytase. Also, the top phase of ATPE did not show any protein bands as discussed earlier.
**Fig 2 SDS PAGE**

a) Purification steps of phytase using chromatography protocol.
Lane 1-Sample from crude extract; Lane 2-Sample from G25 column; Lane 3-Sample from Phenyl sepharose CL-4B column; Lane 4-Sample from Sephacryl column; Lane 5-Molecular standard markers

b) Purified enzyme by ATPE containing PEG 6000+8000- citrate (10.5 /20.5 %)
Lane 1-Top phase of ATPE system; Lane 2-Bottom phase of ATPE system; Lane 3-Molecular standard markers

The other known methods [12, 35] studied for the recovery and purification of phytase from different sources are compiled in Table 6. It clearly shows that the chromatography method seems to be less suitable for downstream processing of phytase and improvements are especially needed with respect to yield, purity, and energy consumption. In comparison, ATPE of phytase from *A. niger* NCIM 563 as shown here represents a simple and efficient process that has considerably improved upon all the above features.
### Table 6 Recovery and purification of phytase using different separation techniques

<table>
<thead>
<tr>
<th>Type</th>
<th>Source of phytase</th>
<th>Purification method</th>
<th>Number of steps</th>
<th>Phytase Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td><em>Escherichia coli</em></td>
<td>ASF, DS, IEC, HIC &amp; GF</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella terrigena</em></td>
<td>ASF, DS, IEC &amp; GF</td>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td>Yeast</td>
<td><em>Schwanniomyces castellii</em></td>
<td>Conc, Anion exchange &amp; GF</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Candida krusei WZ-001</em></td>
<td>UF, IEC, HIC &amp; GF</td>
<td>4</td>
<td>6.00</td>
</tr>
<tr>
<td></td>
<td><em>Pichia. anomala</em></td>
<td>AP, IEC and DEAE-Sephadex</td>
<td>3</td>
<td>20.0</td>
</tr>
<tr>
<td>Plant</td>
<td>Oat</td>
<td>ASF, AP, IEC &amp; GF</td>
<td>7</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>Faba bean</td>
<td>ASF, AP, IEC and GF</td>
<td>7</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td><em>A. ficuum</em></td>
<td>UF, IEC &amp; chromatofocusing.</td>
<td>3</td>
<td>58.0</td>
</tr>
<tr>
<td></td>
<td><em>A. oryzae</em></td>
<td>UF, IEC 2 types and GF</td>
<td>4</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td><em>A. niger SK 57</em></td>
<td>UF, IEC 2 types, GF &amp; CF</td>
<td>5</td>
<td>26.0</td>
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<tr>
<td></td>
<td><em>A. niger ATCC 9142</em></td>
<td>UF, IEC, GF &amp; CF</td>
<td>4</td>
<td>26.0</td>
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<tr>
<td></td>
<td><em>Thermomyces lanuginosus</em></td>
<td>Lyophilization, DS, DEAE sepharose &amp; GF</td>
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<td>3.44</td>
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<tr>
<td>Fungal</td>
<td><em>A. ficuum NTG 23</em></td>
<td>Conc, IEC, DEAE cellulose, CM cellulose &amp; GF</td>
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<td>23.0</td>
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<tr>
<td></td>
<td><em>A. niger NCIM 563</em></td>
<td>ASF, Desalting, HIC, and GF</td>
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<tr>
<td></td>
<td>(Present work)</td>
<td>ATPE</td>
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<td>98.5</td>
</tr>
</tbody>
</table>

Acetone precipitation-AP, Ultra filtration-UF, Concentration-Conc, Desalting-DS, IEC-Ion exchange chromatography; HIC Hydrophobic ion chromatography; GF-Gel filtration; ASF-Ammonium sulphate fractionation; CF-Chromatography focusing
3.8. Thermostability studies

Thermostability is prerequisite for the successful application of phytase in animal feed because of exposure at 60–90°C during pelleting process for a few min. The poor thermostability of existing phytase enzymes is still a major concern for animal feed applications. Engineering of phytase and search for the determinants of its thermostability is of current research interest [7].

Phytase from *A. niger* is thermo-labile and it is assumed that the fermented koji be dried and then used in animal feed. In practice, the step of drying at high temperature and the presence of proteases lowers the phytase activity in the dried product. Our results show that the chromatography purified phytase is less thermo-stable at 60°C and 80°C as compared to ATPE purified phytase which exhibits maximum of 90% and 93% activities after 60 and 1 min respectively (Fig 3).

**Fig 3** Thermostability studies

Residual activity of phytase purified by chromatography separation (---■---, ---▲--- and ---○---) and ATPE (---□---, ---Δ--- and ---○---) at 60°C, 70°C and 80°C, respectively.
In fact, ATPE purified phytase exhibits 4 times better thermostability profile at 60°C as compared to chromatography purified process and is therefore likely to withstand the high temperature required for product formulation. The improvement in the thermostability of enzymes has earlier been reported [17] and ascribed to presence of salts and PEG which increases the heat transfer resistance and consequently protects the enzyme from heat.
4. Conclusion

Development of a viable process for phytase recovery and purification with techno-economic feasibility is necessary as the available methods have several limitations. This is the first report to show phytase extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE. The speed and simplicity of this eco-friendly process with high throughput, improved thermostability and recycling of polymer are additional advantages. The results presented in this work show that the ATPE technique has considerable potential for the commercial development of an efficient process for separation and purification of phytase obtained from *A. niger*. 
References


