This chapter is an introduction to the thesis and starts with the rationale and significance behind the work, carried out in this thesis followed by an overview of phytase production and purification. Although P is a basic component and energy conduit of life, it does not have a cycle to constantly replenish its supply. So dephosphorylation of phytate, a P locking molecule, by phytase is a crucial process. This chapter describes recent findings concerning the production, biochemical properties, molecular characteristics, and expression of phytases. Several potential applications of the phytases in animal nutrition, human health, and synthesis of lower myo-inositol phosphates are also summarized.
1. General 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.

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. P is non-renewable and at the current extraction and usage rate, the existing phosphate reserves will be exhausted in next 80 years.

Use of phytase in animal feed will seize the anti-nutritional effects of phytate, decrease environmental pollution, increase availability of starch, protein, amino acids, calcium and P and abolish the addition of inorganic phosphate in animal feed. They are also imminent candidates for production of special isomers of different lower phosphate esters of myo-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers. The FDA has approved “generally recognized as safe (GRAS)” petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996. All these factors have concurrently made it as the third largest feed enzyme.
Elemental P (Fig. 1) exists in two major forms- white and red P but due to its high reactivity, P is never found as a free element on Earth. The P cycle is the biogeochemical cycle that describes the movement of P through the lithosphere, hydrosphere, and biosphere. It is widely distributed in many minerals, mainly phosphates. Phosphate rock is a non-renewable natural resource, mainly found in sedimentary and igneous deposits. Its sustainable production and management is of critical importance. Most of the world phosphate production is used in agriculture.

P is essential to all known life forms. It is the second most abundant mineral in the human body, surpassed only by calcium. It makes about 1% of today body weight and is largely confined to the skeleton in addition to an important part of the active structure of the muscles, central nervous systems and the energy circuits. P compounds are important ingredients in high-grade detergents, cleaning agents, dental creams, toothpastes, flame retardants, stabilizer of plastics, corrosion inhibitors, dispersion agents in paints and primers and metal surface treatment.

Living cells also use phosphate to transport cellular energy in the form of adenosine triphosphate (ATP). Nearly every cellular process that uses energy obtains it in the form of ATP. ATP is also important for phosphorylation, a key regulatory event in cells (Fig. 2)


**Fig. 2 High energy P bonds**

**Effects of P –**

- In ecosystem an excess of P can be problematic, especially in aquatic systems, resulting in eutrophication which sometimes lead to algal blooms.
- Excess phosphate can lead to diarrhoea and calcification (hardening) of organs and soft tissue, and can interfere with the body's ability to use Fe$^{2+}$, Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$.
- Hypophosphatemia is an electrolyte disturbance in which there is an abnormally low level of phosphate in the blood
- Osteomalacia (deficient calcification of bones; rickets)
- Anorexia (lack of appetite)
- Reduced performance (growth, milk yield or egg production)
- Pica is an appetite for objects not fit as food. Cattle develop an appetite for bones, which often contain *Clostridium botulism*.
- Lethargy, muscle weakness, seizures, erythrocyte deformity, hemolysis.

Despite its importance, P production, utilization and recycling is a slow process due to absence of gaseous phase and thus is therefore well-known as an imperfect cycle. Existing
phosphate reserves will be exhausted in next 80 years. All animal diets must contain adequate amounts of this element. So to meet their P requirements, inorganic P especially dicalcium phosphate is supplemented in diet of livestock and poultry animals. This has made it as the third most expensive nutrient in poultry production after energy and protein.

1.2 Phytate

Phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate; IP6) is the principal storage form of P, inositol, and variety of minerals in plants, representing approximately 75–80% of the total P in plant seeds [28, 52].

Structure of IP6:

IP6 bears six phosphate groups on one six-carbon molecule with low molecular weight of 660 and molecular formula C$_6$H$_{18}$O$_{24}$P$_6$ (Fig. 3). On the basis of Andersons structure [5], the systematic name for IP6 is myo-inositol-1,2,3,4,5,6-hexakisphosphate.

Occurrence

IP6 is a primary storage of inositol and storage form of P in plant seeds that are used as animal feed ingredients (oilseed meal, cereal grains and legumes) [93]. The total P, Phytate-P and IP6 content of common poultry feed are presented in Table 1. Most foods of plant origin contain 50-80% of their total P as phytate [53].
**Fig. 3** Primary structure for myo-inositol hexakisphosphate

**Table 1** Total P and Phytate P of common poultry feedstuffs [138]

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Total P (%)</th>
<th>Phytate P (%)</th>
<th>% of total P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cereals/ Millets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>0.39</td>
<td>0.25</td>
<td>64</td>
</tr>
<tr>
<td>Rice</td>
<td>0.15</td>
<td>0.09</td>
<td>60</td>
</tr>
<tr>
<td>Wheat</td>
<td>0.44</td>
<td>0.27</td>
<td>61</td>
</tr>
<tr>
<td>Sorghum</td>
<td>0.3</td>
<td>0.22</td>
<td>73</td>
</tr>
<tr>
<td>Barley</td>
<td>0.33</td>
<td>0.20</td>
<td>61</td>
</tr>
<tr>
<td>Bajra</td>
<td>0.31</td>
<td>0.23</td>
<td>74</td>
</tr>
<tr>
<td><strong>Oilseed meals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groundnut meal</td>
<td>0.60</td>
<td>0.46</td>
<td>74</td>
</tr>
<tr>
<td>Soyabean meal</td>
<td>0.88</td>
<td>0.56</td>
<td>64</td>
</tr>
<tr>
<td>Cotton seed meal</td>
<td>0.93</td>
<td>0.786</td>
<td>82</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>0.90</td>
<td>0.45</td>
<td>51</td>
</tr>
</tbody>
</table>
Physiological nature of phytase

Phytate can exist in a metal-free form or in a metal–phytate complex, depending on the pH of the solution and the concentration of metal cations (Fig. 4A). At acidic pH, protonation of the phosphate groups of phytate generates the metal-free form. At neutral pH, in contrast, deprotonation of the phosphate groups of phytate enhances the affinity for divalent metal cations and thus phytate forms metal–phytate complexes with divalent metal cations, mostly Mg$^{2+}$ and Ca$^{2+}$ [23, 91].

![Fig. 4 Effects of pH and divalent metal cations on physiological nature of phytate](image)

A Phytate exists as a metal-free phytate or a metal–phytate complex, depending on the pH and divalent metal cations. The extent of binding is dependent upon both pH and divalent metal cations to phytate ratios. In addition, at acidic pH and high cation concentration, a metal–phytate complex is formed due to direct electrostatic interaction.

B Divalent metal cations specifically bind to the phosphate groups of phytate, depending on the ionic radii of the metal cations. The formation of the bidentate metal complex prefers metal cations with large ionic radii.
In the metal–phytate complex, divalent metal cations with large ionic radii, such as Ca$^{2+}$ (0.99 Å) and Sr$^{2+}$(1.12 Å), bind two oxianions from the phosphate groups of phytate in a bidentate fashion [95]. However, divalent metal cations with small radii, such as Mg$^{2+}$ (0.65 Å), Fe$^{2+}$ (0.74 Å), and Zn$^{2+}$ (0.71 Å), bind in a monodentate fashion within two oxygen atoms from the phosphate groups of phytate (Fig. 4B). Therefore, bidentate metal-complex formation prefers divalent metal cations with large ionic radii [66].

**Negative aspects of phytate**

Table 2 presents an overview of the negative interactions of phytate with nutrients and the mode of actions for the negative effects of phytate [76].

**Table 2** Negative interaction of phytate and nutrients in food

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral ions (zinc, iron,</td>
<td>Formation of insoluble phytate-mineral complexes leads to decrease in mineral</td>
</tr>
<tr>
<td>calcium, magnesium,</td>
<td>availability</td>
</tr>
<tr>
<td>manganese and copper)</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Formation of nonspecific phytate-protein complex, not readily hydrolysed by</td>
</tr>
<tr>
<td></td>
<td>proteolytic enzymes</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Formation of phytate carbohydrate complexes making carbohydrate less</td>
</tr>
<tr>
<td></td>
<td>degradable. Inhibition of amylase activity by complexing with Ca$^{2+}$ ion</td>
</tr>
<tr>
<td></td>
<td>and decrease of carbohydrate degradation</td>
</tr>
<tr>
<td>Lipid</td>
<td>Formation of ‘lipophytin’ complexes, may lead to metallic soaps in gut lumen,</td>
</tr>
<tr>
<td></td>
<td>resulting in lower lipid availability</td>
</tr>
</tbody>
</table>
Effect of mineral uptake

Six reactive groups in the molecules of IP6 make it a strong chelating agent that binds cations Ca\(^{2+}\), Mg\(^{2+}\), Fe\(^{2+}\), Zn\(^{2+}\). The order of the ability of the mineral cations to form complexes with phytate in vitro has been found to be: Cu\(^{2+}\) > Zn\(^{2+}\) > Cd\(^{2+}\) at pH 3–7 [108]. Dietary phytate forms an insoluble phytate-mineral complex. These complexes are not readily absorbed by the human gastrointestinal tract, which reduces the bioavailability of minerals. Moreover, the small intestine of the human is devoid of phytate degrading enzyme and also the microbial population in the upper part of the digestive tract is limited [62].

Effect on protein digestibility

Phytate forms a strong complex with some proteins and resists their proteolysis. In general, the interaction of phytate with protein is dependent on pH. At a pH value lower than the isoelectric point of proteins [68], phosphoric acid groups of phytate bind with the cationic group of basic amino acid, e.g., arginine, histidine, lysine, and form binary protein–phytate complexes. They are insoluble complexes that dissolve only below pH 3.5. Such complex formations may affect the protein structures that can hamper enzymatic activity, protein solubility and protein digestibility.

Effect on carbohydrate utilisation

Phytate may bind with starch either directly, via hydrogen bonds, or indirectly via proteins associated with starch [120].

Effect on lipid utilization

Phytate forms ‘lipophytins’ (complexes with lipid and its derivatives), along with other nutrients. Lipid and Ca phytate may be involved in the formation of metallic soaps in gut lumen of poultry, which is a major restraint for energy utilisation derived from lipid sources [77].
Environmental perspective

Bound P (18-88% of total P content) in fact exists as phytate which is already present in animal feed. But this phytate P is not utilized by monogastric animals like poultry and pigs due to lack of intrinsic phytase in their gastrointestinal tracts. Phytate in addition acts as an antinutrient by chelating various cations such as Ca$^{2+}$, Fe$^{2+}$, Zn$^{2+}$ and Mg$^{2+}$ and thereby reducing their bioavailability. This unutilized phytate is the origin of P pollution as it builds up in areas of livestock production leading to eutrophication and algal blooms [103].

Degradation of phytate

The dephosphorylation of phytate is a prerequisite for improving nutritional value because removal of phosphate groups from the inositol ring decreases the mineral binding strength of phytate. This results in increased bioavailability of essential dietary minerals [126].

Non-enzymatic hydrolysis of phytates normally happens under high temperature conditions. By autoclaving at 121°C for 1h, Phillippy et al [112] studied the hydrolysis of IP6 (InsP6) and found that at pH 1.0, 2.0, 4.0, 6.0, 8.0, and 10.8, the percentages of InsP6 decomposed were 67.7, 76.8, 89.6, 81.9, 65.8, and 45.1%, respectively. The hydrolysis products were a variety of isomers of InsP1 to InsP5 (Myoinositol mono-, bis-, tris-, tetrakis-, and pentakis-phosphate). They also found that in the pH ranges of 1.0–10.8, the lower the pH, the more even distribution of inositol phosphate isomers. Based on this property, Chen et al [22] prepared reference standards of myoinositol phosphates by heating InsP6 solution which contains 2M HCl at 140°C for 1h, obtaining a total of 27 peaks representing InsP2–InsP6 isomers. During the process, the decomposition percentage of InsP6 is 95.3%. 
Enzymatic hydrolysis of phytate

Phytases are chemically known as myo-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolase, and catalyze the sequential release of phosphate from phytate. Phytase sequesters orthophosphate groups from the inositol ring of IP6 to produce free inorganic P, along with a chain of intermediate myo-inositol phosphates (inositol pentaphosphate to inositol monophosphate) [34]. Phytase not only releases the P from plant-based diets but also makes available calcium, magnesium, protein and lipid. Thus, by releasing bound P in feed ingredients of vegetable origin, phytase makes more P available for bone growth and protects the environment against P pollution [11].

1.3 Phytase

In recent years, considerable efforts have been made to improve nutritive value of animal feedstuff through supplementation with exogenous enzyme. Currently used feed enzymes are divided into two main groups, the hemicellulases and phytases. Phytases myo-inositol hexaphosphate phosphorhydrolase) hydrolyze IP6 to myo-inositol and inorganic phosphates through a series of myo-inositol phosphate intermediates, and eliminate its anti-nutritional characteristics.

Source

In general, there are four possible sources: plant phytase, microbial phytase (fungal and bacterial phytase), phytase generated by the small intestinal mucosa and gut-associated micro floral phytase. Generally, phytase activity of animals is negligible compared to their plant and microbial counterparts [146]. Most of the scientific work has been done on microbial phytases, especially on those originating from filamentous fungi such as Aspergillus ficuum, Mucor piriformis and Cladosporium species [132]. Although some plants such as wheat and barley are rich in intrinsic
phytase, because of a narrower pH spectrum of activity and low heat stability their phytase activity is less effective than microbial phytases. Additionally, the bio-efficacy of plant phytases was only 40% compared to microbial phytases [156].

**Diversity of phytases**

Since the first phytase was found by Suzuki et al [134] in 1907, many different phytases from a variety of sources have been discovered and described. The International Union of Biochemists [63] currently distinguishes between three classes of phytase enzymes depending on the position (3, 6 or 5) on the inositol ring where the dephosphorylation is initiated as shown in Fig. 5.

1. **3-phytases** - (EC 3.1.3.8) yield 1, 2, 4, 5, 6-pentakisphosphate, does not always completely dephosphorylate IP6 and are normally produced by microorganism.

2. **6-phytases** - (EC 3.1.3.26) give 1,2,3,4,5- pentakisphosphate as the first product along with Pi and always completely dephosphorylate IP6 and are present in plants [100, 119].

3. **5-phytases**- (EC 3.1.3.72) from *Medicago sativa*, *Phaseolus vulgaris*, and *Pisum sativum* initiate phytate hydrolysis at the fifth phosphate group.

   However, there are some exceptions: soybean phytase is a 3-phytase [109] and *Escherichia coli* phytase is a 6-phytase [48].
Based on biochemical properties and amino acid sequence alignment, phytases can be categorized into two major classes [104] (Fig. 7)

1. **Histidine acid phosphatase** – This class shows broad substrate specificity and hydrolyzes metal-free phytate at the acidic pH range and produces myo-inositol monophosphate as the final product.

2. **Alkaline phytase** – This class exhibits strict substrate specificity for the calcium–phytate complex and produces myo-inositol triphosphate as the final product. Phylogenetic analysis clearly shows that alkaline phytases are not a subfamily of HAPs but are indeed novel phytases (Fig 5). Despite considerable differences between alkaline phytases and HAPs, only limited knowledge on the biochemical and catalytic properties of alkaline phytases is currently available. More focus has been on acidic phytases because of their applicability in animal feed and broader substrate specificity than those of alkaline phytases. On the basis of their catalytic properties, phytases are classified as histidine acid phosphatases (HAP), β propeller phytase (BPP), and purple acid phosphatases (PAP) [99].
Fig. 6 Schematic illustrations of substrate hydrolysis by histidine acid phosphatases (HAPs) and alkaline phytases [128]

Fig. 7 Phylogenetic analysis of various HAPs and alkaline phytases [102]
Market trend and manufacture

Recent market trends have clearly shown that enzymes have emerged as big feed supplements. Feed enzymes (protease, xylanase, phytase, amylase, cellulase, lipase, β-glucanase) are the newest segment of the $5 billion animal nutrition market, which is increasing fast. Presently, only about 6% of manufactured animal feeds contain enzymes, against 80±90% for vitamins, which is considered as the largest animal nutrition category. The first phytase product, which entered the feed market in 1991, was manufactured by Gist Brocades (now DSM) and sold by BASF under the trade name Natuphos. Natuphos is available as powder, granulate, or liquid formulation.

Table 3 Commercial phytase preparations

<table>
<thead>
<tr>
<th>Company</th>
<th>Country</th>
<th>Phytase source</th>
<th>Production strain</th>
<th>Trademark</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB Enzymes</td>
<td>Germany</td>
<td>A. awamori</td>
<td>T. reesi</td>
<td>Finase</td>
</tr>
<tr>
<td>Alko Biotechnology</td>
<td>Finland</td>
<td>A. oryzae</td>
<td>A. oryzae</td>
<td>SP,TP, SF</td>
</tr>
<tr>
<td>Alltech</td>
<td>USA</td>
<td>A. niger</td>
<td>A. niger</td>
<td>Allzyme phytase</td>
</tr>
<tr>
<td>BASF</td>
<td>Germany</td>
<td>A. niger</td>
<td>A. niger</td>
<td>Natuphos</td>
</tr>
<tr>
<td>BioZyme</td>
<td>USA</td>
<td>A. oryzae</td>
<td>A. oryzae</td>
<td>AMAFERM</td>
</tr>
<tr>
<td>DSM</td>
<td>USA</td>
<td>P. lycii</td>
<td>A. oryzae</td>
<td>Bio-Feed phytase</td>
</tr>
<tr>
<td>Fermic</td>
<td>Mexico</td>
<td>A. oryzae</td>
<td>A. oryzae</td>
<td>Phyzyme</td>
</tr>
<tr>
<td>Finnfeeds International</td>
<td>Finland</td>
<td>A. awamori</td>
<td>T. reesi</td>
<td>Avizyme</td>
</tr>
<tr>
<td>Genecour International</td>
<td>USA</td>
<td>P. simplicissimum</td>
<td>P. funiculosus m</td>
<td>ROVABIO</td>
</tr>
<tr>
<td>Roal</td>
<td>Finland</td>
<td>A. awamori</td>
<td>T. reesi</td>
<td>Finase</td>
</tr>
<tr>
<td>Novozymes</td>
<td>Denmark</td>
<td>A. oryzae</td>
<td>A. oryzae</td>
<td>Ronozyme</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Roxazyme</td>
</tr>
</tbody>
</table>
Later, other products from different companies appeared, but only a limited number of commercial phytase products are currently available. These first phytases produced on commercial scale were either derived from fungal strains mutated via standard means or by using recombinant DNA technology. Several major animal nutrition companies are getting involved in this area very actively and various products under different trade names are already available as shown in Table 3 [16]. The FDA has approved “generally recognized as safe (GRAS)” petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996. The expressed phytase genes are of fungal origin and originate in most cases from the genus *Aspergillus*. At present, all phytase preparations authorized in the EU as feed additives are produced by recombinant strains of filamentous fungi.

**Ideal phytase and its designing**

The phytase that has the desirable characteristics for application in animal feed industry can be called an ‘ideal phytase’, which should be active in the stomach, stable during animal feed processing and storage, and easily processed by the feed manufacturer for its suitability as an animal feed additive. It should satisfy the following points

1. Phytase should not be detected at the end of the small intestine. This is necessary because in this way the phytase produced by genetically modified organisms should not enter the environment [65].

2. It should be effective in releasing phytate-P in the digestive tract.

3. It should be stable to resist proteases (trypsin and pepsin)

4. It should be able resist inactivation by heat during feed pelleting and storage

5. Low cost of production.

Finally, a phytase produced in high yield and purity by a relatively inexpensive system is attracting for food industries worldwide. It is now realized that any single phytase may never be
‘ideal’ for all feeds and foods. For example, the stomach pH in finishing pigs is much more acidic than that of weanling pigs [115]. Thus, phytase with optimum pH close to 3.0 will perform better in the former than in the latter. For poultry, an enzyme would be beneficial if it is active over broad pH range, that is, acidic (stomach) to neutral pH (crop) [121]. Phytases used for aquaculture application require a lower temperature that is optimum than the swine or poultry [116]. The choice of an organism for phytase production and development is, therefore, dependent upon the target application using directed evolution and protein engineering. All these features are not present within a single phytase, and therefore, based on the sequence of the available phytases, a consensus phytase could be designed [78, 79, 80].

Fig. 8 Designing of ideal phytase
Genetic engineering techniques such as site directed mutagenesis could be employed for further ameliorating the properties. The strategies used for the designing and developing of an ideal phytase are presented in Figure. 8.

1. **Immobilization**- Immobilizing phytase for application in food, feed and pharmaceutical industry and biosensor.

2. **Modification**- Active site modification for enhanced thermostability and efficient catalysis of phytase by incorporating vanadium in active site for peroxidase activity.

3. **Site directed mutagenesis**- Modification for enhanced phytase thermostability and protease resistance

4. **Transgenic expression**- Expression in plants and animal for improving their nutrition and growth.

5. **Protein engineering**- Engineering phytase for enhanced thermostability and pH stability

6. **Scale up**- Economical and large scale phytase production

7. **Stability**- Understanding the role of glycosylation in phytase stability

The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under submerged fermentation condition and have shortcomings especially with regard to obtaining diluted product, sensitivity to heat, inactivation under low pH conditions present in the stomach of animals [3] and high product recovery costs. These disadvantages need to be alleviated while at the same time producing phytase with high yield and purity
1.4 Microbial Production of phytase

Screening and assay

Several screening programmes have been carried out aiming at the isolation of different groups of bacteria yeast and fungi having extra-cellular phytase activity. Lissitskaya et al [87] screened micro-organisms producing phytase using museum and soil samples. It was found that moulds metabolized P more effectively than bacteria. Chen [21] developed a bioassay method for the screening for extra-cellular phytase-producing micro-organisms. Washed cells of Corynebacterium glutamicum were used as indicator strain. About 71% soil isolates had phytase activity above 0.01 U/ml. Gargova et al [42] used a two-step procedure to screen some 200 fungi for phytase production. A simple and rapid method has been described for determining the microbial phytase. The method consisted of determining the inorganic orthophosphate released on hydrolysis of sodium phytate at pH 5.5 [36]. Bae et al [8] developed a method for detecting phytase activity. Differential agar media were used for the detection of microbial phytase activity and the disappearance of precipitated calcium or sodium phytate was as an indication of enzyme activity. This technique, however, was unable to differentiate between phytase activity and acid production by ruminal bacteria.

Production technique

Phytases can be produced from a host of micro-organisms including bacteria, yeasts and fungi (Table 4). Submerged fermentation (SmF) has largely been employed as the production technology. However, in recent years solid state fermentation (SSF) has gained much interest for the production of phytase. Techniques of SmF as well as SSF have been employed for the production of phytases. Type of strain, culture conditions, nature of the substrate and availability of the nutrients are critical factors affecting the yield and should be taken into consideration for selecting a particular production technique. For example, a filamentous fungus in SmF is exposed
to hydrodynamic forces but in SSF the surface of the solid particles acts as the matrix for the culture.

Several bacterial strains (wild or genetically modified) such as *Lactobacillus amylovorus*, *E. coli*, *B. subtilis* *B. amyloliquefaciens*, *Klebsiella sp.*, etc., have been employed for phytase production. The fermentation technique employed is SmF with glucose and yeast extract as main carbon and nitrogen source widely used. Sreemula et al [131] evaluated 19 strains of lactic acid-producing bacteria of the genera *Lactobacillus* and *Streptococcus* for the production of extracellular phytase. A number of them exhibited the enzyme activity in the fermentation medium but *Lactobacillus amylovorus* B4552 produced the maximum amounts of phytase, ranging from 125±146 units/ml in SmF using glucose and inorganic phosphate. Sunitha et al [133] optimized the medium for recombinant phytase production by *E. coli* BL21 using response surface methodology. A 23 central composite experimental design was used to study the combined effects of the medium components, tryptone, yeast extract and NaCl. The optimized medium with glucose showed a highest phytase activity of 2250 U/l. Phytase production using yeast cultures has generally been carried out in SmF systems. The strains used include *Schwanniomyces castellii*, *Pichia*, *Arxula adeninivorans* and *Candida kruzei*. Galactose and glucose were the preferred carbon sources. Phytase production from *P. anomala* has been extensively studied using RSM.

The available phytase preparations used as feed additives are of fungal origin, produced by recombinant strains under submerged fermentation condition. There is no defined medium for optimum production of phytase from different microbial sources especially fungi because each fungus has its own special conditions and specific substrates for maximum enzyme production especially in SSF. But the reports are few because of the low productivities and difficulties associated with operating and up scaling SSF conditions as seen in Table 4.
<table>
<thead>
<tr>
<th>Microbial Strain</th>
<th>pH&lt;sub&gt;opt&lt;/sub&gt;</th>
<th>Temp&lt;sub&gt;opt&lt;/sub&gt;</th>
<th>Fermentation</th>
<th>Carbon source</th>
<th>Nitrogen source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Filamentous fungi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus SRRC 322</em></td>
<td>5</td>
<td>37</td>
<td>SmF</td>
<td>Hylon Starch</td>
<td>NaNO&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>5.5</td>
<td>30</td>
<td>SmF</td>
<td>Glucose starch</td>
<td>-</td>
</tr>
<tr>
<td><em>A. ficuum</em></td>
<td>5</td>
<td>30</td>
<td>SmF</td>
<td>Corn starch,glucose</td>
<td>NaNO&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
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<td><em>A. oryzae</em></td>
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<td>37</td>
<td>SmF</td>
<td>Glucose</td>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>27</td>
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<td>Corn starch,glucose</td>
<td>NaNO&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Glucose</td>
<td>NH&lt;sub&gt;3&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Starch,glucose,Wheat bran</td>
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<td>Wheat bran</td>
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<td>Glucose</td>
<td>NaNO&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>SmF</td>
<td>Starch,Glucose</td>
<td>Peptone</td>
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<td>45</td>
<td>SSF</td>
<td>Sesame oil cake,glucose</td>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Glucose</td>
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<td>77</td>
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<td>Glucose</td>
<td>NH&lt;sub&gt;3&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Caesin,Peptone</td>
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<td>-</td>
<td>Tryptone</td>
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<td>Glucose</td>
<td>Yeast Extract</td>
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</table>
Mutagenesis

Comparatively few reports are published regarding the improvement of phytase production via mutagenesis. Chelius and Wodzinski [20] during the strain improvement studies of *A. niger* NRRL 3135 by UV radiation, isolated a phytase catalytic mutant producing 3.3-fold higher phytase (phyA) than the wild type strain. The production of mutant phyA was highly repressed 60% by the inorganic phosphate (0.006%, w/v), however, their approach was limited by lack of specificity and sensitivity to discriminate between phytase and acid-phosphatase activity during primary screening process.

Transgenic studies

Although phytases are widely distributed in nature, the production in wild-type organisms is far from an economically viable level. Hence, cloning and expression of phytase genes in suitable host organisms is necessary in order to reach higher productivities. As the cost effectiveness of phytase production is a major limiting factor for its application, different heterologous expression systems and hosts have been evaluated. These are plants, bacteria, and fungi including yeast. As expected, each system bears some unique advantages, along with certain limitations.

1. **Plants**- The *A. niger* phyA gene has been successfully expressed in tobacco seeds [107] or leaves [141] and soybean cells [83]. But the difficulties of thermostability and phytase performance under adverse conditions are still a major concern.

2. **Fungi**- Phytase genes from *A. niger*, *A. terreus*, *A. fumigatus*, *E. nidulans*, and *M. thermophila* have all been expressed and secreted as active enzymes by *A. niger*. While fungal systems produce and secrete active phytases, other proteins, including proteases, are often produced at relatively high levels. Thus, there may be a need for further purification or inhibition of proteolysis that adds to the production cost.
3. **Yeast**- Though there are several reports of heterologous gene expression of bacterial and mold phytases in yeast expression systems, there are very few on homologous or heterologous expression of yeast phytase in microbial systems. *P. pastoris* is a potential host to express high levels of *A. fumigatus* phytase [122].

4. **Bacteria**- The obstacle in using bacterial systems to produce fungal phytases is their inability to sufficiently glycosylate the expressed proteins to the extent necessary for activity. Thus, inactive *A. niger* PhyA protein was expressed intracellularly in *E. coli* [110] and extracellularly in *Streptomyces lividans*. The glycosylation of this protein or the expression host affect the properties of the expressed enzyme in terms of yield or non active state.

### 1.5 Biochemical characterization of phytase

Phytase is an ester-hydrolyzing enzyme with an estimated molecular weight of 35–700 kDa depending upon the source of origin and are usually active within pH range of 4.5-6.0.

**Purification**

Purification studies on phytases were usually performed with an aim to study properties of phytase originating from various microbial sources. They possess distinct aspects in molecular features as well as catalytic properties depending on the source of origin. Only a few phytate-degrading enzymes have been purified to homogeneity or near homogeneity (Table 5).

Purification of phytate-degrading enzymes includes common biochemical techniques such as ammonium sulphate fractionation, acetone precipitation, gel filtration, ion-exchange chromatography, affinity chromatography and hydrophobic interaction. One major problem in the purification of phytate-degrading enzymes especially from plants is the separation of phytate-degrading enzymes from contaminating nonspecific acid phosphatases [72].
The recovery and purification of phytase has been achieved through several steps using different techniques. Boyce and Walsh [1], purified phytase from *Mucor hiemalis*, utilizing five steps (ultrafiltration, diafiltration, ion exchange, gel filtration and hydrophobic interaction), achieving 51% recovery and purification factor of 14.1; Spier et al [130] purified from *Aspergillus niger* phytase in three steps (SP Sepharose, Mono Q and single pass), obtained recovery (6.35%) and purification factor (10.1), whereas Azeke et al [7] obtained two phytases from *Rhizopus oligosporus* in five steps (Acetone Fractionation, Mono-S HR 5/50 Cationic-Exchange Chromatography, 16/60 Sephacryl S-200 HR chromatography, Mono-S HR 5/50 Cationic-Exchange Chromatography, Mono-Q HR 5/5 Anionic-Exchange Chromatography) with recovery: phytase 1 (1.3%) and phytase 2 (1.6%) and purification factor (75, 46), respectively.

**Molecular and biochemical characteristics of phytase**

Phytases are high-molecular-weight proteins ranging from 40-500 kDa. They are monomeric proteins, except for phytase B from *A.niger*, which is a tetramer. Phytases from eukaryotic organisms (yeasts, fungi, plants and animals) are often glycosylated and have higher molecular weights: 85-150 kDa for fungal phytases, around 500 kDa for yeast phytases, and 50-150 kDa for phytases from plants and animal tissues. Average molecular masses of bacterial phytases are smaller than those of fungal phytases (40–55 vs. 85–150 kDa), mainly due to glycosylation differences. Glycosylation has no effect on the specific activity and thermostability of phytases.

Most phytases have an optimal temperature of 44–60°C. In contrast, phytases from *A. fumigatus* and *B. amyloliquefaciens* have an optimum temperature of about 70°C. The phytate-degrading enzymes most resistant to high temperatures reported so far have been isolated from *A. fumigatus* and *Schwanniomyces castellii* [127]. In general, phytase enzymes of microbial origin are more pH and thermostable than their plant counterparts. The stability of most of the plant enzymes decreased dramatically at pH values below 4 and above 7.5, whereas the majority of the
corresponding microbial enzymes are rather stable even at pH values above 8.0 and below 3.0. Most phytases belong to either the acid phytases or the alkaline phytases, depending on their optimal pH for catalytic activity. Generally, the phytases from bacteria have an optimum pH in neutral to alkaline range while in fungi the optimum pH range is 2.5-6.0. All of the fungal, bacterial and plant phytate degrading enzymes investigated so far have acidic pI values with the exception of the A. fumigates enzyme, which has a pI of about 8.6. Bacterial phytases seem to be less acidic than fungal phytases: their pI is generally above 6, whereas fungal enzymes have pI values below 5.5.

Phytases usually show broad substrate spectrum with the highest affinity for phytate. The A. fumigatus, Emericella nidulans and M. thermophila phytases exhibited broad substrate specificity, while phytases of A. niger, A. terreus CBS and E. coli were rather specific for IP6. Broad substrate specificity was reported for phytases of S. castellii and S. thermophile, while cell-bound phytase from P. anomala exhibited broad substrate specificity. Only a few phytases have been described as highly specific for phytate such as the alkaline phytases from B. subtilis. The Km values of the phytases ranged between 10 and 650 µM.

Metal ions have been shown to modulate phytase activity. However, it is difficult to determine whether the inhibitory effect of various metals is due to direct binding to the enzyme, or whether the metal ions form poorly soluble complexes with IP6 and therefore decrease the active substrate concentration. Phytase activities of Bacillus sp were found to be Ca$^{2+}$ dependent. Metal depletion caused by EDTA in them resulted in complete enzyme inactivation owing to a conformational change, as evidenced by the differences observed in the circular dichroism spectra of the holozyme versus metal-depleted enzyme [69]. Both of these enzymes, as well as two other Bacillus phytases were greatly inhibited by EDTA, indicating that a metal ion (Ca$^{2+}$) is needed for the activity the molecular weight and the properties, such as optimum pH, temperature, specificity and Km of phytase previously reported in microbes are summarized in Table 5.
Chapter I Introduction

An effective phytase needs to have a strong resistance to hydrolytic breakdown by digestive proteinases in the digestive tract. Fungal and bacterial phytases show different sensitivities to pepsin and trypsin [123], and the latter seem to have a higher resistance to proteolytic degradation than the former [61]. The protease-sensitive sites of phytases, normally in the exposed loops at the surface of the molecules, may be blocked or modified using site-directed mutagenesis. Fluoride, a well known inhibitor of different acid phosphatase, was also found to be a strong competitive inhibitor of several acid bacterial, fungal and plant phytate-degrading enzymes. The reported inhibitor constants range from 0.1 to 0.5 mm. In contrast, the alkaline phytases from *B. subtilis*, *B. amyloliquefaciens* and lily pollen [9] show no reduction in activity in the presence of fluoride. Furthermore, the hydrolysis product orthophosphate was recognized as a competitive inhibitor of enzymatic phytate degradation. Molybdate, wolframate and vanadate are also known to inhibit phytate-degrading enzymes. It has been suggested that these transition metal oxoanions exert their inhibitory effects by forming complexes that resemble the trigonal bipyramidal geometry of the transition state [152].
<table>
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<tr>
<th>Phytase source</th>
<th>Mol wt (kDa)</th>
<th>Temp ( \text{opt} )</th>
<th>pH ( \text{opt} )</th>
<th>Km (mM)</th>
<th>pI</th>
<th>Specificity</th>
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<td>5.0</td>
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</table>

B-broad spectrum, P- phytate specific
1.6 Kinetics and End Products of IP6 degradation

Phytate-degrading enzymes catalyze the stepwise hydrolysis of myo-inositol hexakisphosphate to orthophosphate and lower myo-inositol phosphates. The reaction intermediates are released from the enzymes and serve as substrates for further hydrolysis. Wyss et al [149] investigated the kinetics of phosphate release and the kinetics of accumulation of reaction intermediates, as well as the end products of IP6 degradation by various phytases. They concluded that all fungal phytases studied released five of the six phosphate groups, the end product being myo-inositol 2-monophosphate when excess enzyme is used. This indicates that all of these phytases have a pronounced stereo specificity and a strong preference for equatorial phosphate groups, whereas they are virtually unable to cleave the axial phosphate group.

Only in rare cases were traces of free myo-inositol or myo-inositol 1-monophosphate detected. *A. fumigatus* phytase readily degraded IP6 to myo-inositol 2-monophosphate, and only myo-inositol bisphosphate (stereoisomer not known) accumulated to some extent. In contrast, *A. niger* and *A. terrus* phytases had to be used at much higher initial activities in order to obtain degradation down to myo-inositol 2-monophosphate, and considerable amounts of myo-inositol tris- and bisphosphates accumulated during the degradation. When *E. coli* phytase was used at an even higher initial activity, there was a pronounced accumulation of myo-inositol tetrakisphosphate during IP6 degradation. Myo-inositol bis- and triphosphates comprised more than 90% of the end products after a 90-min incubation period (with excess enzyme) and almost no myo-inositol monophosphate were detected. Therefore, lower myo-inositol phosphates appear to be less suitable substrates for *A. niger*, *A. terrus* and especially *E. coli* phytases than IP6. The stereoisomer assignment of the reaction intermediates and degradation pathway was not determined for these enzymes. The fact that the end products of IP6 hydrolysis for most phytases is identical do not necessarily means that the degradation pathways for IP6 are identical. 3-Phytase starts hydrolyzing the phosphate esters at the D-3 position, giving rise to D-Ins (1,2,4,5,6)P5 as the first intermediate [29, 45].
starts the hydrolysis at the L-6 (or D-4) position, yielding L-Ins(1,2,3,4,5)P5 as the first intermediate. An alkaline phytase from lily pollen (Scott and Loewus, 1986) was shown to start the hydrolysis of IP6 at position D-5, with two subsequent dephosphorylation steps to yield Ins(1,2,3)P3 as the final product [10]. Inositol triphosphate is also the end product of IP6 hydrolysis for the phytase from \textit{Typha latifolia} pollen [51]. Rat hepatic multiple inositol polyphosphate phosphatase (MIPP) catabolizes inositol hexakisphosphate without specificity towards a particular phosphate group. However, it hydrolyzed Ins(1,3,4,5,6)P5 via Ins(1,4,5,6)P4 to Ins(1,4,5)P3 by consecutive 3- and 6-phytase activities [31]. A detailed characterization of the phytase from the protozoan \textit{Paramecium} by Freund et al [38] revealed that this enzyme degrades IP6 by stepwise dephosphorylation via D/L-Ins(1,2,3,4,5)P5, D/L-Ins(1,2,3,4)P4 and Ins(1,2,3)P3 finally to D/L-Ins(1,2)P2. Appearance of D/L-Ins(1,2,3,4)P4 clearly precedes that of Ins(1,2,3)P3. The slow conversion of inositol triphosphate to inositol bisphosphate indicates that Ins (1,2,3)P3 is the main end product. Powar and Jagannathan [113] showed that \textit{myo}-inositol monophosphate (phosphate position not determined) is the end product for \textit{B. subtilis} phytase. Kinetics, reaction intermediates and degradation pathways of IP6 degradation have not been reported for \textit{Bacillus} phytases, neither is it known whether these enzymes are 3- or 6-phytases. The strong stereo specificity for the equatorial phosphate groups over the axial phosphate appears to be common to all phytases. This might indicate that only the phosphate groups protruding equatorially from the inositol ring can access the catalytic sites of these enzymes.

The pathway of hydrolysis by IP6-degrading enzymes seems to be unique for each species, and these enzymes, IP6, and its derivatives may play a variety of roles in biological systems [2]. To date, there is little knowledge of the sequence in which phytases hydrolyze phosphate groups from phytate and the IP6 derivatives. However, it is known that most characterized phytases hydrolyze IP6 in a stepwise manner, yielding \textit{myo}-inositol pentakis-, tetrakis-, tris-, bis- and mono-phosphate products [71]. Biochemical mechanisms in the phytase involved still need to be further explored.
### Table 6 Structural classification of phytases

<table>
<thead>
<tr>
<th>Phytase family</th>
<th>Unique structural feature</th>
<th>Names</th>
<th>Mol mass</th>
<th>Nature of phytate</th>
<th>Inhibition</th>
<th>Stimulation</th>
<th>Glycosylation</th>
<th>Opt pH</th>
<th>Opt Temp</th>
<th>NCBI structure no</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic Histidine acid phosphatase</td>
<td>N-terminal RHGXRXP C-Terminal HD consensus motif</td>
<td>Phy A</td>
<td>62-128</td>
<td>Metal free</td>
<td>Ca(^{2+})</td>
<td>EDTA</td>
<td>Yes</td>
<td>2.5-5.0</td>
<td>55-60</td>
<td>IIHP</td>
<td>A. niger, A. terreus, M. thermophila</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phy B</td>
<td>270</td>
<td>Metal free</td>
<td>Ca(^{2+})</td>
<td>EDTA</td>
<td>Yes</td>
<td>2.5</td>
<td>55-60</td>
<td>IQFX</td>
<td>A. niger, S. cerevisiaeae, S. pombe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phy C</td>
<td>42-45</td>
<td>Metal free</td>
<td>Ca(^{2+})</td>
<td>EDTA</td>
<td>No</td>
<td>5.0-6.0</td>
<td>40-60</td>
<td>IDKP</td>
<td>E. coli, Lyososomal phosphatase, Prostatic phosphatase</td>
</tr>
<tr>
<td>Acidic Cysteine phosphatase</td>
<td>P loop structure contains HCXXGXXRX(T/S) consensus motif</td>
<td>CP</td>
<td>46</td>
<td>-</td>
<td>Pb(^{2+})</td>
<td>Cu(^{2+}), Zn(^{2+}), Hg(^{2+})</td>
<td>-</td>
<td>4.0-5.5</td>
<td>50-55</td>
<td>-</td>
<td>S. ruminatium</td>
</tr>
<tr>
<td>Alkaline β-propeller phytase</td>
<td>Six blade β-propeller shaped molecule</td>
<td>Phy D</td>
<td>38-45</td>
<td>Ca-phytate</td>
<td>EDTA</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>7.0-8.0</td>
<td>-</td>
<td>-</td>
<td>B. subtilis, B. amyloliquefaciens</td>
</tr>
<tr>
<td>Acidic Purple acid phosphatase</td>
<td>consensus motif DXG/GDXXY/GNH/ED/(\text{ED})VXXH/GHXXH</td>
<td>Plant PAP</td>
<td>55</td>
<td>Fe-Zn center in active site</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Glycine max, M. truncatola</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Animal PAP</td>
<td>35</td>
<td>Fe-Fe center in active site</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>bovine spleen PAP, porcine uterus PAP (uteroferrin)</td>
<td></td>
</tr>
</tbody>
</table>
1.7 Structural classification of phytases

The basic structural features of several phytate degrading enzymes have been established and X-ray crystallographic studies have confirmed that they belong to a class with a novel catalytic mechanism [49]. In both instances, the elucidation of the 3-D molecular structure of different phytate degrading enzymes has enhanced our understanding of the linkage between the molecular structure of the molecule and its catalytic function.

It is now evident that different phytases have evolved to supply the unique nutritional requirements found in various forms of plant, animal and microbial life. At this time, four classes of phosphatase enzymes are known to have representatives that can degrade IP6: (1) HAP, (2) BPP, (3)CP and (4) PAP. Each one of these has unique structural features due to their distinct catalytic apparatus that allows them to utilize IP6 as a substrate in various environments (Table 6).

Several fungal, bacterial and plant phytases belong to the HAPs class of enzymes. All of these phytases share a conserved active site hepta-peptide motif RHGXRXP and the catalytically active dipeptide HD, unique to this class of enzymes [37]. This groups of enzymes catalyses the IP6 hydrolysis in a two-step process: a nucleophilic attack on the phosphorous atom by the histidine in the active site, followed by hydrolysis of the resulting phospho-histidine intermediate [144]. Phytases from *Bacillus* species constitute an exception: These enzymes share a sequence identity of 90–98% each other but are unrelated to HAPs and other phosphatases. Unlike other HAPs, they require Ca$^{2+}$ for activity and show a different pH optimum of 7.0–8.0 [70].

Meanwhile, a phytase isolated from soybean was found to be unrelated to previously characterized microbial or maize (*Zea mays*) phytases, classified as HAPs. This soybean phytase is a PAP, characterized by seven conserved residues (bold) in the five conserved motifs – DXG, GDXXY, GNH(D/E), VXXH and GHXH – involved in the coordination of the dimetal nuclear centre [56, 82]. In contrast, *S. ruminantium* phytase neither contains the conserved RHGXRXP
motif nor is affected by divalent metal ions. The active site is located near a conserved cysteine-containing (Cys241) P loop.

1.8 Crystal Structure of phytases

Crystal structure analyses of a number of phytases have revealed a range of distinct folds for these enzymes and have allowed their biophysical properties to be rationalized in terms of their structure. The crystal structure of *A. ficuum* phytase at 2.5 Å resolution revealed three distinct domains, including a large α-helical domain and β-sheet domain, and a small α-helical domain. The large α-helical domain and small α-helical domain contain five α-helixes and four α-helixes, respectively, and the β-sheet domain contains eight β-sheets [74]. Crystal structure analysis of *Escherichia coli* phytase with a resolution of 2.5 Å also showed two domains. One contains five α-helixes and two β-sheets, and the other includes six α-helixes and nine β-sheets [86]. A three-dimensional model of *A. ficuum* phytase (1IHP) from the National Center for Biotechnology Information’s (NCBI) website is shown in Fig 9a.

The crystal structure of *B. amyloliquefaciens* phytase (TsPhy) at 2.1 Å resolution revealed a six-bladed β-propeller in which each blade consists of a four- or five-stranded antiparallel β-sheet (Fig. 9b, PDB code 1H6L). The enzyme binds seven Ca$^{2+}$: two near the periphery, one in the central channel and four near the ‘top’ of the molecule. Unlike other β-propeller structures, it does not show any conserved sequence repeats in the β-sheet. The crystal structures of TsPhy at 2.1 Å resolution in both the partially and the fully Ca$^{2+}$-loaded states were determined. And the dependence of thermostability of TsPhy on Ca$^{2+}$ was assessed by differential scanning calorimetry.

The binding of two Ca$^{2+}$ to high-affinity Ca$^{2+}$-binding sites results in a dramatic increase in thermostability (with an increase of as much as c. 30°C in the melting temperature), because of the joining of loop segments remote in the amino acid sequence. Three Ca$^{2+}$ bind to the active Ca$^{2+}$-binding sites and create an ideal conformation and charge distribution for the substrate. Substrate
binding to the active site would appear to be followed by occupation of the fourth Ca\(^{2+}\) site to offset the negative charge of the substrate phosphate group already coordinated by lysine and arginine.

**Fig 9** Swiss-Pdb viewer-prepared molecular models from the National Center for Biotechnology Information (NCBI)’s website (http://www.ncbi.nlm.nih.gov), representing three types of phytases: (a) 1IHP, PhyA, a histidine acid phosphatase; (b) 1H6L Ts-Phy, a b propeller phytase; (c) 1U26, SrPhy, a cysteine phytase [151]

*Selenomonas ruminantium* phosphatase (SrPhy) represents a third, dual-specificity phosphatase type with a conserved cysteine (C241) in its so-called P loop. Two distinct crystal packing arrangements have been observed of the complex of SrPhy with the inhibitor myo-inositol hexasulfate. The inhibitor is bound to both ‘standby’ and ‘inhibited’ conformations. In a pocket slightly away from the conserved P loop Cys241 and at the substrate binding site, the phosphate group to be hydrolysed is held close to the -SH group of Cys241. Further, mutagenesis studies verify that the P loop-containing phytase attracts and hydrolyses the substrate (phytate) sequentially via a complicated mechanism [25]. Figure 9c shows 1U26 to underscore the structural differences in these three classes of enzyme.
1.9 Development of effective phytases

Public awareness of the environmental impact of animal agriculture has led to legislation that limits the quantity of P in animal excreta in some parts of the world, and will likely extend to other parts of the world in the near future. Under these conditions, phytase will be widely used in animal diets to improve phytate-P bioavailability and reduce P excretion. Significant progress has been made in phytase research during the last 15 years. However, a limited number of phytases have been reported and studied, and our scientific knowledge of phytases has yet to yield a solution to meet the nutritional and environmental requirements that a real-world solution demands. Further research into identifying new phytases, engineering better phytases and developing more cost-effective expression systems should be continued. Two approaches have been taken to develop effective phytases: identifying new native phytase proteins from microorganisms or plants, and genetically modifying these cloned phytases.

Identification of new native phytase

Phytases are produced in a wide range of plant, bacterial, fungal and animal tissues. Most scientific work has, however, been performed on microbial phytases, particularly those from filamentous fungi such as A. ficuum [43], a fumigatus [105] or Mucor piriformis [57], Rhizopus oligosporous [18] and Cladosporium species [114]. The search for phytases with higher thermostability resulted in the cloning of the phytase gene from A. fumigatus, the purified enzyme of which retains 90% of its initial activity after being maintained at 100°C for 20min. recently, a novel phytase gene from A. niger N-3 was cloned and expressed in Pichia pastoris. The purified enzyme of which retains 45% of its initial activity after being maintained at 90°C for 5min. It showed a greater affinity for sodium phytate than for p-nitrophenyl phosphate. Dual optimum pH values were obtained at 2.0 and 5.5. The activity at pH 2.0 was about 30% higher than that at pH 5.5, which is more similar to conditions in the stomachs of monogastric animals. Two novel
thermostable genes were identified in *A. japonicus* BCC18313 (TR86) and BCC18081 (TR170), respectively. The thermostable nature of this phytases gives it valuable potential for applications [111].

Apart from the phytase genes identified in fungi, others have been cloned and identified in other microbes, motivated by their potential for applications. To find a phytase with high activity at low temperature and neutral pH, two phytases have been isolated from *Pedobacter nyackensis* MJ11 CGMCC 2503 and *Erwinia carotovora var. carotovota* ACCC 10276. The *Pedobacter* phytase belongs to the BPP family and shares very low identity (approximately 28.5%) with *Bacillus subtilis* phytase. Compared with the major commercial phytases and *B. subtilis* phytase, the purified recombinant enzyme from *E. coli* displayed higher activity and hydrolysed phytate from soybean meal with better efficiency at neutral pH and 25°C. These characteristics suggest that this phytase has a great potential as an aquatic feed additive in the rapidly developing aquaculture industry. The *Erwinia* phytase contains a conserved active site hepta-peptide motif RHGXRXP and the catalytically active dipeptide HD that is typical of HAPs and shares a 50% amino acid identity to the *Klebsiella pneumoniae* phytase [58, 59]. And except for potential application in aquaculture, the latter is also attractive for food processing by avoiding damage to the food in gradients at low temperatures [47]. Moreover, owing to its typical properties as a low-temperature-active enzyme, it could be a good model protein to study the relationship between structure and function. The gene appA, encoding a phytase from *Yersinia kristeensenii*, was cloned and heterologously expressed in *P. pastoris*. The data show that the *Y. kristeensenii* phytase is highly pH stable at pH 1.5–11.0 and thermostable, providing significant advantages for processing, transportation, storage and application. Comparison of r-APPA with other well known phytases suggested that the *Y. kristeensenii* phytase would be an attractive enzyme for feed industry use [41]. In addition, phytases from yeast have also been identified and characterized (motivated by their potential as a feed additive for improving the phytate-P digestibility in monogastric animals), such as the marine
Protein engineering of phytase

Although properties of phytases vary, there is no single wild-type enzyme that is perfect or ideal for field applications. Theoretically, an ‘ideal’ phytase should be catalytically efficient, proteolysis-resistant, thermostable and cheap [81]. In reality, phytases possessing all of these qualities may never be found or generated. To obtain enzymes with modified and desired properties, two different strategies are used: rational protein design and directed (molecular) evolution, which are increasingly, applied in a synergistic manner to tailor-design the enzyme for a given process [14, 24].

1. **Thermostability**- Because commercial feeds are often pelleted, a process sing high temperature (60–80°C) and steam, all feed enzymes need to be heat stable to avoid substantial activity loss during this process. The thermostability of an enzyme can be enhanced by multiple amino acid exchanges, each of which slightly increases the unfolding temperature of the protein. The rational approaches for thermostability engineering involve the comparison of the amino acid sequence of the protein of interest with a more thermostable, homologous counterpart, followed by replacement of selected amino acids. The thermostabilization concepts include the introduction of additional disulfide bridges, improvements in the packing of the hydrophobic core, engineering of surface salt bridge networks or helix dipole interactions, changes in helix propensity and changes in entropy [50, 136, 137].

2. **Catalytic activity**- Site directed mutagenesis of amino acid residue 300 was resulted in a high phytase activity by *A. niger* NRRL 3135 at pH 3.0 to 5.0, while a single mutation (K300E) resulted in an enhanced hydrolysis of IP6 at pH 4.0 and 5.0. In this study, the
basic amino acid residue lysine (K) was replaced by acidic residue. However, this replacement with another basic residue, or an uncharged but polar residue, did not significantly alter the activity at pH 4.0; but a replacement with basic residue arginine (R) lowered the activity over the pH range from 2.0 to 6.0 [98].

3. **Proteolysis resistance**- An effective phytase needs to have a strong resistance to hydrolytic breakdown by digestive proteinases in the digestive tract. Fungal and bacterial phytases show different sensitivities to pepsin and trypsin, and the latter seem to have a higher resistance to proteolytic degradation than the former. The protease-sensitive sites of phytases, normally in the exposed loops at the surface of the molecules, may be blocked or modified using site-directed mutagenesis [150]. Site-directed mutagenesis, based on crystal structure of phytases [86], has been used to improve pH profile of *A. niger* PhyA phytase. When expressed in *A. niger*, several fungal phytases were susceptible to proteases. N terminal sequences of the fragments revealed that cleavage invariably occurred at exposed loops on the surfaces of the molecules. Site directed mutagenesis at the protease-sensitive sites of *Aspergillus fumigatus* (S151N and R151L/ R152N) and *Emericella nidulans* phytase (K186G and R187R) yielded mutants with reduced susceptibility to proteases, without affecting the specific activity.

The rapidly growing number of successful phytase engineering studies using rational protein design and directed (molecular) evolution is conducted to improve the desirable enzyme characteristics. Tian et al [135] improved *A. niger* 113 (PhyI1s) activity by studying the effect of amino acid residues near the catalytic active centre or substrate specificity site – as well as some residues far from this site using site directed mutagenesis. Zhu et al [155] improved the thermostability of *Escherichia coli* (AppA) by 23.3% as compared to wild type using error-prone PCR and high-throughput screening. This mutant I408L could be used for the large-scale commercial production of phytases. Phytase from *Penicillium sp.* was studied by Zhao et al [154]
for high thermal stability, low optimal temperature and pH using Mn$^{2+}$-dNTP random mutation method. Two mutants were obtained with improved thermal stability and optimal temperature and pH that retained their high resistance to pepsin. Structure-guided consensus approach was used by Viader-Salvado et al [142] for broadening the pH profiles (2.5-9.0) of β-propeller phytase and it was proposed that P257, D336 play an important role for forming a larger number of hydrogen bonds and this resulted in new properties at pH 5.5 and 7.5. Site directed mutagenesis was employed to obtain mutant (A58E P65S Q191R T271R) of Aspergillus niger phytase (PhyA) with higher thermostability. This study shows that hydrogen bond network and ionic interactions play an important role [153].

1.10 Immobilization

Phytases act sequentially on myo-inositolhexakisphosphate to liberate various lower isomers. Thus, an efficient immobilized bioreactor could be used to produce various isomers of IP6 besides rendering the molecule non-chelator of metal-ions, proteins, etc. Fungal phytase are hydrolytic enzymes with a high catalytic turnover number-typically the range being from 220-1000 per second. Thus these categories of enzymes are ideal for immobilization and construction of packed bed reactors. A few studies have been made on the application and properties of immobilized phytate-degrading enzymes [46, 139]. The pH dependence of the phytate-degrading activity was not influenced by immobilization, whereas stability against heat treatment was enhanced as a consequence of immobilization. In addition, the immobilized phytate-degrading enzymes exhibited good optional and storage stability over a period of several months. Liu et al [88] increased the temperature optima of A. ficuum phytase to 58°C, which was 8°C higher than that of free enzyme. This was done by immobilization in gelatin gels and further hardening with formaldehyde. Apparent Km increased to 3.28mM (Km = 2.34mM for free enzyme) and the residual enzyme activity was 34.6% only.
1.11 Biotechnological applications of phytase

Since the first commercial phytase product Natuphos® was launched in 1991, the market volume has reached ca. 150 million Euros and will likely expand with new applications. The main application is still as a feed supplement to improve P bioavailability in plant feed-stuffs via the enzyme-mediated hydrolysis of phytate. Most importantly, the improved utilization of the phosphate deposits in the feed results in a substantial reduction in the phosphate content in animal manure and hence decreases of phosphate load on the environment in areas of intensive animal agriculture. High dietary P bioavailability reduces the need for supplemental inorganic P such as mono- and dicalcium-phosphate (MCP, DCP).

Because of the strong economic growth in China and India along with the oil price hike, the supply and cost of MCP and DCP has become a practical issue. Furthermore, inorganic phosphate is non-renewable resource, and it has been estimated that the easily-accessible phosphate on earth will be depleted in 50 years. Thus, phytase is an effective tool for natural resource management of P on a global scale.

The ban of dietary supplementation of meat and bone meal, as a cheap source of feed P, in Europe to prevent possible cross-species transfer of diseases such as BSE, has led to a profound change in the feed P management. This has given phytase a new socio-economic impact as a cost effective alternative to ensure animals to obtain adequate available P from the plant-based diets. Being the major storage form of P in seeds, plant phytate was produced in 2000 at a global yield >51 million metric tons. This amount accounts for approximately 65% of the elemental P sold worldwide as fertilizers [89]. Apparently, phytase can turn the plant phytate into a very valuable resource of P by improving its bioavailability for animal nutrition. Denmark and the Netherlands have imposed regulations to promote the use of microbial phytases.
Because of the potential value of phytases for improving the efficiency of P use, biotechnology has led the rapid development of the field to its current stage. With the development of heterologous gene expression, large amounts enzymes could be produced at relatively low cost. The importance of phytases as potential biotechnological tools has been recognized in various fields (Table 7). However, only a limited number of phytases have been reported and studied, and our knowledge of the mechanisms and factors regulating phytase activity is limited. Further research into developing new technologies and identifying the most efficient phytases must continue.

**Phytases in animal nutrition:**

Monogastric animals such as swine, fish, and poultry show negligible or no phytase activity in their digestive tracts. Consequently, phytates cannot be metabolized by the animals, thus creating a need to enhance phosphate and mineral bioavailability via phytase supplementation of animal feed. Of late, phytases are also viewed as environment friendly products, which can reduce the level of phosphate pollution in intensive livestock management areas by avoiding the addition of exogenous phosphate [140]. Undigested phytate of monogastric manure is washed off the farmland that imperils adjacent waterways by eutrophication [27]. The effect of feeding phytase to animals on pollution has been quantitatively determined. If phytase were used in the feed of all of the monogastric animals reared in the U.S., it would release P with a value of 168 million U.S dollars and would preclude $8.23 \times 104$ tonnes of phosphate from entering the environment per annum. The use of phytase as a feed additive has been approved in 22 countries.
**Table 7** Potential applications of phytases

<table>
<thead>
<tr>
<th>Application</th>
<th>Role and Effect</th>
<th>Properties</th>
<th>Challenges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed industry</td>
<td>Increased P utilization, metal bioavailability, decreased P conc. in excrement, Substitutes expensive Di-calcium phosphate</td>
<td>Resistance to low pH, active in the stomach, stable during animal feed processing and storage, low cost of production and easily processed by the feed manufacturer</td>
<td>Lack of desirable properties, High cost of production</td>
</tr>
<tr>
<td>Food industry</td>
<td>Increased P utilization, metal bioavailability, technical improvement of food processing</td>
<td>-</td>
<td>It will be a challenge to minimize the negative effect of phytate on iron and zinc nutrition without losing its potential health benefits</td>
</tr>
<tr>
<td>Myoinositol phosphate</td>
<td>Myoinositol phosphate intermediates used as enzyme stabilizers, enzyme inhibitors, potential drugs, chiral building blocks</td>
<td>-</td>
<td>Further intensive investigations, using diverse phytases, need to be undertaken for designing and producing pharmacologically important lower myo-inositol phosphates</td>
</tr>
<tr>
<td>Aquaculture</td>
<td>Substitute for expensive protein source such as menhaden fish meal and maintains the acceptable levels of P in water</td>
<td>Phytase active at low temperature and broad pH optima is required</td>
<td>Effects of phytase supplementation on various physiological and endocrine parameters like secretion of other enzymes, bile salts, on the immune response, hormone levels including growth hormone, thyroid hormone, insulin etc needs to be studied</td>
</tr>
<tr>
<td>Soil Amendment</td>
<td>Plant growth stimulation by mobilization of soil phytate into inorganic P</td>
<td>Phytase with broad pH optima and catalytic activity</td>
<td>Needs more research on phytase supplementation for boosting the productivity in agriculture and horticulture</td>
</tr>
</tbody>
</table>
The FDA (The Food and Drug Administration) has approved the phytase preparation as GRAS [147]. During the past two decades, there has been significant increase in the use of phytases as feed additive in pig, poultry, and fish diets. In numerous studies, the efficacy of microbial phytases to release phytate-bound P has been demonstrated in various animals [32, 15, 104, 39, 96, 117, 118, 157]. Phytases were also found to enhance the utilization of different minerals. Phytases from different sources have been evaluated individually and in combination for their efficacy as feed additives in poultry [19, 35, 106, 148]. Use of both bacterial and fungal phytases together as feed additive would be another promising alternative in improving the P utilization and alleviation of mineral deficiency, owing to their synergistic activities throughout the gastrointestinal tract of the animals. The use of phytase as a feed enzyme sets certain demands on the properties of the enzyme. Particularly, the enzyme should withstand high temperatures. This is because poultry and pig feed is commonly pelleted, which ensure that the animals have a balanced diet and facilitates the preservation of enzyme-containing product in the feed industry. During the pelleting process the temperatures may temporarily reach 90°C. The first commercial phytase product, which became commercially available 10 years ago, offered animal nutritionists the tool to drastically reduce P excretion of monogastric animals by replacing inorganic phosphates with microbial phytase. Depending on diet, species, and level of phytase supplementation, P excretion can be reduced between 25 and 50% [73].

**Phytases in human nutrition:**

Mineral deficiency of diets, caused by radical changes in food habits, is a major concern for developing countries. Processing and manufacturing of human food is also a possible application field for phytase. Up to now, no phytase product for a relevant food application is on the market. Research in this field focuses on better mineral absorption or technical improvement of food processing. Phytate present in cereal-based and legume-based complementary foods has been found to inhibit mineral absorption [60]. The human small intestine has limited ability to digest
undegraded phytates, resulting in adverse nutritional consequences with respect to metabolic cation imbalances. IP6 containing 12 dissociable protons with pKa values ranging from ~1.5 to 10—is a highly reactive and potent chelator of many mineral ions such as Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, and Fe$^{2+}$. IP6 forms insoluble salts, at normal acidity (pH 3.0–6.8), in the human digestive tract, thereby reducing the bioavailability of these critical mineral nutrients for absorption [30]. Mucosal phytase and alkaline phosphatases, even if present in the human small intestine, do not seem to play a significant role in the phytate digestion, while dietary phytase serves as an important factor in phytate hydrolysis [125]. Haros et al [54] investigated the possible use of phytase in the process of bread making. Different amounts of fungal phytase were added in whole wheat breads, and it was shown that phytase is an excellent bread-making improver. The main achievement of this activity was the shortened fermentation period without affecting the bread dough pH. An increase in bread volume and an improvement in crumb texture were also observed.

**Phytases in aquaculture:**

A major concern in aquaculture is the utilization of dietary phosphates which critically affects fish growth as well as the aquatic environment. An efficient utilization of feed leading to optimum fish growth serves as a benchmark of successful aquaculture worldwide. Studies using phytase as feed additive in aquaculture amply establish that phytase supplementation could enhance the bioavailability of P, nitrogen, and other minerals, thereby decreasing P-load in the aquatic environment [101, 143].

**Role of phytases in soil amendment:**

P is an essential plant nutrient that limits agricultural production on a global scale. Approximately 30–80% of the total P in soils is bound in organic form [55]. Phytate constitutes ~50% of the total organic P pool in the soil and is poorly utilized by plants [4]. Extracellular phytase activities have been reported under phosphate stress conditions, in diverse plant species,
namely, tobacco [90], barley [6], tomato, alfalfa [84], and so on. The ability of plants to use P from low phosphate or phytate containing media and/or from soil is improved when soil/media are inoculated with microorganisms that possess the ability to exude phytase, or when a purified phytase is added.

**Phytases for the production of lower myo-inositol phosphates:**

Lower phosphoric esters of myo-inositol (mono, bis, tris, and tetrakisphosphates) play a crucial role in transmembrane signaling processes and in calcium mobilization from intracellular store in animal as well as in plant tissues [12, 97, 33, 75, 124]. Research interest in this field prompted the need for various inositol phosphate preparations. However, chemical synthesis [13] is difficult. In contrast, an enzymatic synthesis has the advantage of high stereospecificity and mild reaction conditions. The use of phytase has been shown to be very effective in producing different inositol phosphate species.

Different isomers of myo-inositol phosphates have shown pharmacological effects for the prevention of diabetic complications, anti-inflammatory effects [17, 26], and antiangiogenic and antitumor effects [92]. Myo-inositol phosphates are also known to ameliorate heart disease conditions by controlling hypercholesterolemia and atherosclerosis [64], and also prevent renal stone formation [44].

**1.12 Future prospects**

**P: Both a curse and necessity**

Peak P is the point in time at which the maximum global P production rate is reached. P is a scarce finite resource on earth and due to its non-gaseous environmental cycle has resulted in alternative means other than mining being unavailable. According to some researchers, Earth's P reserves are expected to be completely depleted in 50–100 years and peak P to be reached in
approximately 2030. It is this nutrient which is necessary to our well-being -- in fact our food security -- that we don't have in plentiful supply. P is an indispensable resource that has been mismanaged to the point that we are jeopardizing our long-term food and water security. The depletion of P is more relevant to our world today than the depletion of oil is. It is a major component in fertilizer, without which fertilizer will be rendered useless. Without fertilizer, two thirds of the world’s population will starve because the Earth cannot support our demands for food. There are no alternatives to P and no synthetic ways of creating it. Without new sources for high quality mineable P agriculture will face major problems within the next 50-100 years. P is an essential ingredient in animal and plant production; however, too much or too little P can be a problem both for animal production and the environment. Phosphate recycling is essential for the sustainable future of our society as it is inconceivable to continue to simply throw away a non-renewable resource which is essential for life. As the need to conserve the world’s phosphate reserves increases the role of phytase will broaden.

**Role of phytase**

Phytases are now being recognized for their beneficial environmental role in reducing the P levels in manure and minimizing the need to supplement P in diets. Their use as an animal feed additive is growing because it is ecofriendly. They have an immense importance in the feed and food industry because they also improve the nutritional status by degrading IP6, which acts as an antinutritional factor. Also, because lower inositol phosphates and phospholipids play important roles in transmembrane cell signaling and calcium mobilization from intracellular shock, an investigation into the potential role of phytase in this mechanism would be interesting. One area that offers tremendous opportunity is increasing the use of phytase in aquaculture in order to allow the use of low-cost plant meals. Other areas for expanded use range from the use of phytase as a soil amendment and its transformation into a peroxidase.
The growing demand for phytase is amply reflected by the multitude of sources screened for phytases with desired attributes. Identification of various phytases from diverse sources and their expression in heterologous systems need to be worked out not only to enhance the enzyme production but also to decrease the cost of production. Physicochemical properties of phytases, namely, broad pH range to survive under varied pH conditions in animal digestive tract, resistance to proteolytic degradation, thermal stability to resist higher temperatures during feed pelleting and substrate specificity, and so on need thorough evaluations to design versatile “second-generation” phytases with wider applicability. Modification and upgradation of enzymatic properties can be achieved through adoption of genetic and protein engineering methods. Combination of fungal and bacterial phytases as feed additives might improve the bioavailability of P and minerals owing to their synergistic activity in animal digestive system.

1.13 Lacunae and need for more research

There are various reports on phytase production by bacteria, yeast and fungi among which fungal phytases are widely employed in animal feed due to their acid tolerance and higher yield. All available phytase preparations used as feed additives today are of fungal origin and produced by recombinant strains in SmF and are active at pH 5. These preparations are expensive because of diluted product and high product recovery costs. In addition, they have important shortcomings, especially with regard to their sensitivity to heat and inactivation under low pH conditions generally present in the stomach.

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. Hence development of a viable process for phytase recovery and purification with techno-economic feasibility is necessary as the available chromatographic methods have several limitations.
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Hence efforts are needed to alleviate these disadvantages while at the same time producing cost effective phytase with fast and economic downstream processing. SSF provides a more economic alternative for enzyme production and application as compared to SmF. But the reports are few because of the low productivities and difficulties associated with operating and up scaling SSF conditions. Earlier, we have reported phytase production by *Aspergillus niger* NCIM 563 under SSF using wheat bran with no additional nutrients [94]. Micro-organisms produce low levels of phytase and it would be beneficial if these production rates be improved with desirable process features by employing statistical techniques.

In this work, our objective is to evaluate application of statistical methods to increase the phytase activity under SSF and up-scaling to tray fermenter. The present work also reports the use of ATPE for separation and purification of phytase and compares it with the conventional chromatography process.

The same fungus produces two dissimilar phytase Phy I and Phy II under SmF [129]. Many studies on SSF and SmF for phytase have focused on process and fermenter design while the organism has been considered as a black box. The role of the physiological and genetic properties of the microorganisms producing phytase used during growth on solid substrates compared with aqueous solutions has so far been all but neglected. Hence we have tried to correlate different protein secretion in Smf and SSF and these studies can provide new insights to the existing “black box” of SSf/SmF biotechnology for phytase production.
1.14 Objectives of the study

“Studies on phytase from Aspergillus niger NCIM 563 under solid state fermentation and its correlation with submerged phytase I and II” was taken up with the following objectives:

- Production of phytase by Aspergillus niger NCIM 563 under solid state fermentation
- Downstream processing of solid state phytase from A. niger NCIM 563
- Characterization and application of solid state Phy III from A. niger NCIM 563
- Correlation studies of solid state Phy III with submerged (Phy I and II) produced by A. niger NCIM 563
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