Enzymes as catalysts:
Life depends on a series of coordinated chemical reactions. Many of these reactions however proceed too slowly to sustain life and hence proteins with catalytic functions have evolved. An enzyme is a protein that functions as a catalyst. The role of an enzyme (or a catalyst) is to speed up the rate of chemical reactions without undergoing any permanent changes itself. At the end of the reaction, the enzyme remains unchanged [1]. In many industrial processes, conventional chemical catalysts are replaced by commercial microbial enzymes. Enzymes have several advantages over chemical catalysts, such as, ability to function under mild conditions of temperature, pH and pressure, consumption of less energy, no requirement for expensive corrosion-resistant equipment. Enzymes are specific, often stereoselective and do not produce unwanted byproducts. Consequently, there is less need for extensive refining and purification of the target product. Enzyme-based processes are also “environmentally friendly” compared to chemical processes as enzymes are biodegradable and there are fewer associated waste disposal problems. Certain enzymes can operate in two-phase water-organic solvent systems and in non-aqueous organic media, particularly hydrophobic solvents. This is very useful with substrates having limited water solubility [2].

Enzyme classification:
Enzyme classification is based on a system originally established by the Commission on Enzymes of the International Union of Biochemistry (1979) (now known as the International Union of Biochemistry and Molecular Biology - IUBMB). There are six main classes, grouped according to the type of reaction catalyzed:

**Table 1: Classification of enzymes**

<table>
<thead>
<tr>
<th>EC Number</th>
<th>Class of Enzyme</th>
<th>Reaction Catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxidoreductases</td>
<td>Catalyze oxidation/reduction reactions, the transfer of H atoms, O atoms or electrons</td>
</tr>
<tr>
<td>2</td>
<td>Transf erase</td>
<td>Catalyze transfer of a group from one molecule to another</td>
</tr>
<tr>
<td>3</td>
<td>Hydrolases</td>
<td>Catalyze hydrolysis, the cleavage of bonds by addition of a water molecule</td>
</tr>
<tr>
<td>4</td>
<td>Lyases</td>
<td>Catalyze splitting of bonds, other than via hydrolysis or oxidation</td>
</tr>
<tr>
<td>5</td>
<td>Isomerases</td>
<td>Catalyze structural rearrangements of molecules</td>
</tr>
<tr>
<td>6</td>
<td>Ligases or Synthetases</td>
<td>Catalyze the formation of new bonds, e.g. C-N, C-O, C-C and C-S, with breakdown of ATP</td>
</tr>
</tbody>
</table>
Introduction

Each enzyme has a four-figure code for class, subclass, etc. For example, invertase, a hydrolase, is classified as EC 3.2.1.26.

Phytase enzyme:
In this era we are part of an increasingly health conscious society where health promotion is becoming of greater significance, pollution and waste are high on the agenda and the concern with food safety is of supreme importance. The role of animal feed in the production of safe food is recognized worldwide [3] and recent events have underlined its impact on public health, feed and food trade, and food security. Economic and technological advances are driving the development of new feed products, especially products of biotechnology. Enzymes as additives to animal feed have an important role to play in current farming systems. Not only have they improved the digestibility of nutrients, leading to greater efficiency in the production of animal products such as meat and eggs, but they have also improved the quality of the environment by allowing better use of natural resources and reducing pollution by nutrients [4].

Phytases (EC 3.1.3.8 and 3.1.3.26) have been one of the principal enzymes for nutrition, environmental protection and human health during the past two decades. Phytases sequentially cleave orthophosphate groups from the inositol core of phytate, the major storage form of phosphorus (>80%) in cereals, legumes and oilseeds [5, 6, 7]. Cereals, legumes and oilseed crops are grown over 90% of the world’s harvested area and together they serve as a major nutrient source for the animal kingdom [6, 7]. However, this large source of phosphorus can only be utilized by some bacteria [8] and fungi [9] as they have phytase enzyme and can hydrolyze phytate. Monogastric animals, such as, pig, fish and poultry lack phytase and therefore plant phytate remains undigested and the animals suffer from anti-nutritional problems [10]. Again, the ingested phytate by these animals when excreted into the environment causes eutrophication [11]. Under normal physiological conditions, phytic acid chelates essential minerals, such as, calcium, magnesium, iron and zinc. It also binds to proteins, amino acids and inhibits digestive enzymes [12, 6]

Phytase releases inorganic phosphorus and inositol as well as protein, amino acids, trace minerals and other nutrients chelated with phytates. Addition of phytase in pig, fish and poultry supplements can reduce or eliminate the supplementation of inorganic phosphorus in their feeds, improves the utilization efficiency of these nutrients contained in feedstuff, reduces the phytate
phosphorus excretion, and helps in maintaining the environmental balance of the concerned areas [13]. Phytate hydrolysis enhances protein digestibility and mineral availability of plant based foods and consequently improves food quality [13]. Certain myo-inositol phosphates released as by-products of phytate hydrolysis are used in treating multiple sclerosis, Alzheimer’s disease and parkinson’s disease [14]. Some of the myo-inositol phosphates may be health-beneficial with possible functions in antioxidation, anti-tumorigenesis, reducing serum lipids and cholesterol levels, preventing renal calculi via mineral binding and in diabetes [15, 16].

Suzuki et al. first detected phytase activity in rice bran in 1907, but, phytase became commercially available in 1991 [17, 18, 19]. To date, only a handful of commercial phytases are available [18, 19] and there are ongoing research projects aiming at the discovery or improvement of phytases that could become more suitable to animal feed applications than current commercial preparations [17, 20].

1.1. Phytate overview:

Phytic acid is myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate [6, 21] having molecular formula $C_6H_{18}O_{24}P_6$ and a molecular weight of 659.86 [22, 23].

1.1.1. Chemical Structure of Phytic acid:

Johnson and Tate [24] derived the conformational structure of phytic acid from $^{31}$P-NMR analysis and suggested that the phosphate at 2-position is axial and the other five phosphates are in equatorial position. Later Costello et al. [25] supported this energetically favourable conformation.

Fig 1: Structure of Phytic Acid [22]

Costello and co-workers [25] determined that pKa values for six dissociating groups of phytic acid were in strong acid range (pKa 1.1-2.1), one in weak acid range (pKa 5.7), two with pKa
6.80-7.60 and three in very weak acid range (pKa 10.0-12.0). Since phytic acid exists as a strong negatively charged molecule over a wide pH range, it has a strong affinity to form complexes with multivalent cations and positively charged proteins [6].

1.1.2. Occurance, Distribution and Content of Phytic acid:
Phytic acid is formed during maturation of plant seed and in dormant seeds and represents 60-90% of the total phosphate [22, 26]. In small-grain cereals, approximately 90% of the seed phytic acid is found in the aleurone and 10% in the scutellum. In contrast, 90% and 10% phytic acid is found respectively in scutellum and aleurone of maize. In dicotyledons and cotyledons, phytic acid is found in the endosperm. Almost all the phytic acid is present as mixed salts of K⁺, Ca²⁺, Mg²⁺ or Zn²⁺, called phytin or phytate, and are deposited as globoid crystals in single-membrane vesicles together with protein. Phytic acid deposition is restricted to cells that will remain alive through the quiescent phase of seed development, but it is also found in vegetative tissues and in pollen [27]. Depending on the amount of plant-derived foods in human diet and the grade of food processing, the daily intake of phytate can be as high as 4500 mg, on average, 2000-2600 mg for vegetarian diets and 150-1400 mg for mixed diets [22].

Table 2.: Phytate content per mass of dry matter in plant-derived foods [14, 22]

<table>
<thead>
<tr>
<th>Type</th>
<th>Food</th>
<th>Phytate (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereal-based</td>
<td>French bread</td>
<td>0.2-0.4</td>
</tr>
<tr>
<td></td>
<td>Sourdough rye bread</td>
<td>0.01-0.03</td>
</tr>
<tr>
<td></td>
<td>Whole wheat bread</td>
<td>3.2-7.3</td>
</tr>
<tr>
<td></td>
<td>Whole rye bread</td>
<td>1.9-4.3</td>
</tr>
<tr>
<td></td>
<td>Unleavened wheat bread</td>
<td>10.6-3.2</td>
</tr>
<tr>
<td></td>
<td>Maize bread</td>
<td>4.3-8.2</td>
</tr>
<tr>
<td></td>
<td>Unleavened maize bread</td>
<td>12.2-19.3</td>
</tr>
<tr>
<td></td>
<td>Oat bran</td>
<td>7.3-2.1</td>
</tr>
<tr>
<td></td>
<td>Oat flakes</td>
<td>8.4-12.1</td>
</tr>
<tr>
<td></td>
<td>Oat porridge</td>
<td>6.9-10.2</td>
</tr>
<tr>
<td></td>
<td>Pasta</td>
<td>0.7-9.1</td>
</tr>
<tr>
<td></td>
<td>Maize</td>
<td>9.8-21.3</td>
</tr>
<tr>
<td></td>
<td>Corn flakes</td>
<td>0.4-1.5</td>
</tr>
<tr>
<td></td>
<td>Rice (polished, cooked)</td>
<td>1.2-3.7</td>
</tr>
<tr>
<td></td>
<td>Wild rice (cooked)</td>
<td>12.7-21.6</td>
</tr>
<tr>
<td></td>
<td>Sorghum</td>
<td>5.9-11.8</td>
</tr>
<tr>
<td>Legume-based</td>
<td>Chickpea (cooked)</td>
<td>2.9-11.7</td>
</tr>
<tr>
<td></td>
<td>Cowpea (cooked)</td>
<td>3.9-13.2</td>
</tr>
</tbody>
</table>
### 1.1.3. Physiological Role of Phytic Acid:

Phytic acid plays several physiological roles in seeds and grains, such as, i) phosphorus store, (ii) energy store, (iii) source of cation, (iv) myo-inositol source (cell wall precursor), (v) initiation of dormancy [6], (vi) antioxidant during dormancy [28].

Phytic acid shows antineoplastic effect in animal models of both colon and breast carcinomas. The presence of undigested phytic acid in the colon may protect against the development of colonic carcinoma [6, 29].

### 1.1.4. Phytic acid Biosynthesis:

Phytic acid is synthesized from myo-inositol via a series of phosphorylation steps. The exact intracellular location of the intermediates of phytic acid biosynthesis is not yet known. It is hypothesized that phytic acid biosynthesis takes place in the cytoplasm or in the endoplasmic reticulum, after which the phytin particles are transported in vesicles to the protein bodies. In the developing castor bean endosperm, phytin particles are either detected in single-membrane vesicles in the cytosol or found to be associated with endoplasmic reticulum and protein storage vesicles. In the slime mold *Dictyostelium*, a cytosolic route for phytic acid biosynthesis has been found. Other studies have shown that the stepwise phosphorylation of the secondary messenger myo-inositol-1,4,5-trisphosphate to phytic acid can occur in the nucleus [27].
myo-Inositol

Fig 2(a): Structure of myo-inositol. The numbering of the carbon atoms in the ring is given following the D-conversion.

Fig 2(b): Biochemical pathways [27, 30, 31]

Numbers at arrows indicate the following enzymatic activities:
(1) D-myoinositol 3-P1 synthase (MIPS); (2) D-Ins 3-P1 phosphatase, or Ins monophosphatase; (3) D-Ins 3-kinase or Ins kinase; (4) Ins P or polyP kinases; (5) Ins 1,3,4,5,6 P5 2-kinase or phytic acid-ADP phosphotransferase; (6) phytases and phosphatases; (7) Ins P6 or
pyrophosphate-forming kinases; (8) pyrophosphate-specific phosphatases; (9) pyrophosphate-containing Ins PolyP-ADP phosphotransferases; (10) phosphatidylinositol (PtdIns) synthase; and (11) PtdIns and PtdIns P kinases followed by PtdIns P-specific phospholipase C.

1.1.5. Antinutritional Effects of Phytic Acid:
Phytic acid acts as an antinutrient factor as it chelates essential minerals and proteins altering their solubility, functionality, digestibility and absorption and thus becoming a threat to human and animal nutrition. As it behaves like a highly negatively charged ion in a broad pH range, it has a great affinity for food components with positive charge(s), like minerals, proteins and trace elements [22]. The formation insoluble phytate-mineral complexes in the intestinal tract prevent mineral absorption, thereby, reducing the bioavailability of essential minerals [32]. Zinc appears to be the most affected trace element. Rimbach and Pallauf [33] indicated that graduated supplementation of phytic acid had a negative effect on apparent zinc absorption and life weight gain of growing rats.

Phytic acid interacts with proteins over a wide pH range, forming phytate-protein complexes due to electrostatic interactions. Ionic binding occurs between the basic phosphate groups of phytic acid and protonated amino acid (lysyl, histidyl, arginyl) residues [34]. Phytic acid also interacts with some important digestive enzymes, such as, trypsin, pepsin, α-amylase and galactosidase, resulting in a decrease in their activity [8].

1.1.6. Phytic Acid & Environmental Pollution:
Monogastric animals (pig, fish, poultry) cannot hydrolyze the phytate within their meal (cereals, legumes, oilseeds) and therefore, a large amount of phytate-bound phosphorus remains unavailable to them. However, undigested phytate-phosphorus is excreted through the faeces and spread as manure into the soil [35]. The soil microorganisms having phytase activity then act upon it and releases phosphorus which accumulates in the surrounding soil causing soil pollution. Since phosphorus is a necessary nutrient for animal growth, supplementation of inorganic phosphorus with the feeds is required. Inadequate phosphorus supply with diet causes phosphorus deficiency leading to weak immune system, bone breakage, loss of appetite, reduction in fertility and loss in live weight gain due to low feed efficiency. The unabsorbed inorganic phosphorus also excreted in soil causing soil phosphorous pollution. Eutrophication is
seen in the areas of extensive livestock production when run-offs of soil are mixed with nearby fresh water streams, lakes and near coastal areas. Eutrophication can cause cyanobacterial blooms, hypoxia and death of aquatic animals followed by production of nitrous oxide, a potential green-house gas [36, 37].

Fig 3.: Phytic acid phosphate cycle [38]

(a) Inorganic phosphate (Pi) is absorbed by plant roots from the soil fluid and translocated by the xylem and the phloem to all parts of the plant. Only a limited amount of the soil Pi is available to the plant because the mobility of Pi is low and it has high affinity for organic and inorganic compounds, and for soil particles. (b) Phytic acid is the major phosphate storage compound in seeds. (c) In agricultural systems, seeds are used either for plant
production or as feed for livestock production. In particular, feeding of non-ruminant animals causes the excretion of large amounts of undigested phytic acid, because the digestive system of these animals lacks phytases. (d) Up to 80 % of phosphorus supplied via fertilizers becomes fixed in the soil.

1.2. Phytase overview:
Phytases or phytate-degrading enzymes belong to a special class of phosphomonoesterases termed myo-inositol hexakisphosphate phosphohydrolases and catalyze stepwise release of phosphate residues from phytate [22].

1.2.1. Sources of Phytase:
Phytases have been isolated from microorganisms, such as, fungi, yeast, bacteria and protozoa [5] and plants and also in some animal tissues [39].

1.2.1.1. Phytases from Microorganisms:
1.2.1.1.1. Fungal Phytases:
Among microorganisms, fungi, especially Aspergillus sp. are considered as the major phytase producer. A. niger and A. ficuum are commonly used in the commercial phytase production [40]. Shieh and Ware [41] screened more than 2000 organisms from 68 soil samples and identified Aspergillus niger as the most active phytase producer. There are several reports of production of phytase from Aspergillus sp., namely, A. niger [42-50], A. ficuum [51, 52], A. oryzae [53], A. fumigatus [54], A. japonicus [55], A. niveus [56]. The genus Aspergillus (A. niger in particular) is favoured for phytase production due to its GRAS status, great secretory potential, in-depth knowledge about its cultivation and high yield and acid tolerance of the produced phytase [17]. Phytase from A. niger (PhyA) was well characterized and commercialized. It was encoded by a 1.4 kb DNA fragment and was a monomer having molecular weight of 80 kDa and two optimal pH at 2.5 and 5.0-5.5, optimal temperature at 55-60°C and had a high affinity for phytate [57].

Phytase from A. fumigatus showed a 66% sequence similarity with phytase (PhyA) from A. niger and its thermotolerance was better [58, 59]. The enzyme had a broad pH range and was highly active against inositol phosphates with low degree of phosphorylation [60, 61]. However, its specific activity against phytate was low [62]. Peniophora lycii PhyA phytase has also been commercialized. It has an optimal pH at 4.0-4.5 and optimal temperature at 50-55°C and had
dimeric conformation [63]. It seems to be susceptible to thermal treatments and proteases [64] or low pH. *Cladosporium sp.* FP-1 produces a low molecular weight phytase (32.6 kDa) which was not glycosylated and had an optimal pH and temperature at 3.5 and 40°C, respectively [65]. Phytases from thermophilic fungi *Myceliophthora thermophila* and *Talaromyces thermophilus* [66, 67] exhibit a high degree of sequence homology to other fungal phytases from *A. niger*, *A. terreus* or *A. fumigatus*. Phytase from thermophilic fungus *Thermomyces lanuginosus* [68] is more thermostable and showed better catalytic efficiency and higher transition temperature than that of the *A. niger* phytase. Chadha *et al.* [69] reported of a phytase from thermophilic fungus *Mucor pusillus* to be active in a wide pH range of 3-7.8.

### 1.2.1.1.2. Yeast Phytases:

Nakamura *et al.* [70] surveyed 738 strains of yeast and found significant levels of phytase activity in 35 species with a wide range of optimum pH and temperature. *Arxula adeninivorans* can utilize phytate as sole source of phosphate and secreted phytase having optimal pH and temperature around 4.5-5.0 and 75°C, respectively [71]. Phytase from soil-isolated yeast *Candida krusei* WZ-001 had two different subunits with molecular masses of 116 and 31 kDa and had an optimal pH at 4.6 and optimal temperature at 40°C [72]. Phytase activity has also been detected in *Pichia anomala* [73], *Saccharomyces cerevisiae* [74] and *Schwanninomyces castellii* [75]. These enzymes were active in the acidic pH range with an optimal temperature at 60-74°C.

### Table 3: Microbial sources of phytase

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Names</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td><strong>Aspergillus niger</strong></td>
<td>[42-50, 57]</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td><em>A. oryzae</em></td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td><em>A. ficuum</em></td>
<td>[51, 52]</td>
</tr>
<tr>
<td></td>
<td><em>A. japonicus</em></td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td><em>A. niveus</em></td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td><em>Peniophora lycii</em></td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td><em>Cladosporium Sp. FP-1</em></td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td><em>Myceliophthora thermophila</em></td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td><em>Talaromyces thermophilus</em></td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td><em>Thermomyces lanuginosus</em></td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td><em>Rhizomucor pusillus</em></td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td><em>Penicillium funiculosum</em></td>
<td>[76]</td>
</tr>
</tbody>
</table>
1.2.1.1.3. Bacterial Phytases:

*Escherichia coli* AppA phytase had a molecular mass of 42 kDa, an acidic optimal pH, high specific activity for phytic acid and is resistant to pepsin hydrolysis [60]. For this reasons *E. coli* AppA phytase is more effective than *A. niger* phytase in releasing phytate-phosphorus in diets for swine and poultry [97]. A novel *E. coli* phytase gene (App2) had been isolated from pig colon and expressed in *Pichia pastoris* [98]. The phytase of *E. coli* has been reported to be periplasmic. The only bacteria showing extracellular phytase activity are those of the genera *Bacillus* and *Enterobacter*. *Bacillus* phytases were monomers with a molecular mass of 38-47 kDa, optimal pH in the neutral range and optimal temperature at 55-70°C [99].
Yanke et al. identified [81] several phytase-producing anaerobic bacteria in cattle rumen and found *Selenomonas* species to be the highest producer followed by *Mitsuokella multiacidus*. Phytase from *Pseudomonas syringae* had a molecular mass of 45 kDa, high specific activity, pH optimum at 5.5 and temperature optimum at 40°C [83]. Kim et al. [84] isolated a novel phytase from *Citrobacter braakii* with optimal pH and temperature at 4 and 50°C, respectively and higher specific activity against phytic acid than other phosphorylated substrates. Phytases had also been isolated from *Obesumbacterium proteus* [85], soil bacterium *Klebsiella spp*. ASR1 [86] and several species of the *Bifidobacterium* genera [88].

1.2.1.4. Phytases from other microorganisms:
Existence of phytase in protozoan *Paramecium tetraurelia* had been reported [89]. The enzyme was a hexamer of 240 kDa with optimal pH of 7.0 and no requirements for divalent cations for activity. Cheng [90] had sequenced a phytase gene from the psychrophile *Shewanella oneidensis* MR-1 that showed a 30% peptide sequence identity with that of *Bacillus spp* phytase.

1.2.1.2. Phytases from Plants:
Most of plant phytases had optimal pH between 4.5-6.0 and optimal temperature between 38-55°C. However, there were wide variations of plant phytases in kinetics (K<sub>m</sub>: 30-300 µM; K<sub>cat</sub>: 43-704 s⁻¹; specific activity: 43-636 U/mg protein) [5]. The phytase from lily pollen showed an optimal pH of 8 and an optimal temperature of 55°C. This enzyme was activated by calcium and inactivated by EDTA and had a narrow substrate specificity [94]. Hageman [92] had isolated a phytase gene from germinating soybean that displayed optimal pH at 4.5-5.0 and optimal temperature at 58°C.
Phytase activity has been detected in cereals (e.g. wheat, spelt, rye, barley, triticale), legumes and oilseeds [100] or highly consumed fruits and vegetables like avocado and scallion leaves [93].

1.2.1.3. Phytases from Animal Tissues:
Phytase detected in brush border vesicles of poultry by Maenz [95] showed an optimal pH of 5.5-6.0. Phytase in the hybrid stripped bass showed an optimal pH of 3.5-4.5 [101]. Phytases have also been detected in tissues of several other animal species [102] and many of these enzymes displayed an optimal pH in the neutral to alkaline range.
1.2.2. Classification of Phytase:
Phytases can be grouped based on their pH optima (acid or alkaline phytases), catalytic mechanisms (histidine acid phosphatases, β-propeller phytase, cysteine phosphatases or purple acid phosphatases) or stereospecificity of phytate hydrolysis (3- or 6- phytase).

1.2.2.1. Acid or Alkaline Phytase:
As their names suggest, these phytases have optimum pH either in the acidic (pH 5.0) or in the alkaline (pH 8.0) range, respectively [39]. Acidic phytase producer are *Escherichia coli*, *Pantoea agglomerans* and *Enterobacter cloacae*. Alkaline phytases can be found in lily pollen and *Bacillus* sp. [103].

1.2.2.2.a. Histidine Acid Phosphatases (HAP):
This group of phytases has RHGXRXP as the N-terminal active site motif and HD as the C-terminal motif [104]. Catalytic site was formed upon proper folding of the amino acid sequence to position together. Both prokaryotic and eukaryotic HAPs possess positively charged active site at acidic pHs to facilitate substrate binding. The best characterized prokaryotic HAPhy (histidine acid phytase) is *E. coli* phytase [105, 5] and its 3-D molecular structure is available [106]. In eukaryotes, HAPhys have been cloned in a number of fungal isolates and in maize [107]. The most widely studied fungal phytases are from *A. niger* and *A. fumigatus* [5]. Glycosylation and disulfide bridges are two important factors in determining and maintaining the structure of HAPhys [5]. Based on substrate specificity, all the known microbial HAPhys are divided into two classes. The first class has broad substrate specificity but a low specific activity for phytate, while the second class has narrow substrate specificity and a high specific activity for phytate [60].

1.2.2.2.b. β- Propeller Phytase (BPPhy):
Molecular structure of this group of phytases consists of β-sheets and resembles a six bladed propeller [80, 108]. A thermostable phytase from *Bacillus amyloliqufacience* falls into this class [80]. This group of enzyme requires Ca$^{2+}$ for both catalytic activity and thermostability. The calcium ions are thought to facilitate the binding of phytate by generating a favourable electrostatic environment [5]. Catalytic mechanism of BPPhys requires one “affinity site” that attracts the substrate and an adjacent “cleavage site” that hydrolyzes the phosphate group [108].
Fig 4.: Crystal structure of a phosphorylated phytase from Aspergillus fumigatus, a representative model of histidine acid phosphatases [109]

Fig 5.: Cadmium inhibited crystal structure of phytase from Bacillus amyloliquefacience, a representative model of β-propeller phytase [110]

Fig 6.: Crystal structure of Selenomonas ruminantium phytase, a representative model of cystein phosphatases [111]
1.2.2.2.c. Cysteine Phosphatases (CP):
This group of phytases have HCXXGXXR(T/S) as active site motif and have substantial similarities with protein tyrosine phosphatase, a member of cystein phosphatase superfamily. The active site forms a loop that functions as a substrate binding pocket and its depth determines substrate specificity [112]. Phytase of *Selenomonas ruminantium* is a member of this class [82].

1.2.2.2.d. Purple Acid Phosphatases (PAP):
All members of this class contain a unique set of seven metal-liganding amino acid residues (D, D, Y, N, H, H, H) that are contained in a shared pattern of five common consensus motifs (DxG/ GDx2Y/ GNH (E, D)/ Vx2H/ GHxH) [113]. This is a large class of phosphatases with known representatives in plants, mammals, fungi and bacteria [113, 114]. A binuclear metallic center containing two irons is found in animal PAPs, while in plant PAPs, the second iron ion is replaced by either a zinc or magnesium ion [114].

1.2.2.3. 3- & 6- phytase:
3-phytases initiates phytate hydrolysis from the D-3 position of the myo-inositol ring and 6-phytase preferentially initiates phytate dephosphorylation at the L-6 (D-4) position.

1.2.3. Fermentative Production of Phytase:
1.2.3.1. Submerged Vs Solid State Fermentation:
Phytase enzyme can be produced by submerged (SmF) [42, 52, 53, 115-117] as well as solid state fermentation methods (SSF) [45, 42, 46, 48, 50]. In SmF, the organisms are cultivated in liquid media in aerated tubes, flasks or fermentors. This method has advantages of low labour and space requirements, uniformity and ease of control. However, phytases produced by SmF are expensive due to product dilution, use of recombinant strains and high product recovery costs [50]. SSF holds tremendous potential for the production of enzymes. SSF can be defined as a fermentation that takes place in the absence or near absence of free water, on a solid or semisolid substrate or in a nutritionally inert support [118]. This system offers numerous advantages over SmF system including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments and process control, low power requirement and less contamination problem [119]. Filamentous fungi are the most important group of microorganisms used in SSF process owing to their physiological, enzymological and biochemical properties. The hyphal mode of fungal growth and their good
tolerance to low water activity and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates and for the utilization of available nutrients. The basic mode of fungal growth is a combination of apical extension of hyphal tips and the generation of new hyphal tips through branching. The hyphal mode of growth gives the filamentous fungi the power to penetrate into the solid substrates. The cell wall structure attached to the tip and the branching of the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of SmF, which makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. Penetration increases the accessibility of all available nutrients within particles. Fungi cannot transport macromolecular substrates, but the hyphal growth allows a close contact between hyphae and substrate surface. The fungal mycelium synthesizes and excretes high quantities of hydrolytic exoenzymes. The resulting contact catalysis is very efficient and the simple products in close contact to enter the mycelium across the cell membrane to promote biosynthesis and fungal metabolic activities [120]. Therefore, use of filamentous fungi in SSF for the production of phytase is advantageous, as with high volumetric productivity, one can obtain the final product in concentrated form, therefore lowering the need of downstream processing. Also, the fermented solid can be extracted immediately, dried and used directly in animal feed preparations [17, 121, 122].

1.2.3.2. Environmental Parameters Affecting Phytase Production:

   a) Substrate:

   The selection of a suitable substrate for enzyme production in SSF depends on several factors; key factors being cost and availability of the substrate [122]. The energy required and the physical support for a fungus to grow and to produce the desired metabolite(s) is primarily provided by the substrate [123]. An ideal substrate provides carbon, nitrogen, minerals, and growth factors to the microorganisms and also has the ability to absorb water to promote growth [124]. So, there is a need of screening several solid wastes or agricultural residues for this purpose. Wastes are the rejected materials, which have very less value in human society. Due to natural fermentation, it releases hazardous gases in the environment, which are responsible for
environmental pollution. Moreover, wastes are responsible for major economic loss. Bioconversion of these wastes to commercially important enzymes will provide useful economic products and will solve the disposal problem and will minimize the environmental pollution hazards [119]. Some of the solid substrates utilized for the production of phytase are, wheat bran [56, 69, 50, 42, 46, 43, 48], rice bran [45], groundnut cake, mustard cake [125], coconut oil cake, palm kernel cake, groundnut oil cake and sesame oil cake [126], citric pulp [46], sugarcane bagasse [127], cassava bast [55] etc..

b) Time of Fermentation:
Time of fermentation is an important parameter for the production of any enzyme in SSF. The duration of incubation is generally dependent on the growth rate and enzyme production pattern of the strain [128]. Optimum fermentation time for phytase production by A. ficuum and P. funiculosum was at 198.3 hours and 120 hours, respectively [51, 74]. There were several reports of optimum phytase production by different strains of A. niger at 96 hours [46], 72 hours [44, 45], and 7th day of fermentation [125]. Optimum fermentation time for phytase production by A. niveus was 6th day of fermentation [56].

c) Fermentation Temperature:
Temperature of incubation is another important factor for the growth of the organism and enzyme production. Temperature control during SSF can be quite challenging due to static nature of the process and poor thermal conductivity of the solid substrates [122]. Gautam et al. [77] found that fermentation temperature influenced the rate of phytase production by A. ficuum and R. oligosporus during SSF and 30°C promoted highest phytase production for both the fungi. Several reports are available on better production of phytase at 30°C [42, 48, 50, 46]. Cha et al. found 35°C fermentation temperature was optimum for phytase production from A. niger [45]. Badamchi et al. produced phytase from A. ficuum at 30°C in packed bed solid-state bioreactor [51]. Boger et al. produced optimum phytase from A. ficuum at 26±1°C [43]. El-Gindy et al. and Moreira et al. found 40°C and 26°C fermentation temperature were optimum for phytase production, respectively, from A. niveus and A. japonicus [56, 55].
d) **Hydration of Substrate:**

In SSF, moisture levels play a critical role as microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture content [123]. The optimum moisture content for growth and substrate utilization not only depends upon the organism but also on the substrate used for cultivation [120]. At too low moisture levels, the substrate is less accessible, as it does not swell and microbial growth is reduced and hence enzyme production. However, too high moisture levels result in reduction in substrate porosity, lowering the oxygen diffusion rates and gaseous exchange. Consequently, the rate of substrate degradation is reduced and there is also an increased risk of microbial contamination [2]. The control of water activity in the substrate is essential for mass transfer of water and solutes across the cells and it can be used to modify the metabolic production and excretion of the microorganisms [118]. If the water content and constant substrate volume is increased, the air content of the substrate is reduced [77]. Bhavsar *et al.* reported optimum hydration to be 1:2, during the SSF of wheat bran by *Aspergillus niger* [50].

e) **Particle Size and Amount of Substrate:**

The size and shape of substrate particles influence the accessibility of the nutrients to the microorganisms [129]. Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. However, too small a substrate particle may result in substrate agglomeration, interfering with microbial respiration/ aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration/ aeration efficiency due to increased inter-particle space, but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process [119]. There is a report that particle size of lupin flour affected both phytase activity and phytic acid hydrolysis by *A. niger* in SSF and better results were obtained with medium and coarse particles (>1.7 mm) [44]. Spier *et al.* reported optimum particle size of the substrate citric pulp to be 0.8-2 mm for phytase production by *A. niger* [130]. Moriera *et al.* used 0.8-2 mm sized cassava bast for optimum phytase production by *A. japonicus* [55]. There were reports of using 5 grams of dry substrates in 250 ml Erlenmeyer flask for phytase production by *A. niveus, Rhizopus* spp. and *R. pusillus* [56, 126, 69]
**f) Inoculum Age:**

Age of inoculum is one of the key factors that strongly influence microbial growth and activity. Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. Enzyme production attains its peak when the nutrients available to the biomass are balanced. Under misbalanced conditions between nutrients and proliferating biomass, enzyme synthesis decreased and it is true with all the microorganisms [126]. Generally researchers used spore suspension as inoculum for phytase production in SSF by *Aspergillus sp.* [125, 126, 42, 46, 43, 48] though Papagianni *et al.* reported the effectiveness of vegetative inocula over spore suspension for phytase production in SSF by *A. niger* [131]. Krishna and Nokes [132] found a strong correlation between phytase yield and age of vegetative inoculum.

**1.2.3.3. Nutritional Parameters Affecting Phytase Production:**

According to Pirt [133] there are five essential conditions, such as, an energy source, nutrients, absence of inhibitors, a viable inoculum and suitable physico-chemical conditions, for growth of microorganisms in a culture. Enzyme productivity is affected by the presence or absence of carbon, nitrogen and mineral supplements [134]. Since, some of the nutrients may be available in sub-optimal concentrations or even absent in the substrate; their supplementation is required with substrate [119]. For this purpose, the input of carbon and nitrogen sources should be regulated based on the requirement of each of these elements in minimum quantities to produce a certain quantity of biomass or metabolite [2]. Thus, optimization of nutritional variables is an important step in the development of a fermentation process. Phytase production is highly influenced by the media components, in particular, the chemical composition of the different ingredients, the quality of carbon and nitrogen sources, in addition to minerals and metal ions, particularly phosphorus [17].

**a) Carbon Source:**

A carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance. In most fermentation it also serves as the energy source. Carbohydrates are traditional carbon and energy sources for microbial fermentations, although alcohols, alkenes and organic acids may be used [2]. Glucose is the most utilized carbohydrate source in the fermentation industry [135]. For this reason, several experiments were performed to study the effect of glucose on phytase production by filamentous fungi [51, 42, 76, 126]. Glucose was the
preferred carbon source for phytase production by *Rhizopus sp.* [126]. Badamchi *et al.* [51] optimized glucose concentration for phytase production by *A. ficuum* by response surface methodology. Sucrose and starch were preferred as carbon source, respectively, by *A. niger* and *A. ficuum* [42, 43]. Dextrin was utilized by Soni and Khire [116] for phytase production from *A. niger* NCIM 563. It was reported that molasses at 10% (w/v) concentration enhanced phytase production from *Penicillium funiculosum* NRC467 using crushed fava bean as substrate [76].

**b) Nitrogen Source:**
An adequate supply of nitrogen is required to obtain rapid growth of the organism. In filamentous fungi 15% of the mycelial dry weight may be composed of nitrogen and the media should include an appropriate source [17]. *Aspergillus* species are capable of utilizing a wide range of nitrogen-containing compounds as nitrogen source, such as, ammonia, nitrate, nitrite, purines, amides and amino acids [136]. Bogar *et al.* reported maximum phytase production with ammonium sulphate [43]. El-Gindy *et al.* studied the effect of supplementation of yeast extract, malt extract, corn steep liquor, cane molasses, beet molasses, crude whey and whey permeate with substrate on phytase production by *Malbranchea sulphurea* and *A. niveus* [56]. There were reports that ammonium nitrate gave best production of phytase [46, 126]. Gulati *et al.* observed maximum phytase production with ammonium dihydrogen phosphate by *Mucor indicus* [78]. Gunasree *et al.* observed maximum phytase production with sodium dihydrogen phosphate by *A. niger* [42].

**c) Phosphorus Source:**
Extracellular fungal phytase production was negatively correlated with the concentration of inorganic phosphate in the growth medium [42]. Low concentrations of phosphorus in the media limited mycelia growth but phytase production was maximal. Conversely, an increase in phosphate in the medium promoted the growth of the fungi, but phytase synthesis decreased considerably [17]. The most commonly used phosphorus sources for phytase production were KH$_2$PO$_4$ and K$_2$HPO$_4$ [23]. Bogar *et al.* reported inhibition of phytase production by *A. ficuum* NRRL 3135 above 10 mg per g DM [43]. Mandviwala *et al.* observed inhibition of phytase production above 10 mg phosphate per 100 gm wheat bran by *A. niger* NCIM 563 [125]. Similar effect was observed by *Aspergillus ficuum* on semi-solid substrate using soyabean meal [137]. Inhibition of phytase production at higher phosphate concentration was also observed in *A.*
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ficuum [138] and A. carbonarius [139]. Kim et al. [140] found that both phytase and phosphatase synthesis by Aspergillus sp 5990 were maximal at low phosphate concentrations (<50 mg/L) but decreased at higher concentration of phosphate (>100 mg/L). Vats and Banerjee [11] observed that at 0.05% (w/v) final concentration of phosphate, phytase production by A. niger decreased abruptly and no production occurred at 0.1% (w/v) concentration or above. Recently, Makarewicz et al. [141] studied the regulation of phyC gene expression in the strains of the genera Bacillus and found that the gene expression was strictly dependent on phosphate starvation.

d) Metal Salts:

Metal salts significantly affect the progress and efficiency of fermentation and secretion of active enzymes by microorganisms. Metal salts also affect the ability of microorganisms to attach to various substrates [142] by affecting the dynamics of cell membranes, cell viability, permeability, membrane fluidity, stability and signaling systems [143]. All micro-organisms require certain mineral elements for growth and metabolism [144, 145]. Normally, cobalt, copper, iron, manganese, molybdenum, zinc, calcium, magnesium, phosphorus, sulphur and chloride ions are added as specific salts to the medium [2]. For the yeasts such as Schwanninomyces castellii [75], Arxula adeninivorans [71], Pichia Spartinae and P. rhodanensis [70], the production media included metal ions such as Mg$^{2+}$, Mn$^{2+}$, K$^+$, Ca$^{2+}$, Fe$^{3+}$. Hassouni et. al [127] reported stimulatory effect of Ca$^{2+}$ and Mg$^{2+}$ on phytase production in SSF. Activation of microbial phytases in presence of metal ions was studied by Vats & Banerjee [11].

1.2.3.4. Statistical Approach for Fermentative Production of Phytase:

Response Surface Methodology (RSM), a most widely used statistical technique for bioprocess optimization, is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for optimum conditions of factors for desirable responses [146, 147]. Statistical approaches involving multivariable optimization are nowadays gaining importance for process optimization studies in biotechnology [148] as single-variable optimization methods are tedious and also can lead to misinterpretation of results because the interaction between different factors is overlooked [149]. Some of the popular choices of RSM include the Plackett- Burman design (PBD) [150, 151], the Box-Behnken design [152], the central composite design (CCD) [153, 154] and the Graeco-Latin square design [155]. Among
them, Box-Behnken design (BBD) is more efficient and easier to arrange and interpret in comparison with others [156]. Also it permits estimation of the best fit parameters of the quadratic model, building of sequential designs and detects lack of fit of the model [157]. Several researchers used CCD model for optimization of production parameters and achieved a 5.25-fold, 10-fold, 3.4-fold and 9-fold increased phytase production, respectively [158, 159, 51, 160]. Boger et al. optimized fermentation medium for phytase production by A. ficuum NRRL 3135 using CCD and obtained 50% increased phytase production [43]. There are several reports on optimization of process parameters for phytase production using BBD. A phytase from A. niger FS3 had an overall 4.3-fold improvement in production after optimization [161]. Phytase production from A. niger NCIM 612 improved by 3.08-fold after optimization of medium components using PBD and BBD [50]. In addition, cultural conditions for phytase production from Rhizomucor pusillus and Schizophyllum commune were also optimized using BBD and optimization resulted in a 2.98-fold and 30% increased phytase production [162, 69]. BBD was also used to optimize the production medium for a moderately halophilic bacterium Pseudomonas AP-MSU2 [163].

1.2.3.5. Purification and Characterization of Phytase:

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. To analyze the function, structure and interactions of the protein of interest, it has to be purified first. Separation may be done by exploiting differences in protein size, physicochemical properties, binding affinity and biological activity of proteins. As phytase is mostly extracellular when produced by SSF, it can be easily extracted with water or a buffer. In bulk protein purification, ammonium sulphate precipitation is a common first step to isolate the protein. Then chromatographic resolution using ion exchange, gel filtration, and/or chromatofocusing are done followed by molecular weight determination.

1.2.3.5.1. Purification of Phytase:

Casey and Walsh used DEAE-Sepharose CL-6B followed by Sephacryl S200 and PBE 90 after ultrafiltration to achieve a total 24.89-fold purification and 26% phytase yield [49]. After ammonium sulphate precipitation, Greiner used a series of combination of chromatographic techniques to obtain a 469-fold purification with 16% phytase recovery [48]. Sariyska et al [47] and Vijayaraghavan et al [164] used a simple 2-step chromatographic resolution to achieve 30-
fold and 4.75-fold purification respectively. However, Dvorakova et al [165] loaded crude filtrate onto Sephadex G-100 followed by DEAE-cellulose and Mono Q HPLC and achieved 4.1-fold purification. Phytase from both *M. sulfurea* and *A. niveus* were partially purified by ammonium sulphate precipitation and ion exchange chromatography on bentonite (2.5%) and 5.05 and 14.44-fold purification was achieved, respectively [56]. Gulati *et al.* reported to obtain a 21.9-fold purified phytase from *M. indicus* by simple two step anion exchange and gel filtration chromatography [78].

**1.2.3.5.2. Molecular Weight Determination of Phytase:**

Molecular mass of the biologically active enzyme was determined either by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) or molecular sieve filtration or by the combination of both. Most fungal phytases are monomeric proteins [165]. A phytase accumulating in maize seedlings during germination is a dimeric enzyme consisting of two 38 kDa subunits [91]. An unusual large molecule (700 kDa), together with a small peptide (10-13 kDa) were found in *Klebsiella aerogenes* [87]. Bhavsar *et al* [50] and Greiner *et al* [48] determined molecular weight of a fungal phytase as 87 kDa and 85 kDa respectively, by gel filtration using Sephacryl S-200. Casey and Walsh [49] determined the molecular mass of purified phytase to be 84 kDa by SDS-PAGE. Purified phytase of *Sporotrichum thermophile* was a homopentameric glycoprotein having molecular mass of 456 kDa [13].

**1.2.3.5.3. Temperature and pH Optima and Stability:**

Most fungal phytases have a pH optimum between 4.5-5.6. In contrast, *A. fumigatus* phytase has a broad pH optimum; at least 80% of the maximal activity is observed at pH values between 4.0-7.3 [6]. Phytases from various *Aspergilli* show optimum temperature in the range 40–65°C [166]. Phytase from *A. japonicus* URM 5633 had optimum pH at 3.6 and optimum temperature at 60°C. Phytase was stable in the pH range of 2.4-3.0 at 30-70°C [55]. The partially purified phytase from *A. niger* NCIM 563 was optimally active at 55°C and pH 6.0. The enzyme retained 75% activity over a wide pH range 2.0–9.5 and retained 20% residual activity at 60°C after 1 h [50]. The temperature and pH optima of partially purified phytase from *M. sulfurea* and *A. niveus* were 70°C and 5.6, respectively. Thermostability over a wide range of temperature (40-75°C) and pH stability over a wide range of pH (4.6-5.6) was observed for both the organism [56]. *M. thermophila* phytase showed pH optimum at 5.0 and temperature optimum at 45-50°C.
phytase was stable at pH range 5-6.5 [127]. Escobin-Mopera et al. [167] observed a 15% reduction in enzyme activity at 60°C after 10 min of a phytase from Klebsiella pneumoniae 9-3B. These authors obtained highest enzyme activity at 50°C, which was stable even after 1 h of exposure. pH optimum of the phytase was at pH 4.0, with broad pH stability from 2.0 to 7.0. Phytase from R. oryzae had a lower optimum temperature (45°C) and dual pH optima at 1.5 and 5.5 [79]. Chadha et al. [69] reported of a partially purified phytase from Rhizomucor pusillus which was optimally active at 70°C and pH 5.4, though the enzyme showed approximately 80% activity over a wide pH range, 3.0–8.0. Phytase from A. niger 11T53A9 had two distinct pH optima, one at pH 5.0 and another at pH 2.8. The phytase did not lose any activity in the pH range from 3.0 to 8.0 within 14 days. The purified enzyme is also stable at low pH values, retaining full activity after incubation at pH 2 for 24 h at 4°C and 95% after incubation at pH 2.0 at 37°C [48]. Casey and Walsh [49] reported of a phytase from A. niger ATCC 9142 that possessed a temperature optimum of 65°C, and a pH optimum of 5.0. Thermo-stability profiling illustrated that the purified phytase retained 22% residual activity after 3 min at 80°C.

1.2.3.5.4. Effect of Metal Salts on Phytase Activity:
Metal ions have been shown to modulate phytase activity. Study on the effect of metals salts on phytase activity is of great importance considering application of the enzyme. Bhavsar et al. reported stimulation of A. niger phytase activity in presence of 5 mM Fe^{2+/3+}, Ca^{2+}, Ba^{2+} [50]. Fe^{3+} enhanced the phytase activity of A. niveus while Mg^{2+}, Mn^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+} and Fe^{2+} inhibited its activity [56]. Mn^{2+}, Ca^{2+}, Mg^{2+} ions are known to stimulate the phytase of A. niger 92 [165] whereas Cu^{2+}, Hg^{2+}, Zn^{2+} ions were inhibitory to phytases [165, 50]. Greiner et al. reported strong inhibition of A. niger phytase by Fe^{2+/3+} [48]. Zn^{2+}, Fe^{2+} and Fe^{3+} enhanced the phytase activity of M. sulfurea while Na^{+}, Li^{+}, Mg^{2+}, Hg^{2+}, Mn^{2+} and Cd^{2+} inhibited its activity [56]. Escobin-Mopera et al. observed that phytase activity was slightly stimulated by Ca^{2+} whereas moderately stimulated in the presence of Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+} and F^{-} ions and inhibited by Zn^{2+} and Fe^{2+} [167]. Phytase activity from A. japonicas was stimulated in the presence of Na^{+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, Mg^{2+}, Fe^{2+}, Ca^{2+} at 10 mM and of Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+} at 100 mM. However, Fe^{2+} (10 and 100 mM concentrations) significantly stimulated phytase activity [55]. Complete inhibition of a Rhizopus oryzae phytase was observed in presence of Fe^{2+}, Ni^{2+} and Cu^{2+}. The phytase showed stimulatory effect in presence of Zn^{2+}.  

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Phytase activity increased by 5.5- and 2.5-fold in presence of 5 mM Ba$^{2+}$ and Ag$^+$, respectively indicating insensitivity of the phytase to heavy metal ions [79]. However, it is difficult to determine whether the inhibitory effect of various metal ions is due to their direct binding to the enzyme or due to formation of poorly soluble complexes with phytic acid and as a result, active substrate concentration decreased [6].

**1.2.3.5.5. Substrate Specificity and Kinetic Parameters of Phytase:**

Wyss et al. [60] divide phytase into 2 classes in terms of substrate specificity: 1) phytases with narrow substrate specificity, eg., *A. niger*, *A. terreus*, *E. coli* phytases and 2) phytases with broad substrate specificity, eg., *A. fumigates*, *Emericella nidulans*, *Myceliophthora thermophila* phytases. A *A. niger* 92 phytase shows broad substrate specificity hydrolyzing 4-nitrophenyl phosphate and ATP with a relative activity of 35% and 86% respectively [165]. However, a phytase from *A. niger* 11T53A9 had a high affinity towards sodium phytate only. The kinetic parameters for the hydrolysis of sodium phytate were determined to be $K_m = 54 \text{ μmol}^{-1}$ and $k_{cat} = 190 \text{ sec}^{-1}$ at pH 5.0 and 37°C [48]. Non-phytate phosphorylated compounds, such as, ADP, ATP, p-nitrophenyl phosphate, phenyl phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate, α- and β- glycerophosphate and 3-glycerophosphate are frequently hydrolyzed by phytases [6]. A phytase from *R. pusillus* showed comparable activities against phytic acid, pNP, sodium phosphate, AMP, ADP, ATP, NADPH$_2$, phosphoenol pyruvate and riboflavin phosphate [69]. Broad substrate specificity had been observed in *Emericella nidulans* and *M. thermophila* also [67]. Bhavsar et al. observed the $K_m$ and $V_{max}$ of a phytase from *A. niger* were 0.156 mM and 220 lm/min/mg for sodium phytate [50]. In case of a *R. oryzae* phytase, more than 2.4-fold higher activity on ATP was observed as compared to that on sodium phytate. The phytase manifested a $K_m$ and $V_{max}$ of $2.42 \times 10^{-4}$ mM and $6.46 \times 10^3$ mMs$^{-1}$, respectively [79]. Extracellular phytase produced by Aspergillus niger ATCC 9142 showed $K_m$ and $V_{max}$ values of 100 μM and 7 nmol/s, respectively. Substrate specificity studies showed that the enzyme displayed slightly higher activity when assayed with calcium phytate than sodium phytate and displayed significantly lesser activity when assayed using nonphytate- based phosphorylated substrates [49]. Phytase from Thermomyces lanuginosus showed $K_m$ 4.55 μM and $V_{max}$ 0.833 μM/min/mg against sodium phytate as substrate [78]. A phytase from *M. indicus* showed $K_m$ value of 200 nmolL$^{-1}$ with sodium phytate as substrate [78].
1.2.3.6. Immobilization of Phytase:

Enzyme immobilization facilitates the efficient recovery and reuse of costly enzymes and enables their use in continuous, fixed-bed operation. Immobilized enzymes are easier to handle and to separate from the product, thereby minimizing or eliminating protein contamination of the product. Additionally, immobilized enzymes are often more stable than the free ones, allowing the repeated reuse of the biocatalyst [168]. Among the various methods of immobilization (adsorption, covalent binding, entrapment and membrane confinement), entrapment in Ca-alginate beads is favoured as it is cheap, simple, non-toxic, mechanically stable and high yielding [169, 170]. Phytase has been immobilized in different ways using different matrices, such as, on NHS-activated Sepharose [171], on CnBr-Sepharose [172], on epoxy activated Sepabead [173], on solid gelatin particles crosslinked with glutaraldehyde [174], on allophonic synthetic compounds and montmorillonite nanoclays [175], on polyacrylamide gels [176] etc.. Quan et al. have attempted the immobilization of a phytase-producing *Candida crusei* cells on calcium alginate gel-beads for the preparation of specific myo-inositol phosphates [5]. Celem et al. reported of a soybean sprout phytase immobilized on epoxy-activated sepabead EC-EP to retain 44% of its initial activity after 7 cycles of reuse at 60 °C [173]. Jin In et al. reported the reusability of a yeast phytase for 5 cycles which was immobilized by entrapping the yeast cells in calcium alginate beads [177]. Several researchers found that the temperature and pH optima of both the free and immobilized phytase were same [173, 177, 178]. Celem et al. reported of an immobilized phytase having pH optimum at 5.5 [173]. Liu et al. studied the effect of immobilization on thermal stability of *A. ficium* phytase. The optimum temperature was increased to 58°C, which was 8°C higher than that of the free enzyme [174]. Greiner and Konietzny described the covalent immobilization of *E. coli* phytase on NHS activated Sepharose (R) high performance which enhanced thermostability [171]. Increase in $K_m$ and/or $V_{max}$ value after immobilization is very common [171, 173, 174, 178, 179]. The variations in both $K_m$ and $V_{max}$ values of the enzyme upon immobilization may be due to the noncovalent interactions of the immobilized enzyme molecule with the carrier surface which in turn induced an inactive conformation to the enzyme molecules [173]. Singh and Satyanarayana applied immobilized phytase in dough and the bread made with phytase contained reduced level of phytic acid and a high-soluble phosphate [180]. Enzyme immobilization has been employed by Vieira and Nogeira
for the development of a flow injection spectrophotometric procedure to determine the amount of ortho-phosphate, phytate and total phosphorus in cereal samples and by Mak et al. for the development of novel biosensors to measure phytic acid and phytase activity [5].

1.2.3.7. Application of Phytase:

a) Feed Application:

The major application of phytase is still as a feed supplement for monogastric animals to improve phosphorus bioavailability in plant feed-stuffs via the enzyme-mediated hydrolysis of phytate. Phytate is the major phosphorus storage form in cereal, legumes and oilseed meals that are mainly used as feed of monogastric animals (pig, fish, poultry). But due to lack of phytase enzyme, monogastrics cannot utilize the phytate-bound phosphorus. Phosphorus is an essential nutrient to biological systems and is one of the most important minerals in animal nutrition. It is the second most abundant element in an animal’s body after calcium, with 80% of phosphorus found in bones and teeth and the remainder located in the body fluids and soft tissue [181]. Phosphorus plays key role in major metabolic processes like, development and maintenance of skeletal tissue, maintenance of osmotic pressure and acid base balance, energy utilization and transfer, protein synthesis, transport of fatty acids, amino acid exchange, growth and cell differentiation, appetite control, efficiency of feed utilization and fertility [182]. Therefore, inadequate phosphorus supply with diet can cause phosphorus deficiency leading to weak immune system, bone breakage, loss of appetite, reduction in fertility and loss in live weight gain due to low feed efficiency [183]. Therefore, to maintain animal welfare and productivity, livestock producers traditionally supplement inorganic phosphate with the feeds for pigs and poultry which is very costly [7]. Phytase supplementation with pig and poultry feed increases phosphorus bioavailability as a result of phytate hydrolysis and also reduces the need of costly inorganic phosphate addition. Role of phytase in the management of natural resources of phosphorus and thereby saving inorganic phosphates makes it an important feed enzyme worldwide as inorganic phosphate is a non-renewable resource [5]. The FDA (The Food and Drug Administration) has awarded phytase preparation a GRAS (Generally Regarded As Safe) status [104]. Phytase supplementation also significantly improves digestibility of protein and utilization of calcium and zinc [184, 185, 186, 19]. Jondreville et al. [187] reported that 500 units of Natuphos® replaced 30 mg of Zn as sulphate given in a maize-soya-bean meal based diet [5].
Finase® phytase added to corn-soybean pig diet converted approximately one-third of the unavailable phosphate to an available form [188]. In a similar way, experiments with Allzyme® phytase and Natuphos® phytase additions to pig and chicken diets indicated that phytase improved the bioavailability of phytate phosphorus for pigs and broiler chickens [189]. Chantasartrasamee et al. reported that digestion of pre-mixed chicken feed with the enzyme koji at approximately 500 U/kg feed in the presence of 1% each of trypsin and taurochlorate increased the phosphate and protein available for animal consumption [53].

b) Application of Phytase as Environmental Pollution Reducer:

Monogastric animals (pig, fish, and poultry) cannot utilize the dietary phytate-bound phosphorus in their feed (cereals, legumes and oilseed meals) due to lacking phytase enzyme. To meet up their phosphorus requirement, they are also provided with inorganic phosphate supplementation with feed. The excess phosphorus along with the undigested phytate is excreted with feaces and accumulates in soil causing phosphorus pollution. This can be eliminated by phytase supplementation with feed of monogastrics. Phytase releases phytate-bound phosphorus by phytate hydrolysis and it can then be taken up by those animals. As a result, the need of phosphorus supplementation as well as feacal phytate excretion is reduced, consequently, reducing environmental phosphorus pollution. In Holland, A. niger phytase has been successfully introduced as a feed supplement, leading to a 30-40 % reduction in phosphate pollution [188]. Sukumar et al. studied the effect of phytase enzyme incorporation in the feed of layer chicken and found significant (p<0.01) reduction of phosphorus excretion in the droppings of birds [190].

c) Food Application:

High levels of dietary phytate ingestion negatively affect the absorption of trace elements such as iron and zinc in the digestive tract. Supplementation of phytase in human foods is proved to be an effective way for reducing negative effect of phytate on mineral utilization [5]. Porres et al. reported effectiveness of phytase in reducing bread phytate and improving iron bioavailability [191]. The phytase-mediated dephytinization of infant formulas, infant cereals or complementary foods has been studied and it was found that the effectiveness in improving trace element nutrition is greater if the protein supply is from legume seeds instead of milk [5, 192]. Addition of phytase to several plant phytates were reported to release significant amount of inorganic phosphate. Some of the plant phytate sources used are – chick pea, corn, green pea, ground nut
and pearl pea [164], wheat flour, sesame oilcake, soymilk and soybean meal [180, 193, 194, 68]. Jin In et al. reported the use of harvested *S. cerevisiae* CY cells for the degradation of phytate in the soybean-curd whey. Over 60% of phytate in the soybean-curd whey were found to be degraded linearly with cells of 27.16 U/g-phytate dosage as phytase source after a hydrolysis time of 2 h [177].

Development of low-phytate crops via impairing the biosynthesis of phytic acid by disruption of the inositol polyphosphate kinases or other mutations could be another alternative to reduce the negative effects of phytate [38, 195, 5], but it was reported that low-phytate maize and soybean have reduced yield and seed germination [196, 197, 5].

Phytase supplementation also improves the nutritional quality of food by enhancing protein digestibility and other mineral availability (calcium, magnesium, copper, cobalt, and manganese) through phytate hydrolysis.

**Table 4. List of Some Commerially Available Microbial Phytases [19, 198]**

<table>
<thead>
<tr>
<th>Company</th>
<th>Phytase Source</th>
<th>Production Strain</th>
<th>Trademark</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB Enzymes</td>
<td><em>Aspergillus awamori</em></td>
<td><em>Trichoderma reesei</em></td>
<td>Finase</td>
</tr>
<tr>
<td>Alko Biotechnology</td>
<td><em>A. oryzae</em></td>
<td><em>A. oryzae</em></td>
<td>SP, TP, SF</td>
</tr>
<tr>
<td>Alltech</td>
<td><em>A. niger</em></td>
<td><em>A. niger</em></td>
<td>Allzyme phytase</td>
</tr>
<tr>
<td>BASF</td>
<td><em>A. niger</em></td>
<td><em>A. niger</em></td>
<td>Natuphos</td>
</tr>
<tr>
<td>Biozyme</td>
<td><em>A. oryzae</em></td>
<td><em>A. oryzae</em></td>
<td>AMAFERM</td>
</tr>
<tr>
<td>Phermic</td>
<td><em>A. oryzae</em></td>
<td><em>A. oryzae</em></td>
<td>Phyzyme</td>
</tr>
<tr>
<td>Roal</td>
<td><em>A. awamori</em></td>
<td><em>T. reesei</em></td>
<td>Finase</td>
</tr>
<tr>
<td>Novozyme</td>
<td><em>Peniophora lycii</em></td>
<td><em>A. oryzae</em></td>
<td>Ronozyme&lt;sup&gt;@&lt;/sup&gt;, Roxazyme&lt;sup&gt;@&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Phytases may find application in food processing for production of functional foods [39] due to its ability to hydrolyze phytate, a chelating agent of dietary minerals. Technical improvements by adding phytases during food processing have been reported for production of plant protein isolates, corn wet milling and the fractionation of cereal bran [22]. Fujita et al. [199] reported the use of an *A. oryzae* mutant strain with high phytase activity for sake brewing that yielded higher amount of alcohol when compared to the wild type strain. Saito et al. [200] proposed a novel use of phytase for separation of soybean β-conglycinin and glycinin.

Phytases are also used in bakery industry. Supplementation of phytase with the dough improves nutritional value of bread by increasing mineral absorption through phytate hydrolysis [201]. The
supplementation of commercial fungal phytase (3.1.3.8) from *Aspergillus niger* in the dough ingredients containing fiber formulation leads to an acceleration of the proofing, an improvement of the bread shape, a slight increase of the specific volume, and also confers softness to the crumb. These improvements in bread quality were suggested to be associated with an indirect impact of phytase on α-amylase activity [14].

**d) Application of Phytase in the Preparation of Myo-inositol phosphates:**
Myo-inositol phosphates play major role in transmembrane signaling and mobilization of calcium from intracellular reserves [6]. Certain levels of dietary inositol phosphates may be health-beneficial with possible functions in antioxidation, anti-tumorigenesis, reducing serum lipids and cholesterol levels, preventing renal calculi via mineral-binding and in diabetes [15, 16, 5]. Chemical synthesis of these compounds is difficult, requiring protection and deprotection steps [202, 6]. Phytase acts on phytate and convers it to lower myo-inositol phosphates. Therefore, phytase can be used for the industrial production of myo-inositol phosphates via phytate hydrolysis.

**e) Application in Pulp and Paper Industry:**
Madhavan *et al.* [203] suggested role of thermophilic phytases as powerful additives in pulp and paper industry. Phytase could act synergistically with xylanase in crude multienzyme preparation from xylanase-producing microorganisms like *Streptomyces cupidosporus* [204] that are used for the treatment of pulp.

**f) Application of Phytase for Peroxidase Preparation:**
The combination of phytase with vanadate produced an effective semi-synthetic peroxidase. A cross-linked enzyme aggregate of 3-phytase was transformed into peroxidase by incorporation of vanadate [205, 198] which showed similar efficiency and asymmetric induction as the free enzyme and can be reused at least three times without significant loss of activity. Phytases from *Aspergillus ficuum, A. fumigatus* and *A. nidulans* catalyzed the enantioselective oxygen transfer reactions when incorporated with vanadium [198].

**Objective of the work:**
In this era we are part of an increasingly health conscious society where health promotion is becoming of greater significance, pollution and waste are high on the agenda and the concern
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with food safety is of supreme importance. The role of animal feed in the production of safe food is recognized worldwide and recent events have underlined its impact on public health, feed and food trade, and food security. Economic and technological advances are driving the development of new feed products, especially products of biotechnology. Enzymes as additives to animal feed have an important role to play in current farming systems. Not only have they improved the digestibility of nutrients, leading to greater efficiency in the production of animal products such as meat and eggs, but they have also improved the quality of the environment by allowing better use of natural resources and reducing pollution by nutrients. Phytases (EC 3.1.3.8 and 3.1.3.26) have been one of the focal enzymes for nutrition, environmental protection and human health during the past two decades. Phytases sequentially cleave orthophosphate groups from the inositol core of phytate, the major storage form of phosphorus (>80%) in cereals, legumes and oilseeds. However, this large amount of phytate-phosphorus can only be utilized by some bacteria and fungi as they have phytase enzyme and can hydrolyze phytate. Monogastric animals, such as, pig, fish and poultry lack phytase and therefore plant phytate remains undigested and the animals suffer from anti-nutritional problems. Again, the ingested phytate by these animals when excreted into the environment causes eutrophication and phosphorus pollution. Under normal physiological conditions, phytic acid chelates essential minerals, such as, calcium, magnesium, iron and zinc. It also binds to proteins, amino acids and inhibits digestive enzymes.

Phytase releases inorganic phosphorus and inositol as well as protein, amino acids, trace minerals and other nutrients chelated with phytates. Addition of phytase in pig, fish and poultry supplements can reduce or eliminate the supplementation of inorganic phosphorus in their feeds, improves the utilization efficiency of these nutrients, reduces phytate phosphorus excretion and consequently reduces phosphorus pollution and helps in maintaining the environmental balance of the concerned areas. Phytate hydrolysis enhances protein digestibility and mineral availability of plant based foods and consequently improves food quality. Certain myo-inositol phosphates released as by-products of phytate hydrolysis are used in treating Multiple Sclerosis, Alzheimer’s disease and Parkinson’s disease. Some of the myo-inositol phosphates may be health-beneficial with possible functions in antioxidation, anti-tumorigenesis, reducing serum lipids and cholesterol levels, preventing renal calculi via mineral binding and in diabetes.
Even though phytase supplementation effectively improves nutritional quality while decreasing phosphorus waste, the high cost of the phytase enzyme preparations, thermostability at elevated temperatures during pelleting and stability at different gastrointestinal pH are some issues that makes phytase an active area of research. To date, only a handful of commercial phytase products are available. Ongoing research projects continue to focus on the discovery or improvement of phytases that could become even more suitable to animal feed applications than current commercial preparations.

In this study, phytase was produced from *Aspergillus niger* NCIM 612 by solid state fermentation utilizing agricultural residue as substrate. Agricultural residues are wastes having very less value in human society. They are responsible for a major economic loss besides environmental pollution by releasing hazardous gases due to natural fermentation. Bioconversion of these wastes to a commercially important enzyme like phytase is encouraging regarding value addition of waste, bioprocess economy, solve of disposal problem and minimization of environmental pollution hazards. To improve the yield of phytase, some environmental factors along with some nutritional factors were optimized. The phytase produced was then partially purified and immobilized. Application of partially purified phytase was studied with food and chicken feed. The following work plan has been outlined:

1. Optimization of environmental conditions for production of the enzyme,
2. Optimization of nutritional conditions for production of enzyme,
3. Optimization of metal salts,
4. Partial purification of the enzyme,
5. Application of partially purified enzyme for food and feed.

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