CHAPTER 1

General Introduction and Literature Review

1.1. INTRODUCTION

Phytic acid (myo-inositol 1, 2, 3,4,5,6 hexakisphosphate) is the main storage form of phosphorus (P) in cereals, legumes and oil seed crops, the major source of nutrient for human and animals (Yao *et al.* 2013). A large portion of phytic acid (PA) in seeds is in the form of calcium/ magnesium phytate, which is highly stable. Although PA serves as the major source of energy and P for seed germination, the bound P is poorly available to monogastric animals. PA also acts as an antinutritional agent due to its interaction with other food ingredients. Under normal physiological conditions PA chelates essential minerals such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}. Phytates also reduce digestibility of proteins, starch and lipid by inhibiting amylase, trypsin, acid phosphatase and tyrosinase (Olazaran *et al.* 2010, Coulibaly *et al.* 2011, Lei *et al.* 2013).

Phytase (myo-inositol hexakisphophate phosphohydrolase) is the enzyme that catalyses the hydrolysis of PA to inorganic monophosphate and lower myo-inositol phosphate, and in some cases to free myo-inositol (Kerovuo *et al.* 1998, Haefner *et al.* 2005, Maldonado *et al.* 2014). Phytase is widespread in nature; occurring in plants, animals and in a variety of microorganisms. Phytases from these sources exhibit variations in structure and catalytic mechanism and consequently, have been categorized into cystein phytases (CPs), histidine acid phosphatases (HAP), β-propeller phytases (BPPs) and purple acid phosphatases (PAPs) (Jorquera *et al.* 2008, Maldonado *et al.* 2014). Moreover, the ExPASy enzyme database (http://www.expasy.ch/ enzyme/) classifies phytases into three different groups: 3-phytase (alternative name, 1-phytase; EC 3.1.3.8), 4-phytase (alternative name, 6-phytase; EC 3.1.3.26), and 5-phytase (EC 3.1.3.72) (Olazaran *et al.* 2010). This classification is based on the carbon ring position where removal of phosphate groups from phytate is initiated. Most bacterial, fungal and plant phytases belong to HAP family that is characterized by conserved active site motifs RHGXRXP and HD. This group of enzymes catalyzes the PA hydrolysis in two steps: a nucleophilic attack of histidine in the active site of the enzyme to the phosphoester bond of PA and protonation of the leaving phosphate group by the aspartic acid

residue of the HD motif (Afinah *et al.* 2010). During the last decade, a number of phytases have been characterized from various microorganisms such as *Aspergillus fumigatus*, *Aspergillus niger*, *Obesumbacterium proteus*, *Citrobacter braakii*, *Bacillus subtilis*, *Escherichia coli*, *Pichia anomala* and *Yersinia intermedia* and corresponding genes have been isolated, cloned, modified and expressed in different hosts (Bhavsar and Khire 2014).

The ruminants digest PA through the action of phytases produced by the anaerobic gut fungi and bacteria present in their rumenal microflora. However, monogastric animals such as pigs, poultry and fishes utilize phytate P poorly because they are deficient in gastrointestinal tract-phytases and therefore an inorganic, non-renewable and expensive mineral supplement is used in their diet to meet their nutritional requirement of P (Rao *et al.* 2009). The unutilized phytate P from plant feed is excreted as an environmental pollutant in areas of intensive live-stock production (Singh *et al.* 2011). Excessive P in soil runs off to lakes and the sea, causing eutrophication and also stimulating growth of aquatic organisms that may produce toxic products injurious to human beings (Vats *et al.* 2005). Therefore, the enzymatic hydrolysis of PA into less-phosphorylated myo-inositol derivatives in the intestine of the monogastric animals is desirable. Attempts have been made to enzymatically hydrolyze PA to improve the nutritional value of feed and to decrease the amount of P excreted by animals (Rodehutscord *et al.* 2006).When used as additives in feed of monogastric animals, phytases not only enhance utilization of phosphate and reduce phosphate output in manures but also increase mineral uptake (Singh *et al.* 2011).

Phytases belonging to class histidine acid phosphatase have been used successfully as a feed additive. Although, the commercial production of phytase is currently focused on the fungal histidine acid phytase from *Aspergillus* species, studies have suggested bacterial phytase as more promising because of their thermostability, higher substrate specificity, greater resistance to proteolysis and better catalytic efficiency. The substrate specificity property of the enzyme is highly desirable to prevent hydrolysis of other phosphate compounds so that they remain available for animal uptake (Nielson *et al.* 2013). Present study aims at isolation, purification, characterization, molecular cloning and expression of bacterial phytase from environmental samples.

1.2. OBJECTIVES

1. To isolate the phytase-producing bacteria from different sources and determine the cellular localization of the enzyme.

2. To determine the taxonomic status of the selected phytase-producers by biochemical and molecular methods.

3. To purify the enzyme from selected phytase-producers and determine their kinetic properties.

4. To optimize growth conditions for increased phytase production.

5. To amplify and clone the gene for the phytase enzyme(s) showing novel biochemical characteristics and sequence analysis.

6. To characterize the function of the gene product.

1.3. LITERATURE REVIEW

1.3.1. PHYTIC ACID AND PHYTATE

Plant foods comprise an important part of human and animal diets by providing carbohydrates, proteins, minerals, vitamins, fibres, various antioxidants and phytochemicals. Yet those same healthy foods also contain components such as phytate, oxalate, tannins, which have been called 'anti-nutritional' elements (Bohn *et al.* 2008).

PA (myo-inositol 1,2,3,4,5,6-hexakis phosphate) and mixed cation salt of PA, phytate, are a group of organic phosphorous (P) compounds found widely in nature. In terrestrial ecosystem, it is synthesized by plants during maturation of plant seeds and grains and therefore is a common constituent of plant derived food. It comprises 80% of the total phosphorous of cereal and legumes, the major source of nutrient for human and animal (Afinah *et al.* 2010, Coulibaly *et al.* 2011). They are also strongly complexed in soils, representing an important class of organic P, which is only poorly available to plants (Wang *et al.* 2013). Since the pioneering work of Harrison and Mellanby (1939) on cereal consumption and rickets, research has determined that phytates are responsible for a number of antinutritional effects, such as reduction of bioavailability of iron, calcium, magnesium, selenium and other di- and trivalent cations through chelation of these minerals (Jorquera *et al.* 2008). Despite its potential drawbacks, PA has some potential benefits as it is similar in some ways to a vitamin, and metabolites of PA serve as secondary messengers in cell signaling (Jenab *et al.* 2002).

1.3.1.1. Chemistry of phytic acid

Phytic acid [myo-inositol 1,2,3,4,5,6-hexakis phosphate, $C_6H_6[OPO(OH)_2]_6$] is a phosphorylated cyclic sugar alcohol commonly found in plant seeds. The prefix "*myo*" refers to the conformation of the hydroxyl groups on the inositol ring. Inositol phosphates have an inositol ring and minimum one phosphate group. According to the set of rules suggested by Posternak (1965), there are nine possible configurations of the inositol ring (Fig.1.1).

Myo-inositol is the major nutritionally relevant form of inositol, which is always numbered according to the D configuration with the numbering initiated at the atom that is esterified in inositol phospholipids. This is recommended by the International Union of Pure

and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB, 1989) to avoid confusions regarding *myo*-inositols and enzymes related to them.

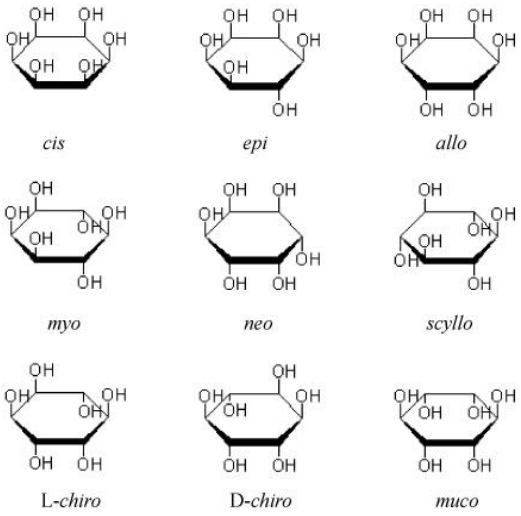

Fig. 1.1: The nomenclature of the nine stereoisomers of inositol (Haworth projections). Seven have a mirror axis in the molecule and numbering of the carbon atoms can be performed either counter-clockwise (D) or clockwise (L). Only the *chiro* form has specific D and L conformations.
Source: Bohn *et al.* 2008.

Myo-inositol (1,2,3,4,5,6) hexakisphosphate has six phosphate groups attached to the inositol ring. The prefix "hexakis" (instead of "hexa") is indicative of the fact that the phosphates are not internally connected (Johnson and Tate 1969). Consequently, the compound is a polydentate ligand and a chelator, which can bind to more than one coordination site of the metal atoms. Each of the phosphate groups is esterified to the inositol ring and together they can bind up to 12 protons in total (Torres *et al.* 2005). Trivial names for D-myo-inositol (1,2,3,4,5,6) hexakisphosphate are IP6, InsP6 or PA (PA). In the pH range of 0.5~10.5, PA maintains the sterically stable conformation with one axial and five equatorial groups (Fig. 1.2) (Bohn *et al.* 2008).

The chelating effect of the phosphate groups causes PA to bind readily to mineral cations, especially to Ca^{2+}, Mg^{2+}, K^{+}, Cu^{2+} and Zn^{2+}, which have a high affinity for inositol phosphates (Bohn *et al.* 2008). In presence of PA and excess metal ions, the phytate salts are formed (Torres *et al.* 2005). Commonly the alkali metal (Na, K) salts of PA are soluble but those formed with alkaline earth and transition metals are insoluble; in some cases it is bound to proteins and starches (Fig. 1.3) (Coulibaly *et al.* 2011). The solubility of phytate is also influenced by pH (Bohn *et al.* 2008).

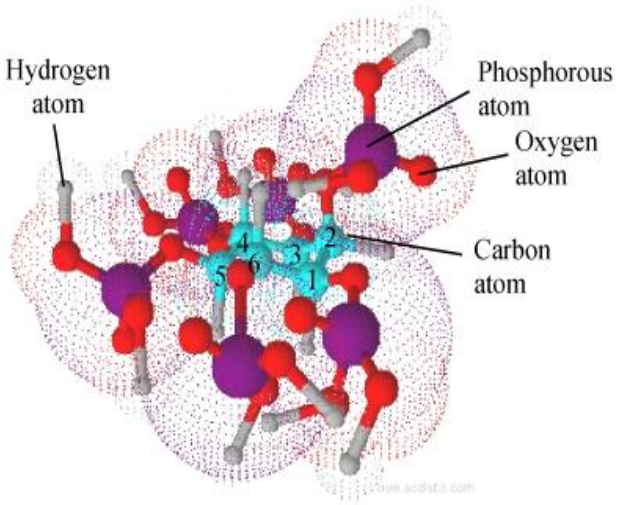

Fig. 1.2: *Myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate in boat formation showing the 5 equatorial and the 1 axial group. Red oxygen, purple phosphorous and grey hydrogen atoms form phosphate groups on each of the blue carbon atoms that have been numbered according to the IUPAC-IUB recommendation. The special relationships between the atoms have been indicated with dots. Source: Bohn *et al.* 2008

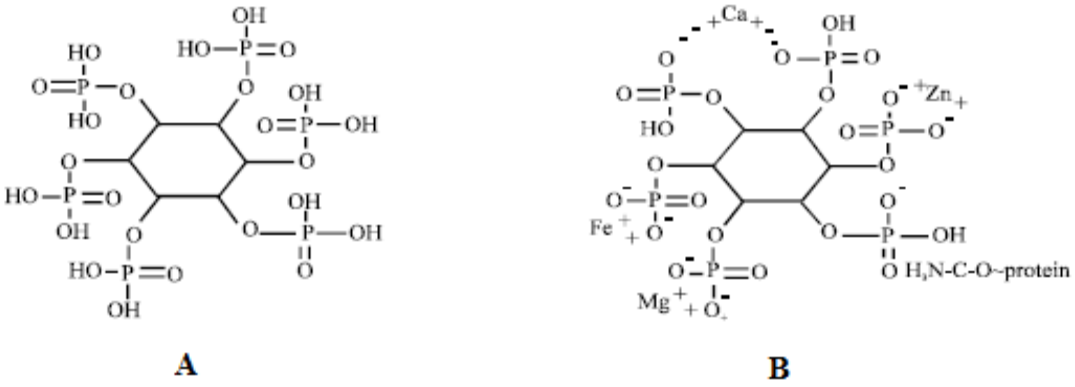

Fig. 1.3: A. Structure of PA. B. Structure of PA with the different possibilities to interact with both metal cations as with protein residues. Source: Coulibaly *et al.* 2011

1.3.1.2. Storage and functions of phytic acid

The amount of phytates in grains, nuts, legumes and seeds is highly variable; their levels in different foods depend on several factors like growth conditions, harvesting techniques, processing methods, testing methods and even the age of the food being tested. For example, it was observed that PA is much higher in foods grown using modern high-phosphate fertilizers than those grown in natural compost (Burgess and Gao 2002, Afinah *et al.* 2010). It accumulates in seeds during maturation and represents up to 60-90% of total phosphate in dormant seeds (Coulibaly *et al.* 2011).

Though phytate is found primarily in seeds (Ravindran 1995), its location within the seed differs among plants. The accumulation site of PA in monocotyledonous seeds (wheat, millet, barley, rice, etc.) is the aleurone layer, except corn as 90 % of the phytate in corn is found in the germ portion of the kernel (O'Dell 1976). In most oilseeds and grain legumes, phytate is concentrated within subcellular inclusions called globoids found throughout the kernel; but in soybean seeds, no specific location for phytate has been found (Ravindran 1995). Location of phytate and its chemical associations with other nutrients influence its availability.

In seeds, phytate serves many purposes, such as, phosphate reserve; an energy store for the germinating seed; a competitor for ATP during the rapid biosynthesis of phytate near seed maturity when seed metabolism is inhibited and dormancy is induced; an immobilizer of divalent cations needed for the control of cellular processes and that are released during germination upon the action of intrinsic plant phytases; and a regulator of readily available seed inorganic phosphate (Pi) level (Cosgrove 1980). It has also been postulated that the phytates protects the seeds against the destructive effects of oxygen and iron and helps to regulate calcium and potassium flows (Veiga *et al.* 2006). In higher animals lower inositol phosphates are involved in stress responses, membrane biogenesis, intracellular signaling and other metabolic processes mainly acting as a precursor compound as well as a chelator and P donor/acceptor (Storcksdieck *et al.* 2007).

On the other hand, PA is considered as an anti-nutritient, because of its ability to complex or bind with mineral cations in seed or diet, rendering these bound cations partially or completely unavailable to the animals. Each PA molecule has 12 replaceable protons or reactive sites; six of which are strongly acidic (pK of 1.5 to 2.0), two are weakly acidic (pK of ~ 6.0) and four are very weakly acidic (pK of 9.0 to 11.0) (Angel *et al.* 2002). This means that at pH normally encountered in feeds and in the digestive tract, phytate will carry a strong negative charge and is capable of binding divalent cations (e.g. Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+} etc.) in very stable complexes, thereby reducing the availability of these complexed minerals as well as that of phosphate to the animal. Such ability of the metal ions to inhibit hydrolysis of phytate depends on the stability of the complex, the pH of the solution, and the phytate to mineral molar ratio (Angel *et al.* 2002). PA may also form complexes with proteins and starches which may again lead to reduced availability of these nutrients from the diet. Proteins are able to bind directly with PA through electrostatic charges at low pH or through salt bridges formed at high pH (Coulibaly *et al.* 2011) while starch binding occurs as a result of hydrogen bond formation (Angel *et al.* 2002). Tompson (1986) showed that ternary complexes of protein, PA and carbohydrate might also form. PA can also inhibit some

digestive enzymes, such as pepsin, amylase and trypsin which are necessary for protein degradation in stomach, starch into sugars and protein degradation in small intestine, respectively (Dvorakova 1998).

Besides that, phytate is relatively unavailable to humans and other monogastric animals that largely lack the enzyme phytase (Lei *et al.* 2007). It is also an environmental pollutant in poultry and swine populations, where the unutilized phytate P from plant feeds is excreted as a pollutant in areas of intensive live-stock production (Hosseinkhani *et al.* 2009). Excessive P in soil runs off to lakes and the sea, causing eutrophication i.e. stimulating growth of planktonic vegetation such as algal blooms and cyanobacterial blooms. These may lead to hypoxia and death of aquatic animals and production of nitrous oxide which is a potential greenhouse gas (Vats *et al.* 2005). In the laboratory, it has been observed that PA in faeces inhibits polymerase chain reactions (PCR), as a result preventing PCR-based diagnostic tests for detecting microorganisms in livestock (Thornton and Passen, 2004).

Although there are many adverse effects of high intake of phytate in humans, many beneficial effects have also been found. Phytate exerts beneficial effects in the gastrointestinal tract and other target tissues through its chelating ability (Fig. 1.4) (Jenab and Thompson, 2002).

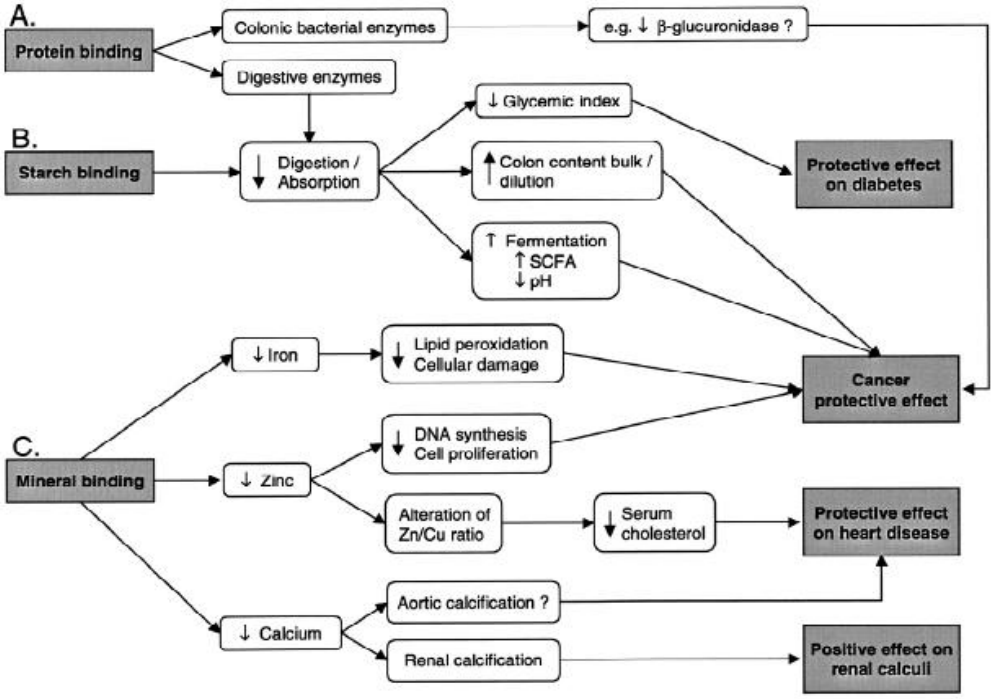

Fig. 1.4: Possible mechanisms of action of PA. Adapted from Jenab and Thompson 2002.

1.3.2. PHYTASE

The generic term phytase [*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate phosphohydrolase] indicates a class of phosphatases with the *in vitro* capability to release at least one phosphate from PA or phytate. Phytases catalyze the hydrolysis of phosphate ester bonds of the PA molecule yielding Pi and a series of lower phosphoric esters (Lei *et al.* 2007, Rao *et al.* 2009, Mullaney *et al.* 2011). Phytase activity leads to releasing phosphates, lower inositol phosphates and also potentially chelated minerals. Hydrolysis of PA is an important reaction for energy metabolism, metabolic regulation and signal transduction pathways in cellular systems (Vats and Banerjee 2004).

The importance of phytase was established with the fact that inspite of the abundance and divergence of phosphatases, these enzymes are virtually unable to hydrolyse phosphomonoesters in PA and phytases are solely responsible for the dephosphorylation of antinutritional and indigestible phytate to digestible P (Fu *et al.* 2008).

1.3.2.1. Phytase classification

The detailed characterization of several phytase enzymes has revealed that nature did not develop a single catalytic mechanism to dephosphorylate phytate.

- Based on the catalytic mechanism, four distinctly different classes of phytases are recognized: histidine acid phosphatase, β-propeller phytase, cysteine phosphatase, and purple acid phosphatase (Fig. 1.5) (Mullaney *et al.* 2007, Lei *et al.* 2013).

Histidine acid phosphatase (HAP): Enzymes from this class are the most abundant in nature and also widely studied and utilized to date. Phytases belonging to this class of enzymes are known to occur in animals, plants and microorganisms (Oh *et al.* 2004, Mullaney *et al.* 2007). It is observed that all HAPs share two common active site motifs, RHGXRXP and HD. The catalytic mechanism of hydrolysis by HAPs is proposed to be as follows: the histidine residue in the conserved motif makes a nucleophilic attack on the carbon of interest and an aspartic acid in the C-terminal of the enzyme stabilizes the leaving group by acting as a proton donor (Ostanin and van Etten 1993). A water molecule is consumed in the hydrolysis of the intermediate, but the phospho-histidine interaction is stable enough for crystallization, as it has been done e.g., with a heat stable HAP phytase from *Aspergillus fumigatus* (Xiang *et al.* 2004). Another term Histidine Acid Phytase (HAPhy) has been coined to designate those HAPs that can specifically accommodate phytate as their substrate, usually causing hydrolysis

of metal-free phytates in acidic pH-range (Oh *et al.* 2004). Studies revealed that there are two classes of HAPhys: one with broad substrate specificity but a lower specific activity for PA and the other having narrow substrate specificity but a high specific activity for phytate (Wyss *et al.* 1999). Evidence from site-directed mutagenesis studies established that certain amino acid residues are important for the substrate specificity and also the pH profile in fungal HAPhys (Mullaney *et al.* 2002). Though not directly involved in the catalytic mechanism of HAPhys, a conserved eight-cysteine motif appears to be essential for maintenance of the proper molecular structure necessary for the enzyme activity in fungal phytases (Mullaney and Ullah 2005). This HAP class is again subdivided into 3 different subclasses (PhyA–PhyC), based on amino acid sequence homology and biochemical properties (Oh *et al.* 2004). HAPhys are known in both prokaryotes and eukaryotes, but phytase from both sources show little sequence homology other than the conserved active sites. Among prokaryotic HAPhys, the best characterized one is the *Escherichia coli* phytase (Greiner *et al.* 1993) and in case of eukaryotes HAPhys have been found in maize and in a number of fungal isolates among which most widely studied are from *A. niger* and *A. fumigatus* (Oh *et al.* 2004). Today, the major application for HAPhys is in the hydrolysis of phytate in cereal and grains in animal feed. The enzyme from *Aspergillus niger* has been advanced for use as an animal feed additive. Future applications extend from the development of plant cultivars that require less P fertilizer to modification for use as a peroxidase.

β−Propeller Phytase (BPP): The 3D structure of a BPP molecule resembles a propeller with six blades and hence the name (Ha *et al.* 2000). A wide range of catalytic functions has been ascribed to proteins possessing the β-propeller molecular architecture (Pons *et al.* 2003). A novel calcium dependent phytase from *Bacillus* sp. showing this configuration has been cloned and characterized, which requires Ca^{2+} for both activity and thermostability (Kim *et al.* 1998). There are two phosphate-binding sites in this type of phytases, a cleavage site for substrate hydrolysis and an affinity site to bind the substrate (Shin *et al.* 2001). β-propeller phytases show an optimum pH in alkaline range, have narrow substrate range, require calcium for activity and only remove three phosphates from PA to yield inositol trisphospahte as a final product. No commercial applications are available so far for BPP; but it has been advanced as an animal feed additive and as a means to promote plant growth under phosphate limiting conditions.

Purple acid phosphatase (PAP): PAPs have representatives in various organisms and contain binuclear Fe(III)-Me(II) centres, where Me is Fe, Mn or Zn (Schenk *et al.* 2000). PAPs are widespread in mammals, fungi, bacteria, and plants (Kuang et al. 2009).

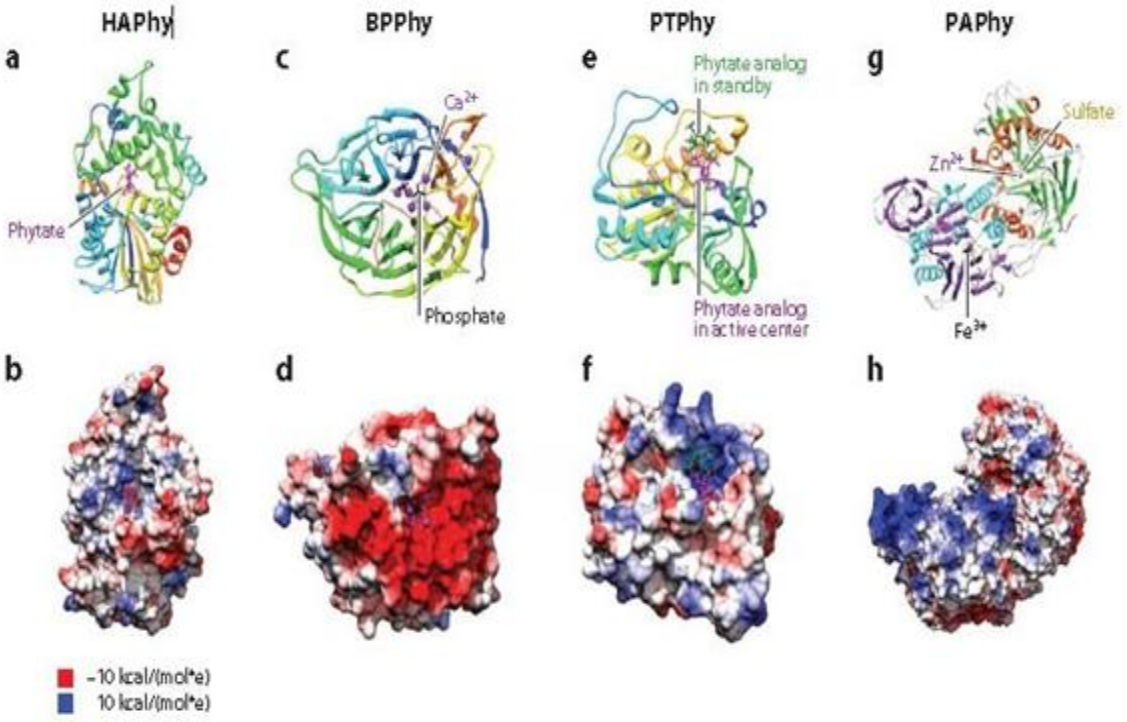

Fig. 1.5: Crystal structures of representatives of each of the four structural classes of phytases. Secondary structure (first row) and surface colored by electrostatic potential (second row) are displayed. Coulombic surface coloring (Chimera) was used to calculate electrostatic potentials with charges of -10kcal/(mol^{4}e) (red) and 10kcal/(mol^{4}e) (blue) using a dielectric constant of 4.0. Images are not shown to scale. (a, b: 1DKQ) Histidine acid phytase (HAPhy), *Escherichia coli* AppA in complex with phytate (magenta) in the active center. (c,d: 1H6L) β-propeller phytase (BPPhy), *Bacillus amyloliquefaciens* phytase in complex with Ca^{2+} (magenta) and phosphate (black). Four Ca^{2+} ions are involved in catalysis and creation of a favorable electrostatic potential; three stabilize the enzyme. (e,f: 1U25,1U26) Protein tyrosine phytase (PTPhy), *Selenomonas ruminantium* phytase in complex with *myo*-inositol hexasulfate (phytate analog) in the standby position (green) and in the active center (magenta). The inositol hexasulfate has five axial and one equatorial sulfates, in contrast to the more general five equatorial and one axial phosphates of phytate seen in crystal structure of HAPhy. (g,h: 2QFR) Purple acid phytase (PAPhy), *Phaseolus vulgaris* (kidney bean) PAP (no PAPhy structure is available) in complex with sulfate (yellow). PAPhy are generally homodimeric and contain two metal ions involved in catalysis and creation of a favorable electrostatic potential. Shown here are Fe^{3+} (black) and Zn^{2+} (magenta). One chain is colored cyan and purple (active site on reverse of the molecule), the other orange and green. Images created with Chimera using Protein Data Bank accessions as indicated. Source: Lei *et al.* 2013

Interestingly, while only a few copies of PAP-like genes are present in mammalian and fungal genomes (Mullaney and Ullah 2003, Flanagan *et al.* 2006), multiple copies are present in plant genomes. In plants, PAPs with phytase activity may play a role in seed and pollen germination (Kuang *et al.* 2009). As compared to fungal phytases, seed phytase has a relatively low specific activity for PA and this property may be advantageous in plant seed where a slow and balanced breakdown of phytate during germination could be efficacious. The first plant phytase PAP, GmPHY, was isolated from the cotyledon of germinating soyabean seedlings, having a relatively low specific activity for PA (Hegeman and Grabau

2001). A tobacco (*Nicotiana tabacum*) root PAP phytase was identified more recently (Kuang *et al.* 2009). PAP is characterized by seven conserved residues in the five conserved motifs – **D**XG, G**D**XX**Y**, G**N**H(D/E), VXX**H** and G**H**X**H** – involved in the coordination of the dimetal nuclear centre (Li *et al.* 2002).

Cysteine Phosphatase (CP): This class of phytase has been reported from an anaerobic ruminal bacterium, *Selenomonas ruminantium* (Chu *et al.* 2004). It showed similarities with the catalytic domain found in the cysteine phosphatase superfamily which is supported by sequence homology studies. The enzyme is found to catalyze dephosphorylation of PA to myo-inositol monophosphate. Its optimum temperature ranged between 50-55°C with optimal pH range of 4.0 - 5.0. Several lead cations enhance activity, while Fe^{2+}, Fe^{3+}, Hg^{2+}, and Zn^{2+} ions strongly inhibited the enzyme. The 3D structure showed that it consists of one large and one small domain. Near the C-terminal edge of the large domain is a shallow pocket containing a two loop structure similar to the active site found in protein tyrosine phosphatase with the catalytically important HCXXGXXR(T/S) (Huang *et al.* 2011, Lei *et al.* 2013).

- According to International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology (IUPAC-IUBMB), phytases are grouped into three classes (3-phytases, 5-phytases and 4/6-phytases), which initiate the dephosphorylation of PA at different positions on the inositol ring and produce different isomers of the lower inositol phosphates (Fig. 1.6).

The 3-phytases (EC 3.1.3.8): The 3-phytases are named so as they initiate the dephosphorylation of PA at the 3 position, yielding 1,2,4,5,6-pentakisphosphate and Pi; subsequent ester bonds in the substrate are hydrolysed at different rates though they do not always completely dephosphorylate PA (Wodzinski and Ullah 1996). It is the largest group of phytases to date. Structurally, most of the 3-phytases show homology to histidine acid phosphatases (HAP) or β-propeller phosphatases (BPP) which in general are found in fungi and bacteria. A well studied phytase from this class is isolated from baker's yeast, *Saccharomyces cerevisiae*, which is extracellular and its expression can be induced when grown in a medium containing PA as the sole phosphorous source (Andlid *et al*. 2004).

The 5-phytase (EC 3.1.3.72): The alkaline phytase from lily pollen is the only 5-phytase (EC 3.1.3.72) detected so far. This enzyme is unique because of another reason, that is, it is the only one in the family of phytases that initiates hydrolysis with an attack of a phosphate group

in the plane of symmetry, thereby creating yet another symmetrical compound (Barrientos *et al.* 1994). The pollen phytase shows optimum activity at pH 8.0 and temperature 55°C (Jog *et al.* 2005). Though its conformation resembles that of an HAP phytase, with the exception of the active site, it shows higher amino acid sequence homology towards multiple inositol polyphosphate phosphatase (MINPP) from humans or rats (Mehta *et al.* 2006).

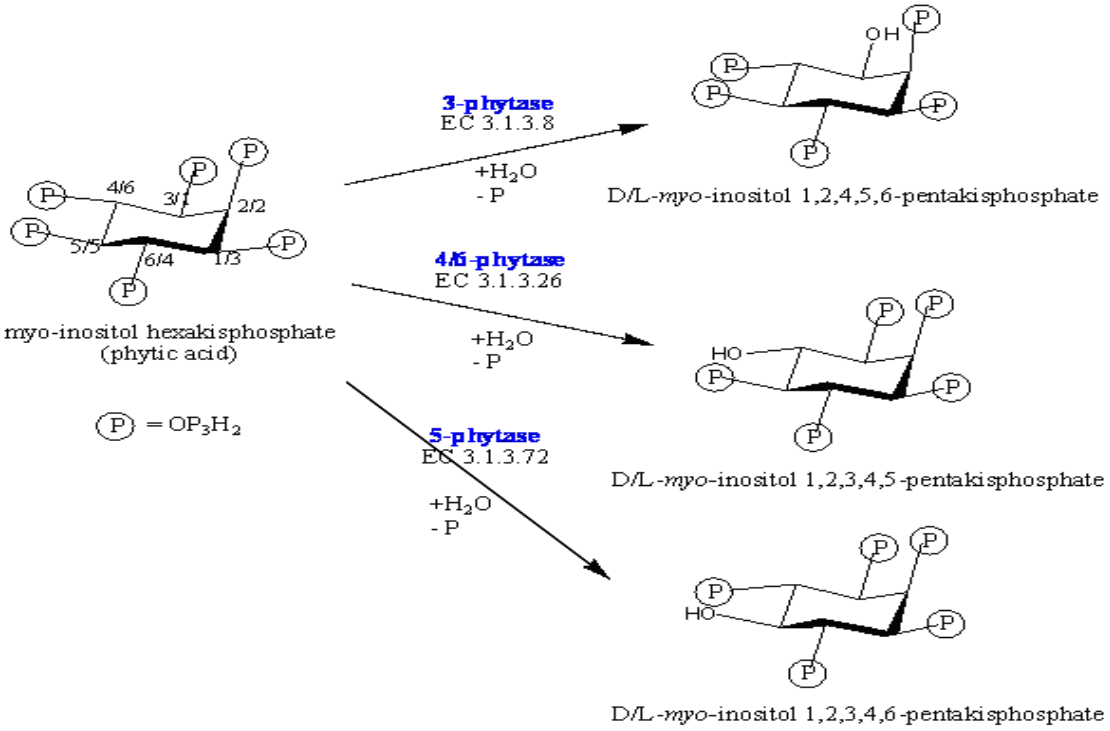

Fig.1.6: The general enzymatic reaction of 3-, 4/6-, and 5-phytases modified from Kerovuo *et al.* (2000). Carbon atoms in the inositol ring are numbered for the D/L –configuration, respectively.

The 4/6-phytases (EC 3.1.3.26): The 4/6-phytases (EC 3.1.3.26) act on the carbon atom next to C5 of the inositol ring; so the official name should be 4-phytase, but traditionally it has been called a 6-phytase. Usually, these phytases always completely dephosphorylate PA (Wodzinski and Ullah 1996). Several structurally different phytases are found in this group such as the purple acid phosphatase (PAP), the ADP phosphoglycerate phosphatase (related to EC 3.1.3.28), a HAP and the acid phosphatase (related to EC 3.1.3.2). Plant phytases are mostly 6-phytases. In general, the 4/6-phytases are the most active in weak acidic environments (pH 4~6) with a temperature optimum in the range 40~60°C. They are usually 50~70 kD and have K_m in the range of 10^{-5}~10^{-4} mol l^{-1} PA (Greiner *et al.* 2001). There are

exceptions, such as a larger phytase (164 kD) from tomato roots (Li *et al.* 2007) and a highly active phytase (K_m 0.5 μmol l^{-1} PA) from wheat (Nakano *et al.* 1999).

➢ On the basis of their pH optima, phytases are broadly divided into two major classes: acid and alkaline phytases (Fig. 1.7).

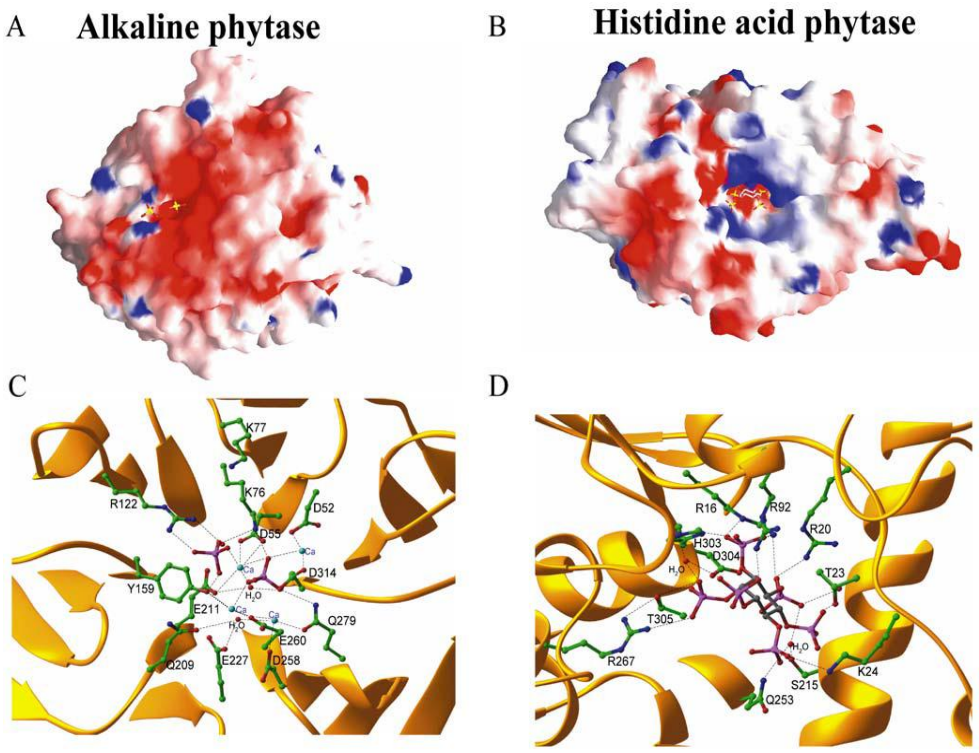

Fig. 1.7: 3-D Electrostatic surface potential of alkaline phytase and HAP in the active-site region. The surfaces of the substrate-binding sites of *Bacillus amyloliquefaciens* (A) and *Escherichia coli* phytase (B) are colored according to their local electrostatic potentials, ranging from −7 kt/e in red to +7kt/e in blue, using GRASPP(Honig and Nicholls 1995). Stick models of two phosphates (A, C) and phytate (B, D) are shown in the substrate-binding site. The positively charged active site of the phytase from *E. coli* prefers metal-free phytate. In contrast, a negatively charged active site of the phytase from *B. amyloliquefaciens* provides a favorable electrostatic environment for the positively charged calcium–phytate complex.

Source: Oh *et al.* 2004

Acid phytases include those enzymes belonging to the histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs) and PTP-like class of phosphatases [the latter of which was identified more recently (Nakashima *et al.* 2007)]. To date, the beta-propeller phytases (BPPs) from *Bacillus* are the only extensively characterized class of alkaline phytase (Lim *et al.* 2007, Viader-Salvado *et al.* 2010, Kumar *et al.* 2014); but there are some known alkaline plant phytases.

1.3.3. PHYTASE PRODUCING ORGANISMS

Phytases have a wide distribution in microorganisms, plants, and some animal tissues (Dvorakova 1998, Yao *et al.* 2011). The role of phytase in each of these organisms is different.

1.3.3.1. Phytase in plants and animals

In plants, phytase is induced during germination to release the stored phytates to provide the growing seedling with Pi and cations, such as K^+, Mg^{2+}, Zn^{2+} and Ca^{2+} and the plant with concomitant free myo-inositol, which is an important growth factor (Gibson *et al.* 2010). The myo-inositol phosphate intermediates are important as secondary messengers and in signal transduction in plants (Loewus and Murthy 2000).

Mostly, 6-Phytases are isolated from grains and oil seeds, but 5-phytases have also been found in beans, peas, alfalfa (Chu *et al.* 2004). Chemically, several plant phytases belong to the HAPs class of enzymes (Wyss *et al.* 1999); but a phytase isolated from soybean was found to be unrelated to previously characterized microbial or maize (*Zea mays*) phytases and was classified as a PAP (Hegeman and Grabau 2001, Li *et al.* 2002).

On the other hand, the role of phytases in animal cells is more obscure. However, a phytase-like enzyme, multiple inositol polyphosphate phosphatase (MIPP), has been found to regulate the cellular activities of PA and Ins $(1,3,4,5,6)P_5$ (Puhl *et al.* 2007). A mammalian phytase, which is a rat hepatic form of MIPP, has been cloned and characterized (Craxton *et al.* 1997). The predicted amino acid sequence of MIPP includes an 18 amino acid region that aligned with approximately 60% identity with the catalytic domain of a fungal inositol hexakisphosphate phosphatase (phytase A); the similarity encompassed conservation of the RHGXRXP signature of the histidine acid phosphatase family (Mehta *et al.* 2006).

1.3.3.2. Fungal phytases

Phytases are wide spreaded among different fungal species. Fungal phytases have been studied extensively as many research works have been performed on them (Table 1.1), particularly those from filamentous fungi such as *Aspergillus ficuum* (Gibson 1987, Shivanna *et al.* 2014), *Aspergillus fumigatus* (Pasamontes *et al.* 1997, Xiang *et al.* 2004), *Rhizopus oligosporus* (Casey and Walsh 2004), *Cladosporium* species (Quan *et al.* 2004), *Mucor racemosus* (Roopesh *et al.* 2006) etc.

Table 1.1: Some of the reported phytase-producing organisms

Producing organisms	Localisation	Reference
Plants and animals		
Germinated Maize	Intracellular	Oh *et al.* 2004
Soybean seeds	Intracellular	Oh *et al.* 2004
Legume seeds	Intracellular	Oh *et al.* 2004
Lilly pollen	Intracellular	Mehta *et al.* 2006
Rat, intestinal mucosa	Intracellular	Yang *et al.* 1991
Rat, liver	Intracellular	Craxton *et al.* 1997
Arabidopsis thaliana	Intracellular	Zhang *et al.* 2008, Kuang *et al.* 2009
Yeasts		
Arxula adininivorans	Extracellular	Olstorpe *et al.* 2009
Fellomyces fuzhouensis		Sano *et al.* 1999
Pichia anomala	Extracellular, Intracellular	Haefner *et al.* 2005, Kaur *et al.* 2010
Pichia farinose		Zhu *et al.* 2012
Candida bombi	Extra- & Intracellular	Olstorpe *et al.* 2009
Candida laurentii	Intracellular	Olstorpe *et al.* 2009
Sporobolimyces sp.	Extracellular	Sano *et al.* 1999
Sterigmatosporus polymorphum	Extracellular	Sano *et al.* 1999
Saccharomyces cerevisiae		Nayini 1984, Olstorpe *et al.* 2009
Filamentous fungi		
Aspergillus niger	Extracellular	Xiong *et al.* 2004
A. flavus	Extracellular	Oh *et al.* 2004
A. terrus	Extracellular	Oh *et al.* 2004
A. ficuum	Extracellular	Shivanna *et al.* 2014
A. oryzae	Extracellular	Yao *et al.* 2011
A. fumigatus	Extracellular	Xiang *et al.* 2004
Cladosporium sp. FP-1	Extracellular	Quan *et al.* 2004
Rhizopus oligosporus	Extra- & Intracellular	Casey *et al.* 2004
Rhizomucor pusillus		Chadha *et al.* 2004
Peniophora lycii		Lassen *et al.* 2001

Some yeast, such as *Saccharomyces cerevisiae*, *Candida tropicalis*, *Torulopsis candida*, *Debaryomyces castelii*, *Debaryomyces occidentalis*, *Kluyveromyces fragilis* and *Schwanniomyces castelii*, have also been shown to produce phytase (Nayini and Markakis 1984, Gontia-Mishra and Tiwari 2013). Despite the high degree of amino acid sequence homology among many fungal phytases, these enzymes often had substantially different catalytic properties. To withstand the high temperature fluctuations during the feed

processings, especially pelleting, there is a constant search for phytases with higher thermostability. Fungal phytases have been proved to be a good candidate in this regard and hence there is a considerable economic interest about these phytases. For example, a phytase from *A. fumigatus* (Pasamontes *et al.* 1997) retains 90% of its initial activity after being maintained at 100ºC for 20 min. By contrast, the phytase from *A. niger* remains only 30% active after being heated to 70°C for 20 min (Pasamontes *et al.* 1997). The thermal stability of fungal phytases is often attributed to highly reversible thermal unfolding, rather than an intrinsic thermostability (Wyss *et al.* 1998). When assessed by protein unfolding and refolding experiments using circular dichroism spectroscopy and protein fluorescence, only *A. fumigatus* phytase was found to refold into a fully active, native conformation even after heat denaturation at 90°C (Wyss *et al.* 1998). Fungal phytases from *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia* sp., and *Trametes pubescens* also showed a high degree of refolding after thermal unfolding, as evidenced by differential scanning calorimetric studies (Lassen *et al.* 2001).

Fungal phytase have also been isolated from thermophilic *Rhiomucor pusillus* and *Thermomyces lanuginosus*. The former has a temperature optimum of 70°C, while the latter retains its activity up to 75°C, has a higher catalytic efficiency at 65°C than other fungal phytases and has a comparably broad pH optimum as *A. fumigatus* phytase (Berka *et al.* 1998, Chadha *et al.* 2004). A novel phytase gene from *A. niger* N-3 was cloned and expressed in *Pichia pastoris*. The purified enzyme from it retained 45% of its initial activity after being maintained at 90°C for 5 min. 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 stomach of monogastric animals (Shi *et al.* 2009). Two novel thermostable genes were identified in *Aspergillus japonicus* BCC18313(TR86) and BCC18081(TR170). The thermostable nature of these phytases has given them valuable potential for applications (Promdonkoy *et al.* 2009). In general, the specific activities of fungal phytases with PA as substrate ranged from 23 to 196 U mg^{-1}; the pH optima varied between 2.5 and 7.0 and were either broad (e.g., pH 4.0 to 7.3 for *A. fumigatus* phytase) or narrow (e.g., pH 6.5 for *E. nidulans* phytase). In contrast to most other fungal phytase, the enzyme from *A. fumigatus* has a broad pH optimum; at least 80 % of the maximal activity was observed at pH values between 4.0 and 7.3 (Wyss *et al.*1999). On the basis of substrate specificity, two classes of fungal phytases could be discriminated, phytases with broad substrate specificity (*A. fumigatus*, *E. nidulans*, and *M. thermophila* phytases) and phytases which are rather specific for PA (*A. niger*, *A. terreus* 9A1, *A. terreus* CBS phytases) (Wyss *et al.* 1999).

1.3.3.3. Bacterial phytases

A great variety of bacteria have been reported to produce phytases or phytase-like proteins and most of them belong to the γ- proteobacteria group. Phytase has been detected in various bacteria (Table 1.2), e.g. *Yersinia intermedia* (Huang *et al.* 2006), *Pseudomonas* sp.

Table 1.2: Some of the reported phytase-producing bacteria

Species	Localization	Class of phytase	Reference
Bacillus subtilis	Extracellular	BPP	Powar *et al.* 1982
Bacillus subtilis (natto)	Extracellular		Shimizu 1992
B. amyloliquefaciens	Extracellular	BPP	Idriss *et al.* 2002
B. licheniformis			Tye *et al.* 2002
Bacillus sp.			Kim *et al.* 1998
Citrobacter braakii	Intracellular		Kim *et al.* 2006
Dickeya paradisiaca			Gu *et al.* 2009
Escherichia coli	Intracellular-Periplasmic	HAP	Greiner *et al.* 1993
Enterobacter sp.	Extracellular	HAP	Yoon *et al.* 1996
Erwinia carotovora	Extracellular	HAP	Huang *et al.* 2009
Klebsiella pneumonia	Intracellular	HAP	Wang *et al.* 2004
K. aerogenes	Intracellular		Tambe *et al.* 1994
K. terrigena	Intracellular	HAP	Jorquera *et al.* 2008
Lactobacillus pentosus	Extracellular	HAP	Palacios *et al.* 2005
Obesumbacterium proteus		HAP	Zinin *et al.* 2004
Psedomonas syringae		HAP	Cho *et al.* 2005
Psedomonas sp.	Extracellular	HAP,BPP, CP	Jorquera *et al.* 2008
Prevotella sp.			Jorquera *et al.* 2008
Pantoea agglomerans			Greiner *et al.* 2004
Selenomonas ruminantium			Chu *et al.* 2004
Shigella sp.	Intracellular	HAP	Pal Roy *et al.* 2012
Xanthomonas sp.	Intracellular-periplasmic	HAP,BPP,CP	Jorquera *et al.* 2008
Yersinia intermedia	Intracellular	HAP	Huang *et al.* 2006

(Hosseinkhani *et al.* 2009), *B. subtilis* (Powar and Jagannathan, 1982, Olazaran *et al.* 2010), *Klebsiella* sp. (Wang *et al.* 2004), *B. subtilis* (natto) (Shimizu 1992), *E. coli* (Greiner *et al.*

1993, Tai *et al.* 2013, Yao *et al.* 2013), *Enterobacter* sp. 4 (Yoon *et al.* 1996), *Bacillus* sp. DS 11 (later designated as *B. amyloliquefaciens*) (Kim *et al.* 1998) etc. Two bacterial genera, *Pseudomonas* and *Xanthomonas* have been shown to produce different types of phytases in the same species; for example, *X. campestri* and *P. syringae* produce the three types of phytases, that is, HAP, BPP and CP, whereas *X. axonopodis* and *X. oryzae* produce HAP and BPP (Jorquera *et al.* 2008). But no bacterial PAP has been reported so far, though in the whole genome sequences of few mycobacteria and cyanobacteria PAP-like sequences have been indicated (Schenk *et al.* 2000). The only bacteria producing extracellular phytase are those of the genera *Bacillus, Pseudomonas* and *Enterobacter*. *E. coli* phytase is a periplasmic enzyme. Bacterial phytases have pH optima between 4.0 and 7.5; but some bacterial phytases and especially those from *Bacillus* have pH optima at 6.5 - 8.5 (Lei *et al.* 2013). Optimal temperatures of most phytases vary from 37 to 77°C (Yao *et al.* 2011). Marked differences were also noted in substrate specificity, not only with different *myo*-inositol phosphates but also with classic acid phosphatase substrates [phenyl phosphate and *p*-nitrophenyl phosphate (pNPP)], sugar phosphates, and other phosphate compounds. Although, many of the characterized phytases [eg. *Klebsiella pneumoniae* (Wang *et al.* 2004)] exhibit a broad specificity for substrates with phosphate esters but relatively low specificity to phytate, some bacterial phytases showed a narrow substrate specificity, exhibiting high affinity to PA, as reported earlier for *E.coli* (Greiner 1993) and *Shigella* sp. CD2 (Pal Roy *et al.* 2012).

1.3.4. PHYLOGENY

Retrieving the amino acid sequences of phytases found in different fungi and yeasts from various databases like NCBI, DDBJ and EMBL, Gontia-Mishra and Tiwari (2013) analysed the phylogenetic relationships among fungal phytases. They inferred from the phylogenetic tree (Fig. 1.8) that phytases from Ascomycetes belong to 3-phytase class, whereas phytases from Basidiomycetes fall in a different cluster and belong to 6-phytase class. Despite the presence of conserved N-terminal motif (RHGXRXP) and C-terminal motif (HD) (which are present in Ascomycetes), a characteristic of HAPs, the phytase protein sequences of Basidiomycetes like *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia sp.* and *Trametes pubescens*, also exhibit three different conserved regions.

Based on the microbial and environmental sequence database available at the NCBI and CAMERA (Seshadri *et al.* 2007), Lim *et al.* (2007) performed a study on the distribution and diversity of phytase-producing bacteria in various habitats. Representative genes from CP, HAP and BPP were obtained from public databases. CP-like sequences were mainly found in plant pathogenic, enteric and free-living bacteria, whereas HAP-like sequences were found in

plant pathogenic and enteric bacteria. No CP and HAP-like sequences were found in aquatic bacteria. In contrast, BPP-like sequences were found in plants, soil and aquatic bacteria suggesting that BPP phytases can play a major role in the cycling of phytate in both soil and aquatic environments. In relation to PAPs, no bacterial enzyme has been purified, but a search of complete and partial genome sequences in the TIGR Microbial Database indicated that PAP-like sequences may be restricted to a small number of bacteria, such as mycobacteria and cyanobacteria (Schenk *et al.* 2000).

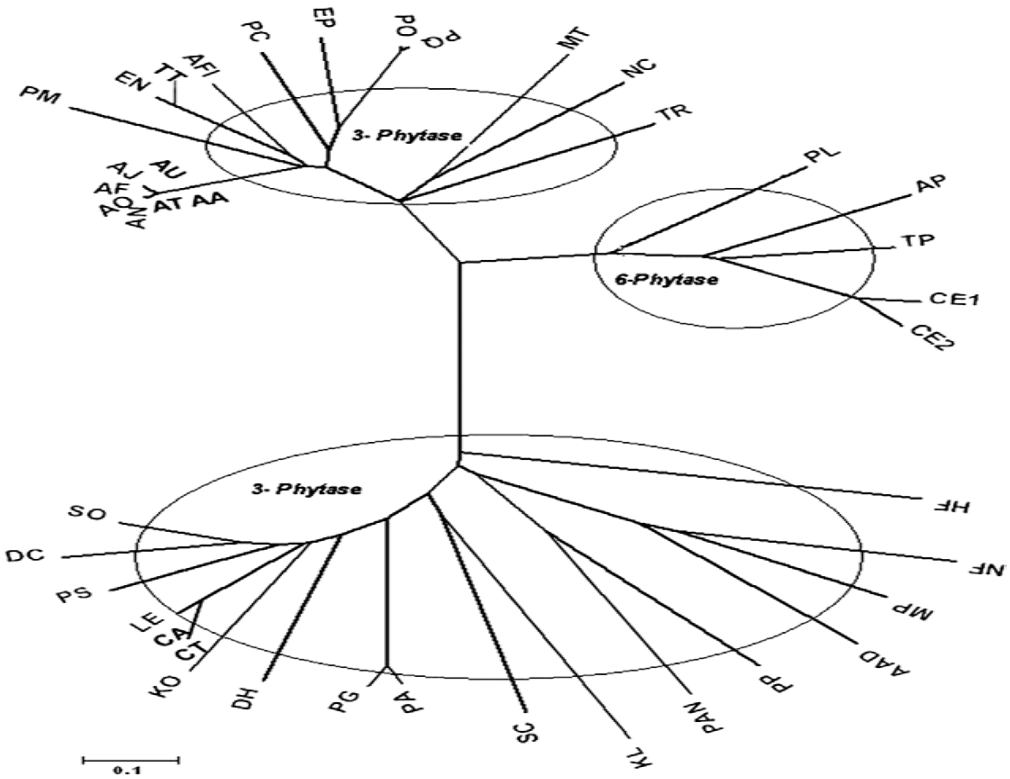

Fig.1.8: Phylogenetic tree of phytase protein found in fungi and yeasts. The following amino acid sequences were analyzed: AA=*Aspergillus awamori* ABA29207, AT=*A. terreus* AAB52507, AF=*A. ficuum* AAG40885, AN=*A. niger* BAA74433, AU=*A. usamii* ABA42097, AO=*A. oryzae* AAT12504, AJ=*A. japonicus* ACE79228, AFl=*A. flavus* XP002376973, PQ=*Penicillium* sp. Q7 ABM92788, PO=*P. oxalicum* AAL55406, PC=*P. chrysogenum* XP002561094, PM= *P. marneffei* AP00218821, *EP=*Eupenicillium parvum*, EN=*Emericella nidulans* AAB96871, TT=*Talaromyces thermophilus* AAB96873, MT=*Myceliophthora thermophila* AAB52508, NC=*Neurospora crassa* AAS94253, TR=*Trichoderma reesei* EGR47873, TP=*Trametes pubescens* CAC48234, AP=*Agrocybe pediades* CAC48160, PL=*Peniophora lycii* CAC48195, CE1=*Ceriporia* sp. CAC8163, CE2=*Ceriporia* sp. CAC8164, SO=*Schwanniomyces occidentalis* ABN04184, DC=*Debaryomyces castellii* ABN04184, DH=*D. hansenii* Q6BM75, PS=*Pichia stipitis* XP001385108, PG=*P. guilliermondii* CAL69849, PA=*P. anomala* FN641803, PAN=*P. angusta* O74677, PP=*P. pastoris* P52291, LE=*Lodderomyces elognisporus* XP001527604, CT=*Candida tropicalis* XP002546108, CA=*C. albicans* XP713452, KO=*Kodamaea ohmeri* ABU53001, SC=*Saccharomyces cerevisiae* EDN64708, KL=*Kluveromyces lactis* CAA83964, AAD=*Arxula adeninivorans* CAJ77470, HF=*Hansenula fabianii* BAH588739, NF=*Neosartorya fischeri* AICXF7, MP=*Monascus purpureus* Q8X1W7 Source: Gontia-Mishra and Tiwari 2013

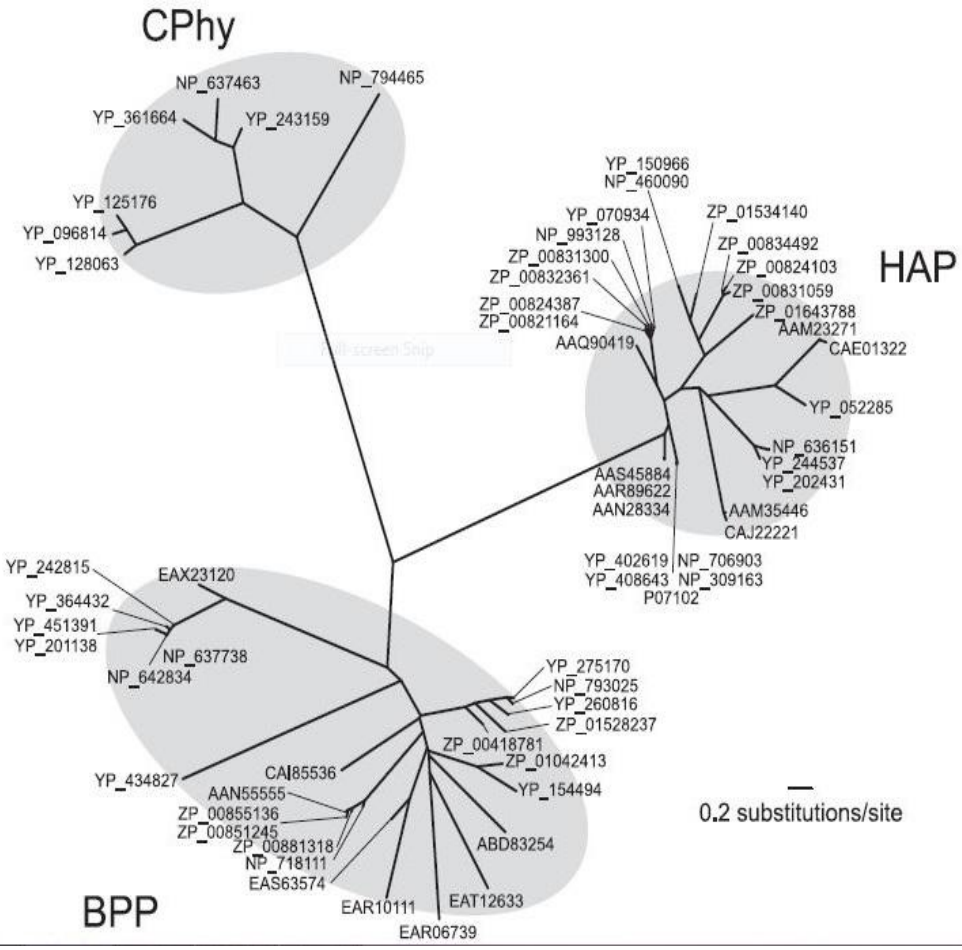

Fig. 1.9: Phylogenetic analysis of representative phytase-like proteins found in the γ-proteobacteria group. Amino acid sequences were aligned using the ClustalW program[169] and the neighbor-joining tree was constructed using Prodist and Drawtree in PHYLIP (*Phylogenetic Inference Package*, version 3.67, available from J. Felsenstein of the Department of Genome Sciences at the University of Washington, Seattle, WA, USA). The selected sequences analyzed were as follow: Cystein phytase-like sequences (**CPhy**): *Legionella pneumophila* str. Lens, YP 128063; *L. pneumophila* str. Philadephia, YP 096814; *L. pneumophila* str. Paris, YP 125176; *Xanthomonas campestris* str. 85-10, YP 361664; *X. campestris* ATCC 33913, NP 637463; *X. campestris* str. 8004, YP 243159; *Pseudomonas syringae* str. DC3000, NP 794465. Histidine acid phosphatase-like sequences (**HAP**): *Obesumbacterium proteus*, AAQ90419; *Yersinia bercovieri* ATCC 43970, ZP 00821164; *Y. mollaretii* ATCC 43969, ZP 00824387; *Y. intermedia* ATCC 29909, ZP 00832361; *Y. frederiksenii* ATCC 33641, ZP 00831300; *Y. pestis* str. 91001, NP 993128; *Y. pseudotuberculosis* IP 32953, YP 070934; *Salmonella enterica* ATCC9150, YP 150966; *Salmonella* Typhimurium LT2, NP 460090; *Serratia proteamaculans* 568, ZP 01534140; *Y. intermedia* ATCC 29909 (2), ZP 00834492; *Y. mollaretii* ATCC 43969 (2), ZP 00824103; *Y. frederiksenii* ATCC 33641 (2), ZP 00831059; *Stenotrophomonas maltophilia* R5513 ZP 01643788; *Klebsiella pneumoniae*, AAM23271; *K. terrigena*, CAE01322; *Erwinia carotovora* SCRI1043, YP 052285; *Xanthomonas campestris* ATCC 33913, NP 636151; *X. campestris* str. 8004, YP 244537; *X. oryzae* KACC10331, YP 202431; *X. axonopodis* str. 306, AAM35446; *X. campestris* str. 85 10, CAJ22221; *Shigella dysenteriae* Sd197, YP 402619; *S. boydii* Sb227, YP 408643; *S. flexneri* str. 301, NP 706903; *Escherichia coli* 0157:H7, NP 309163; *E. coli* K12 AppA, P07102; *E. coli* K12, AAN28334; *Citrobacter braakii*, AAS45884; *C. freundii*, AAR89622. β-propeller phytase-like sequences (**BBP**): *Pseudomonas syringae* 1448, YP 275170; *P. syringae* str. DC3000, NP 793025; *P. fluorescens* Pf-5, YP 260816; *P. mendocina* ymp, ZP 01528237; *Azotobacter vinelandii* AvOP, ZP 00418781; *Idiomarina baltica* OS145, ZP 01042413; *I. loihiensis* L2TR, YP 154494; *Saccharophagus degradans*, ABD83254; *Oceanobacter* sp. RED65, EAT12633; *Alteromonas macleodii* Deep ecotype, EAR06739; *Reinekea* sp. MED297, EAR10111; *Vibrio angustum* S14, EAS63574; *Shewanella oneidensis* MR-1, NP 718111; *Shewanella* sp. MR-4, ZP 00881318; *Shewanella* sp. ANA-3, ZP 00851245; *Shewanella* sp. MR-7, ZP 00855136; *S. oneidensis* PhyS, AAN55555; *Pseudoalteromonas haloplanktis* TAC125, CAI85536; *Hahella chejuensis* KCTC 2396, YP 434827; *Xanthomonas campestris* str. ATCC 33913, NP 637738; *X. axonopodis* str. 306, NP 642834; *X. oryzae* KACC10331, YP 201138; *X. oryzae* MAFF 311018, YP 451391; *X. campestris* str. 85-10, YP 364432; *X. campestris* str. 8004, YP 242815; *S. maltophilia* R551-3, EAX23120.

Source: Jorquera *et al.* 2008

However, the current database is too small to provide adequate coverage of phytases present in the bacterial world (Lim *et al.* 2007). Phytase-like proteins are mostly reported in γ-proteobacteria group (Fig. 1.9), mainly because this group has the most number of whole genome sequences than that for any other cellular organisms (Brown *et al.* 2004). Besides, the γ-proteobacteria are commonly chosen in phylogenetic studies because this group represents a model of bacterial diversification (Lerat *et al.* 2003) and are also sufficiently closely related to reduce the problem of lack of phylogenetic signals and to identify a large set of unambiguous orthologs (Jorquera *et al.* 2008).

1.3.5. CLONING AND EXPRESSION OF PHYTASES

Increasing public concern regarding the environmental impact of high P levels in animal excreta has driven the biotechnological development of phytase and its application in animal nutrition and other beneficial purposes. Although acceptance of any new phytase by mainly the animal industry depends on many factors, such as, effectivity in releasing phytate P in the digestive tract, stability to resist inactivation by heat especially from feed processing and storage and cheap production strategy. The ability of any given phytase to hydrolyze phytate in the digestive tract is determined by its enzymatic properties such as catalytic efficiency, substrate specificity, temperature and pH optima and resistance to proteolysis. For example, as the stomach is the main functional site of these supplemental phytases, a phytase with a low or acidic pH optimum and high resistance to pepsin is certainly desirable. Because dietary ingredients for swine and poultry are often undergo pelleting at 65-80°C with steam, an ideal phytase is desired to be able to withstand the high temperature and steam. Similarly an enzyme that can tolerate long term storage or transport at ambient temperatures is also attractive industrially. Finally, a phytase will not be considered competitive if it cannot be produced in high yield and purity by a relatively inexpensive system. However it is important to realize that any single phytase may never be ideal for all species or in all cases of its application. Thereby an array of phytases is needed for different target applications. Now, two basic approaches have been taken to develop effective phytases, i.e., identifying new native phytase proteins from microorganisms or plants and genetically modifying these cloned phytases.

Many phytases have so far been cloned and expressed in different expression systems to meet some or all of the features mentioned above. Some of the examples are listed in the Table 1.3. Different organisms, both prokaryotic and eukaryotic, have been used for cloning and expression studies of phytase, of which most notable are *E.coli*, *Bacillus subtilis*, *Pichia*

pastoris, Saccharomyces cerevisiae etc. Now, the methylotrophic yeast *Pichia pastoris* has evolved into a highly successful system for the production of phytases. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; the capability of performing many eukaryotic posttranslational modifications, such as glycosylation, disulfide bond formation and proteolytic processing; and the availability of the expression system as a commercially available kit (Cereghino and Cregg 2000).

Until now, expression of *Escherichia coli* phytase was studied in detail in different expression hosts by many researchers. Chen and coworkers (2004) used *P. pastoris* for the heterologous overexpression of the *E. coli* phytase gene *app*A, which was cloned under the control of the *AOX1* promotor and was highly expressed in presence of methanol as the only carbon source. Extracellular phytase activities of almost 5,000 U ml^{-1} were achieved by applying high cell-density cultivation, replacing culture medium prior to methanol induction and using a modified medium composition. Stahl *et al.* (2003) compared the extracellular *Escherichia coli* AppA phytases expressed in *Streptomyces lividans* and *Pichia pastoris*, where both the expressed proteins shared same temperature optimum of 65°C and similar pH optima (3.5-4.0) with higher yield in case of *P. pastoris*. On the other hand, Lee *et al.* (2005) expressed *Escherichia coli* AppA2 phytase in four yeast systems (three inducible yeast systems: *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *P. pastoris*, and one constitutive system: *P. pastoris*). They found that all four recombinant AppA2 phytases had pH optimum at 3.5 and temperature optimum at 55°C and similar efficacy in hydrolyzing phytate–phosphate from soybean meal. An efficient expression system was developed in *B. subtilis* for the large-scale expression of phytase (Kim *et al.* 1999). The phytase gene with a native promoter derived from *B. amyloliquefaciens* was cloned in the *Bacillus* expression vector pJH27 under a strong BJ27 promoter and its expression was optimized. Kim *et al.* (1998) cloned the gene encoding the thermostable phytase from *Bacillus* sp. DS11 in *E. coli* and sequenced. Phytase was produced to 20% content of total soluble proteins in *E. coli* and has similar characteristics as that of native one in thermostability. This was claimed as the first nucleic acid sequence report on phytase from a bacterial strain. Besides, phytase genes from *Obesumbacterium proteus* (Zinin *et al.* 2004), *Dickeya paradisiaca* (Gu *et al.* 2009), *Erwinia carotovora* (Huang *et al.* 2009), *Klebsiella pneumoniae* (Wang *et al.* 2004) were cloned and expressed in *E.coli* which yielded active phytases. *E. coli* is one of the most

widely used expression hosts as the expression of proteins in it is the easiest, quickest and cheapest method. There are many commercial and non-commercial expression vectors available with different N- and C-terminal tags and many different strains which are optimized for special applications. Kim *et al.* (2006) reported expression of the phytase gene from *Citrobacter braakii* in both *E. coli* and *Saccharomyces cerevisiae*. The yeast *Saccharomyces cerevisiae* has been widely used as a host organism to produce heterologous proteins, because its use gives high cell density and easy detection and purification of the recombinant protein. Han and Lei (1999) studied the expression of a phytase gene from *A. niger* in *P. pastoris*. The phyA gene was inserted into the pPICZ alpha A vector with a signal peptide alpha-factor, under the control of *AOX1* promoter. When the recombinant plasmid was transformed into two *P. pastoris* strains: KM71 (methanol utilization slow) and X33 (wild-type), both the strains produced high levels of active extracellular phytase (25- 65 U ml^{-1} of medium). Mayer and coworkers (1999) used *Hansenula polymorpha* strains containing multiple copies of the *A. terreus* phytase gene, two variants of the *A. fumigatus* phytase gene or a consensus phytase gene, respectively to yield very high phytase concentrations in the medium (up to 13.5 g l^{-1}) in high cell-density cultivations with glucose as carbon source. Expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginsus* involved the cloning of the *phy*A gene encoding an extracellular phytase into an expression vector under transcriptional control of the *Fusarium oxysporum* trypsin gene promoter which was then used to transform a *F. venenatum* recipient strain. The secreted recombinant phytase was active between pH 3 and 7.5 and retained activity at 75°C showing higher catalytic efficiency to any known fungal phytase at 65°C as optimum temperature (Berka *et al.* 1998).

It has been observed that recombinant phytases produced in yeasts, such as *P. pastoris*, are hyper-glycoenzymes containing different levels of glycosylation. Actually, in eukaryotes most of extracellular and membrane-associated proteins are glycosylated to some extent, though the role of the glycosylation is still remained unclear (Guo *et al.* 2008). Studies have shown that glycosylation played a vital role in phyase's heat tolerance. Han and Lei (1999) reported that deglycosylation of the *A. niger* phytase expressed in *P. pastoris* and the commercial *A. niger* phytase with Endo H treatment results in less than 20% reduction in their original activities, but reduces their thermostabilities by approximately 50%. So they proposed that glycosylation is not essential to maintain the phytase activity, but it is very important to protect the enzyme from heat denaturation. Moreover, some studies also suggested that although the carbohydrate did not influence the conformation of the polypeptide backbone, *N*-glycosylation is critical to bio-activity and stability of glycoprotein

Table 1.3: Sources, hosts and properties of some of the expressed microbial phytases

Origin	Expression host	Mol. Wt. (kDa)	pH optimum	Temperature optimum (C°)	References
E. coli	*E.coli* BL21 (DE3) *E.coli* BL21	45	4.5	60	Golovan *et al.* 2000
E. coli AppA	*Pichia pastoris* and *Streptomyces lividans*	45	3.5-4 4	65	Stahl *et al*. 2003
E. coli	*Pichia pastoris*				Rodriguez *et al.* 1999
E. coli AppA2	*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Pichia pastoris*	51.5- 56	3.5	55	Stahl *et al*. 2003
Bacillus sp. DS11 *B.amyloliquefaciens*	*E.coli* BL21(DE3) *Bacillus subtilis*	44		70	Kim *et al*. 1998 Kim *et al*. 1999
Bacillus subtilis	*Pichia pastoris*	39	9.0	55	Sayari *et al.* 2014
Citrobacter braakii	*Saccharomyces cerevisiae* and *E.coli*	49			Kim *et al.* 2006
Dickeya paradisiaca	*Escherichia coli*	43	4.5 & 5.5	55	Gu *et al.* 2009
Erwinia carotovora	*Escherichia coli*	45.3	5.5	~40	Huang *et al.* 2009
O. proteus	*Escherichia coli*	45	4.9	40-45	Zinin *et al.* 2004
Klebsiella pneumoniae	*Escherichia coli*	43.4	3.7 & 5.5	37 & 55	Wang *et al.* 2004
Aspergillus japonicus and *Aspergillus niger*	*Escherichia coli*	51	5.5	50	Promdonkoy *et al.* 2009
A.niger	*Saccharomyces cerevisiae*				Xiong *et al.* 2004
A. niger *A. terreus* *S. occidentalis*	*Pichia pastoris* *H.polymorpha* *Candida boidinii*				Han and Lei 1999 Mayer *et al*.1999 Pandey *et al.* 2001

expressed in yeasts, especially enhancing protein stability toward heat and resistance to proteolysis (Guo *et al.* 2008). This higher thermostability of the hyperglycosylated form of the recombinant enzymes has a clear industrial advantage over its non-glycosylated counterparts produced in bacteria.

1.3.6. APPLICATIONS OF PHYTASES

Phytases have many applications in different directions ranging from human to animal, plant and environmental benefits.

Animal nutrition

Supplementation of the animal feed with microbial phytases is an alternative appproach to check the phosphate utilization for better animal nutrition. Many microbial phytases are marketed and used as animal feed supplements such as phytase from *Aspergillus ficuum* (*niger*) as Natuphos; *A. niger* as Allzyme; *A. awamori* as Finase and Avizyme; *A. oryzae* as SP, TP, SF, AMAFERM and Phyzyme; *Peniophora lycii* as Ronozyme, Roxazyme and Bio-Feed phytase. In the aquaculture industry, supplementation of phytase in plant-based feed will reduce the cost of fish feed and also aid in the reduction of P discharge into the aquatic environment, thereby causing less pollution. Studies showed that dietary phytase improves the nutritive value of canola protein concentrate and decreases P output in case of rainbow trout (Forster *et al*. 1999, Cheng *et al.* 2002) and other different species like channel catfish (Li *et al.* 1997), African catfish (Weerd *et al.* 1999) and *Pangasius pangasius* (Debnath *et al.* 2005). The addition of microbial phytase to diets improves the absorption and utilization of P and the bioavailability of Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} in poultry (Selle *et al.* 2007). Phytase in broiler diets increased the coefficient of P retention and reduced the presence of phytate in poultry waste, thus indicating a favourable environmental effect (Pillai *et al.* 2009). Moreover, microbial phytases also have positive effects on pigs' performance and their daily gain (Hill *et al.* 2009).

Human nutrition

Phytases are known to be used in bread-making processes (Haros *et al.* 2001) as it significantly reduces the phytate content in dough as well as shortens the fermentation time and also improves the bread shape, volume and softness of the crumb. Phytases are also used in fractionation of cereal bran which is the most nutritious part of cereal grain. The bran is subjected to a combination of enzymatic treatments using phytate-hydrolysing enzymes and wet milling, followed by sequential centrifugation and ultrafiltration. All fractions obtained have much broader market applications and greater value than the original bran (Greiner *et al.*

2006). Besides these, phytases could play a role in combating iron deficiency in humans because a major cause of this deficiency is poor absorption of iron from cereal and legume-based diets high in PA. So to increase iron bioavailability phytase overexpressing transgenic plants are being developed, eg. phy gene from *Aspergillus fumigatus* was introduced into the rice endosperm for better bioavailability of iron (Lucca *et al.* 2001).

Probiotics

Probiotics are beneficial microorganisms which are commonly consumed as part of fermented foods with specially added active live cultures. Phytases from some yeasts and fungi, eg. *Saccharomyces cerevisiae* (Nayini and Markakis 1984, Cao *et al*. 2007) are reported safe for human and animal consumption. Any of the two approaches could be used for the application of such microorganisms as probiotics; firstly, their pathogenicity should be reduced to a safe level for human consumption, maintaining at the same time their phytase-producing trait and secondly, phytase genes from these microorganisms could be cloned in microorganisms regarded safe for human consumption.

Biofertilizers for plant growth promotion

Phytase- and phosphatase-producing microorganisms were used as seed inoculants to help attain higher P nutrition of plants in the soil containing high phytate P (Yadav *et al.* 2003). Studies are going on the plant growth-promoting effect of phytase-producing fungi and bacteria, eg., phytase-producing bacteria such as *Bacillus mucilaginosus* (Li *et al.* 2007) and *Bacillus amyloliquefaciens* (Idriss *et al.* 2002) promoted growth of tobacco and maize plants, respectively, under P-limiting conditions and the fungus *Sporotrichum thermophile* promoted growth of wheat plant (Singh *et al.* 2010). Extracellular phytase-producing microorganisms can also be incorporated in traditionally used phytate- rich manures such as poultry and fish manure to increase the availability of P and other essential minerals in manure. Another approach could be to introduce phytase expression in traditionally used biofertilizers such as *Rhizobium, Pseudomonas*.

Transgenic plants and animals

The transgenic crops having high phytase expression is helping in improvement of the bioavailability of P in food/feed along with direct supplementation of microbial phytase to animal feed. A transgenic microalga *Chlamydomonas reinhardtii* overexpressing appA phytase gene from *E. coli* was developed to be used as a food additive (Yoon *et al.* 2011). These transgenic plants have also been evaluated as cost-effective bioreactors for recombinant phytase production to meet their industrial demand (Gontia *et al.* 2012). Transgenic plants

overexpressing phytase gene from microorganisms targeted for root specific secretion, eg. transgenic *Arabidopsis* plants expressing phytase gene from *Aspergillus niger* (Mudge *et al.* 2003) and transgenic soybean plants expressing phytase gene from *Aspergillus ficuum* (Li *et al.* 2009), were found to enhance the P nutrition of crop plants. This approach has a potential to improve the efficiency of P fertilization in agricultural fields.

On the other hand, the manure-based environmental pollution is a big problem of the time. To overcome this problem, transgenic animals expressing phytase have been developed such as transgenic mice (Golovan *et al.* 2001a) and transgenic pigs (Golovan *et al.* 2001b) with the phytase appA gene from *Escherichia coli* regulated for expression in salivary glands. Expression of salivary phytase in mice reduced faecal P by 11 % and 75 % less faecal P in case of the transgenic pigs than nontransgenic animals. These results indicate that the introduction of salivary phytase transgenes into monogastric farm animals offers a promising biological approach to lessen the requirement for dietary phosphate supplements and reduce P pollution.

Industrial applications

Phytases have also found their way in various industrial processes, such as in paper manufacture to eliminate plant phytate from various raw materials (Liu *et al.* 1998); in wet milling of maize to obtain phytate-free corn steep liquor (Caransa *et al.* 1988); in whole wheat bread making (Palaciosa *et al.* 2007) and other benefits; in production of myoinositol and its pharmaceutical applications; in production of semi synthetic peroxidase (Velde *et al.* 2000) etc.

1.4. REFERENCES

Afinah S, Yazid AM, Anis Shobirin MH and Shuhaimi M (2010). Phytase: application in food industry. Int Food Res J 17: 13-21.

Andlid TA and Sandberg JV (2004). Metabolism of extracellular inositol hexaphosphate (phytate) by *Saccharomyces cerevisiae*. Int J Food Microbiol 97: 157-169.

Angel R, Tamim NM, Applegate TJ, Dhandu AS and Ellestad LE (2002). Phytic acid chemistry: Influence on phytin-P availability and phytase efficacy. J Appl Poult Res 11: 471-480.

Barrientos L, Scott JJ and Murthy PP (1994). Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. Plant Physiol 106: 1489-1495.

Berka RM, Rey MW, Brown KM, Byun T and Klotz AV (1998). Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl Environ Microbiol 64: 4423-4427.

Bhavsar K and Khire JM (2014). Current research and future perspectives of phytase bioprocessing. RSC Adv 4: 26677-26691.

Bohn L, Meyer AS and Rasmussen SK (2008). Phytate: impact on environment and human nutrition. A challenge for molecular breeding. J Zhejiang Univ Sci B 9: 165-191.

Brown JR and Volker C (2004). Phylogeny of γ-proteobacteria: revolution of one branch of the universal tree. BioEssays 26: 463-468.

Burgess JR and Gao F (2002). The antioxidant effects of inositol phosphates. In Reddy R and Sathe SK (Eds.), Food Phytates. London: CRC Press LLC. pp. 183.

Cao L, Wang W, Yang C, Yang Y, Diana J and Yakupitiyage A (2007). Application of microbial phytase in fish feed. Enz Microb Technol 40: 497-507.

Caransa A, Simell M, Lehmussaari M, Vaara M and Vaara T (1988). A novel enzyme application in corn wet milling. Starch 40: 409-411.

Casey A and Walsh G (2004). Identification and characterization of a phytase of potential commercial interest. J Biotechnol 110: 313-322.

Cereghino JL and Cregg JM (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24: 45-66.

Chadha BS, Gulati H, Minhas M, Saini HS and Singh N (2004). Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J Microbiol Biotechnol 20:105-109.

Chen CC, Wu PH, Huang CT and Cheng KJ (2004). A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. Enz Microb Technol 35: 315-320.

Chu HM, Guo RT, Lin TW, Chou CC, Shr HL, Lai HL, Tang TY and Cheng KJ (2004). Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. Structr 12: 2015-2024.

Cosgrove DJ (1980). The determination of myo-inositol hexakisphosphate (phytate). J Sc Food Agricultr 31: 1253-1256.

Coulibaly A, Kouakou B and Chen J (2011). Phytic acid in cereal grains: structure, healthy or harmful ways to reduce pa in cereal grains and their effects on nutritional quality. Am J Plant Nutri Fertili Technol 1: 1-22.

Craxton A, Caffrey JJ, Burkhart W, Safrany ST and Shears SB (1997). Molecular cloning and expression of a rat hepatic multiple inositol polyphosphate phosphatase. Biochem J 328: 75-81.

Debnath D, Pal AK, Sahu NP, Jain KK, Yengkokpam S and Mukherjee SC (2005). Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. Aquacult Res 36: 180-187.

Dvorakova J (1998). Phytase: Sources, preparation and exploitation. Folia Microbiol 43: 323-338.

Dvorakova J, Volfova O and Kopecky J (1997). Characterization of phytase produced by *Aspergillus niger*. Folia Microbiol 42: 349-352.

Flanagan JU, Cassady AI, Schenk G, Guddat LW and Hume DA (2006). Identification and molecular modeling of a novel, plant-like, human purple acid phosphatase. Gene 377: 12-20.

Forster I, Higgs DA, Dosanjh BS, Rowshandeli M and Parr J (1999). Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11°C fresh water. Aquacult 179: 109-125.

Fu S, Sun J, Qian L and Li Z (2008). *Bacillus* phytases: present scenario and future perspectives. Appl Biochem Biotechnol 151: 1-8.

Gibson DM (1987). Production of extracellular phytase from *Aspergillus ficuum* on starch media. Biotechnol Lett 9: 305-310.

Gibson RS, Bailey KB, Gibbs M and Ferguson EL (2010). A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. Food Nutr Bull 31: 134-146.

Golovan SP, Hayes MA, Phillips JP and Forsberg CW (2001a). Transgenic mice expressing bacterial phytase as a model for P pollution control. Nat Biotechnol 19: 429-433.

Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ and Barney DJ (2001b). Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19: 741-745.

Gontia-Mishra I, Tantwai K, Rajput LPS and Tiwari S (2012). Transgenic plants expressing phytase gene of microbial origin and their prospective application as feed. Food Technol Biotechnol 50: 3-10.

Gontia-Mishra I and Tiwari S (2013). Phylogenetic Analysis of Fungal Phytases. Food Technol Biotechnol 51: 313-326.

Greiner R and Konietzny U (2006). Phytase for food application. Food Technol Biotechnol 44: 125-140.

Greiner R, Konietzny U and Jany KD (1993). Purification and characterization of two phytases from *Escherichia coli*. Arch Biochem Biophys 303: 107-113.

Greiner R, Larsson Alminger M and Carlsson NG (2001). Stereospecificity of myo-inositol hexakisphosphate dephosphorylation by a phytatedegrading enzyme of baker´s yeast. J Agric Food Chem 49: 2228- 2233.

Gu W, Huang H, Meng K, Yang P, Fu D, Luo H, Wang Y and Yao B (2009). Gene cloning, expression and characterization of a novel phytase from *Dickeya paradisiaca*. Appl Biochem Biotechnol 157: 113-123.

Guo M, Hang H, Zhu T, Zhuang Y, Chu J and Zhang S (2008). Effect of glycosylation on biochemical chatacterization of recombinant phytase expressed in *Pichia pastoris*. Enz Microbe Technol 42: 340-345.

Ha NC, Oh BC, Shin HJ, Oh TK, Kim YO, Choi KY and Oh BH (2000). Crystal structures of a novel, thermostable phytase in partially and fully calcium-loaded states. Nat Struct Biol 7: 147-153.

Haefner S, Knietsch A, Scholten E, Braun J, Lohscheidt M and Zelder O (2005). Biotechnology production and applications of phytases. Appl Microbiol Biotechnol. 68: 588-597.

Han Y and Lei XG (1999). Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (*phyA*) in *Pichia pastoris*. Arch Biochem Biophys 364: 83-90.

Haros M, Rosell CM and Benedito C (2001). Fungal phytase as a potential breadmaking additive. Eur Food Res Technol 213: 317-322.

Harrison DC and Mellanby E (1939). Phytic acid and the rickets-producing action of cereals. Biochem J 33: 1660-1680.

Hegeman CE and Grabau EA (2001). A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. Plant Physiol 126: 1598-1608.

Hill BE, Sutton AL and Richert BT (2009). Effects of low-phytic acid corn, low- phytic acid soybean meal, and phytase on nutrient digestibility and excretion in growing pigs. J Anim Sci 87: 1518-1527.

Hosseinkhani B, Emtiazi G and Nahvi I (2009). Analysis of phytase producing bacteria (*Pseudomonas* sp.) from poultry faeces and optimization of this enzyme production. Afri J Biotechnol 8: 4229-4232.

Huang H, Huiying Luo, Wang Y, Fu D, Shao N, Yang P, Meng K and Yao B (2009). Novel low-temperature-active phytase from *Erwinia carotovora* var. *carotovota* ACCC 10276. J Microbiol Biotechnol 19: 1085-1091.

Huang H, Luo H, Yang P, Meng K, Wang Y, Yuan T, Bai Y and Yao B (2006). A novel phytase with preferable characteristics from *Yersinia intermedia*. Biochem Biophys Res Commun 350: 884-889.

Huang H, Zhang R, Fu D, Luo J, Li Z, Luo H, Shi P, Yang P, Diao Q and Yao B (2011). Diversity, abundance and characterization of ruminal cysteine phytases suggest their important role in phytate degradation. Environ Microbiol 13: 747-757.

Idriss EE, Makarewicz O, Farouk A, Rosner K, Greiner R and Bochow H (2002). Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiol 148: 2097-2109.

IUPAC-IUB (1989). Numbering of atoms in myo-inositol. Recommendations 1988. Nomenclature Committee of the International Union of Biochemistry. Biochem J 258: 1-2.

Jenab M and Thompson I (2002). Role of phytic acid in cancer and other diseases. In Reddy N and Sathe S (Eds.), Food phytates. Boca Raton, FL: CRC Press. pp. 225-248.

Jog SP, Garchow BG, Mehta BD and Murthy PP (2005). Alkaline phytase from lily pollen: Investigation of biochemical properties. Arch Biochem Biophys 440: 133-140.

Johnson LF and Tate ME (1969). Structure of phytic acids. Can J Chem 47: 63-73.

Jorquera M, Martinez O, Maruyama F and De La Luz Mora M (2008). Current and future biotechnolical applications of bacterial phytases and phytase-producing bacteria. Microbes Environ 23: 182-191.

Kaur P, Kunze G and Satyanarayana T (2007). Yeast phytases: present scenario and future perspectives. Crit Rev Biotechnol 27:93-109.

Kaur P, Singh B, Böer E, Straube N, Piontek M, Satyanarayana T and Kunze G (2010). Pphy--a cell-bound phytase from the yeast *Pichia anomala*: molecular cloning of the gene PPHY and characterization of the recombinant enzyme. J Biotechnol 149: 8-15.

Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N and Apajalahti J (1998). Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64: 2079-2085.

Kim YO, Kim HK, Baeks YJ and Oh TK (1998). Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. Enzyme Microb Technol 22: 2-7.

Kim YO, Kim HW, Lee JH, Kim KK and Lee SJ (2006). Molecular cloning of the phytase gene from *Citrobacter braakii* and its expression in *Saccharomyces cerevisiae*. Biotechnol Lett 28: 33-38.

Kim YO, Lee JK, Oh BC and Oh TK (1999). High-level expression of a recombinant thermostable phytase in *Bacillus subtilis*. Biosci Biotechnol Biochem 63: 2205-2207.

Kuang R, Chan KH, Yeung E and Lim BL (2009). Molecular and biochemical characterization of AtPAP15, a Purple acid phosphatase with phytase activity, in *Arabidopsis*. Plant Physiology 151: 199-209.

Kumar V, Singh G, Sangwan P, Verma AK and Agrawal S (2014). Cloning, Sequencing, and in silico analysis of β-propeller phytase *Bacillus licheniformis* Strain PB-13. Biotechnol Res Int e841353: 78-89.

Lassen SF, Breinholt J, Ostergaard PR, Brugger R, Bischoff A, Wyss M and Fuglsang CC (2001). Expression, gene cloning, and characterization of five novel phytases from four basidiomycetes fungi: *Peniophora lycii, Agrocybe pediades,* a *Ceriporia* sp., and *Trametes pubescens*. Appl Environ Microbiol 67: 4701-4707.

Lee S, Kim T, Stahl CH and Lei XG (2005). Expression of *Escherichia coli* AppA2 phytase in four yeast systems. Biotechnol Lett 27: 327-334.

Lei XG, Porres JM, Mullaney EJ and Brinch-Pedersen H (2007). Phytase: source, structure and application. In Polaina J and MacCabe AP (Eds.), Industrial Enzymes: Structure, Function and Applications. Dordrecht, Netherlands: Springer. pp. 505-29.

Lei XG, Weaver JD, Mullaney E, Ullah AH and MJ Azain (2013). Phytase, a New Life for an "Old" Enzyme. Annu Rev Anim Biosci 1: 283-309.

Lerat E, Daubin V and Moran NA (2003). From gene tree to organismal phylogeny in prokaryotes: The case of the γ-proteobacteria. PLoS Biol 1: 101-109.

Li D, Zhu H, Liu K, Liu X, Leggewie G, Udvardi M and Wang D (2002). Purple acid phosphatases of *Arabidopsis thaliana* comparative analysis and differential regulation by phosphate deprivation. J Biol Chem 277: 27772-27781.

Li G, Yang S, Li M, Qiao Y and Wang J (2009). Functional analysis of an *Aspergillus ficuum* phytase gene in *Saccharomyces cerevisiae* and its root-specific, secretory expression in transgenic soybean plants. Biotechnol Lett 3: 1297-1303.

Li MH and Robinson EH (1997). Microbial phytase can replace inorganic phosphorus supplements in channel catfish *Ictalurus punctatus* diets. J World Aquacult Soc 28: 402-406.

Li X, Wu Z, Li W, Yan R, Li L, Li J, Li Y and Li M (2007). Growth promoting effect of a transgenic *Bacillus mucilaginosus* on tobacco planting. Appl Microbiol Biotechnol 7: 1120-1125.

Lim BL, Yeung P, Cheng C and Hill JE (2007). Distribution and diversity of phytate-mineralizing bacteria. ISME J 1: 321-330.

Liu BL, Jong CH and Tzeng YM (1998). Effect of immobilizationon pH and thermal stability of *Aspergillus ficuum* phytase. Enz Microb Technol 25: 517-521.

Loewus F and Murthy P (2000). Myo-inositol metabolism in plants. Plant Sci 150:1-19.

Lucca P, Hurrell R and Potrykus I (2001). Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theor Appl Genet 102: 392-397.

Maldonado RF, Mallera A, Bonneil E, Thibault P, Machadoc CB, Ward RJ, Polizeli MLTM (2014). Biochemical properties of glycosylation and characterization of a histidine acid phosphatase (phytase) expressed in *Pichia pastoris.* Prot Express Puri 99: 43-49.

Mayer AF, Hellmuth K, Schlieker H, Lopez-Ulibarri R, Oertel S, Dahlems U, Strasser AWM, van Loon APGM (1999). An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. Biotechnol Bioeng 63: 373-381.

Mehta BD, Jog SP, Johnson SC and PPN Murthy (2006). Lily pollen alkaline phytase is a histidine phosphatase similar to mammalian multiple inositol polyphosphate phosphatase (MINPP). Phytochem 67: 1874-1886.

Mudge SR, Smith FW and Richardson AE (2003). Root-specific and phosphate-regulated expression of phytase under the control of a phosphate transporter promoter enables *Arabidopsis* to grow on phytate as a sole phosphorus source. Plant Sci 165: 871-878.

Mullaney EJ and Ullah AHJ (2005). Conservation of cysteine residues in fungal histidine acid phytases. Biochem Biophy Res Comm 328: 404-408.

Mullaney EJ and Ullah AHJ (2007). Phytases: Attributes, catalytic mechanisms and applications. In Turner BL, Richardson AE and Mullaney EJ (Eds.), Inositol Phosphates Linking Agriculture and the Environment. Cambridge, MA: CABI. pp. 97-110.

Mullaney EJ and Ullah AHJ (2003). The term phytase comprises several different classes of enzymes. Biochem Biophys Res Commun 312:179-184.

Mullaney EJ, Daly CB, Kim T, Porres JM, Lei XG, Sethumadhavan K and Ullah AHJ (2002). Site-directed mutagenesis of *Aspergillus niger* NRRL 3135 phytase at residue 300 to enhance catalysis at pH 4.0. Biochem Biophys Res Commun 297: 1016-1020.

Nakano T, Joh T, Tokumoto E and Hayakawa T (1999). Purification and characterization of phytase from bran of *Triticum aestivum* L. cv. Nourin #61. Food Sci Technol Research 5: 18-23.

Nayini NR and Markakis P (1984). The phytase of yeast. Lebensm Wiss Technol 17: 24-26.

Nielsen AVF, Tetens I and Meyer AS (2013). Potential of phytase-mediated iron release from cereal-based foods: A quantitative view. Nutri 5: 3074-3098.

O'Dell BL and de Boland AR (1976). Complexation of phytin with proteins and cations in corn germ and oilseed meals. J Agri Food Chem. 24: 804-808.

Oh BC, Choi WC, Park S, Kim YO and Oh TK (2004). Biochemical properties and substrate specificities of alkaline and histidine acid phosphatases. Appl Microbiol Biotechnol 63: 362-372.

Olazaran MG, Blanco LR, Trevino JGC, Lopez JAG and Salvado JMV (2010). Expression of a *Bacillus* phytase C Gene in *Pichia pastoris* and properties of the recombinant enzyme. Appl Environ Microbiol 76: 5601-5608.

Olstorpe M, Schnürer J and Passoth V (2009). Screening of yeast strains for phytase activity. FEMS Yeast Res 9: 478-488.

Ostanin K and Van Etten RL (1993). Asp304 of *Escherichia coli* acid phosphatase is involved in leaving group protonation. J Biol Chem 268: 20778-84.

Pal Roy M, Poddar M, Singh KK and Ghosh S (2012). Purification, characterization and properties of phytase from *Shigella* sp. CD2. Ind J Biochem Biophys 49: 266-271.

Palaciosa MC, Harosa M, Sanzb Y and Rosella CM (2007). Selection of lactic acid bacteria with high phytate degrading activity for application in whole wheat bread-making. Food Sci Technol 41: 82-92.

Pandey A, Szakacs G, Soccol CR, Rodriguez- Leon JA and Soccol VT (2001). Production, purification and properties of microbial phytases. Bioresource Technol 77: 203-214.

Pasamontes L, Haiker M, Wyss M, Tessier M and Van Loon AP (1997). Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. Appl Environ Microbiol 63: 1696-1700.

Pillai UP, Manoharan V, Lisle A, Li X and Bryden W (2009). Phytase supplemented poultry diets affect soluble P and nitrogen in manure and manure-amended soil. J Environ Qual 38: 1700-1708.

Pons T, Hernandez L, Batista FR and Chinea G (2000). Prediction of a common beta-propeller catalytic domain for fructosyltransferases of different origin and substrate specificity. Protein Sci 9: 2285-2291.

Posternak T (1965). Cyclitols. San Francisco, CA: Holden-Day, Inc.

Powar VK, and Jagannathan V (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. J Bacteriol 151: 1102-1108.

Promdonkoy P, Tang K, Sornlake W, Harnpicharnchai P, Kobayashi RS, Ruanglek V, Upathanpreecha T and Vesaratchavest M (2009). Expression and characterization of *Aspergillus* thermostable phytases in *Pichia pastoris*. FEMS Microbiol Lett 290: 18-24.

Puhl AA, Gruninger RJ, Greiner R, Janzen TW, Mosimann SC and Selinger LB (2007). Kinetic and structural analysis of a bacterial protein tyrosine phosphatase-like myo-inositol polyphosphatase. Prot Sci 16: 1368-1378.

Quan CS, Tian WJ, Fan SD and Kikuchi J (2004). Purification and properties of a low-molecular-weight phytase from *Cladosporium* sp. FP-1. J Biosci Bioeng 97: 260-266.

Rao DE, Rao KV, Reddy TP and Reddy VD (2009). Molecular characterization, physicochemical properties, known and potential applications of phytases: An overview. Crit Rev Biotechnol 29: 182-198.

Ravindran V, Bryden WL and Kornegay ET (1995). Phytates: Occurrence, bioavailability, and implications in poultry nutrition. Poult Avian Biol Rev 6: 125-143.

Rodehutscord M, Hempel R and Wendt P (2006). Phytase effects on the efficiency of utilization and blood concentration of phosphorus and calcium in pekin ducts. Br Poult Sci 47: 311-321.

Rodriguez E, Porres JM, Han Y and Lei XG (1999). Different sensitivity of recombinant *Aspergillus niger* phytase (r-phyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin *in vitro*. Arch Biochem Biophys 365: 262-267.

Roopesh K, Ramachandrana S, Nampoothiria KM, Szakacs G and Pandey A (2006). Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. Bioresource Technol 97: 506-511.

Sano K, Fukuhara H, Nakamura Y (1999). Phytase of the yeast *Arxula adeninivorans*. Biotechnol Lett 21: 33-38.

Sayari AH, Elgharbi F, Farhat A, Rekik H, Blondeau K and Beja RS (2014). Overexpression and biochemical characterization of a thermostable phytase from *Bacillus subtilis* US417 in *Pichia pastoris*. Mol Biotechnol 56: 839–848.

Schenk G, Korsinczky MLJ, Hume DA, Hamilton S and DeJersey J (2000). Purple acid phosphatases from bacteria: similarities to mammalian and plant enzymes. Gene 255: 419-424.

Schneider G, Lindqvist Y and Vihko P (1993). Three-dimensional structure of rat acid phosphatase. EMBO J 12: 2609–2615.

Selle PH and Ravindran R (2007). Microbial phytase in poultry nutrition. Anim Feed Sci Technol 135: 1–41.

Seshadri R, Kravitz SA, Smarr L, Gilna P and Frazier M (2007). CAMERA: a community resource for metagenomics. PLoS Biol 5: 394-397.

Shi XW, Sun ML, Zhou B and Wang XY (2009). Identification, characterization, and overexpression of a phytase with potential industrial interest. Can J Microbiol 55: 599-604.

Shimizu M (1992). Purification and characterization of phytase from *Bacillus subtilis*. Biosci Biotechnol Biochem 56: 1266-1269.

Shin S, Ha NC, Oh BC, Oh TK and Oh BH (2001). Enzyme mechanism and catalytic property of beta propeller phytase. Structr 9: 851-858.

Shivanna GB and Venkateswaran G (2014). Phytase Production by *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 through Submerged and Solid-State Fermentation. The Sci World J e392615: 23-29.

Singh B and Satyanarayana T (2010). Plant growth promotion by an extracellular HAP-phytase of a thermophilic mold *Sporotrichum thermophile*. Appl Biochem Biotechnol 160: 1267-1276.

Singh B, Kunze G and Satyanarayana T (2011). Developments in biochemical aspects and biotechnological applications of microbial phytases. Biotechnol Mol Biol Rev 6: 69-87.

Stahl CH, Wilson DB and Lei XG (2003). Comparison of extracellular *Escherichia coli* AppA phytase expressed in *Streptomyces lividans* and *Pichia pastoris*. Biotechnol Lett 25: 827-831.

Tai HM, Yin LJ, Chen WC and Jiang ST (2013). Overexpression of *Escherichia coli* phytase in *Pichia pastoris* and its biochemical properties. J Agri Food Chem 61: 6007-6015.

Thornton CG and Passen S (2004). Inhibition of PCR amplification bovine fecal specimens with by PA, and treatment of phytase to reduce inhibition. J Microbiol Meth 59: 43-52.

Tompson LV (1986). Phytic acid: factor influencing starch digestibility and blood glucose response. In Graf E (Ed.), Phytic Acid: Chemistry and Applications. Minneapolis, MN, USA: Pilatus Press. pp. 173-194.

Torres J, Dominguez S, Cerda MF, Obal G, Mederos A, Irvine RF, Diaz A and Kremer C (2005). Solution behaviour of *myo*-inositol hexakisphosphate in the presence of multivalent cations. Prediction of a neutral pentainagnesium species under cytosolic/nuclear conditions. J Inorg Biochem 99: 828-840.

van de Velde F, Könemann L, van Rantwijk F and Sheldon RA (2000). The rational design of semisynthetic peroxidases. Biotechnol Bioeng 67: 87-96.

Vats P and Banerjee UC (2004). Production studies and catalytic properties of phytases (myoinositolhexakis phosphate phosphohydrolases): An overview. Enz Microb Technol 35: 3-14.

Vats P, Bhattacharyya MS and Banerjee UC (2005). Use of phytases (*myo*-inositolhexakisphosphate phosphohydrolases) for combatting environmental pollution: A biological approach. Crit Rev Environ Sci Technol 35: 469-486.

Viader-Salvado JM, Gallegos-Lopez JA, Carreon-Trevino JG, Castillo-Galvan M, Rojo-Dominguez A and Guerrero-Olazaran M (2010). Design of thermostable beta-propeller phytases with activity over a broad range of pHs and their overproduction by *Pichia pastoris*. Appl Environ Microbiol 76: 6423-6430.

Wang Y, Ye X, Ding G and Xu F (2013). Overexpression of *phyA* and *appA* genes improves soil organic P utilisation and seed phytase activity in *Brassica napus*. PLoS ONE 8: e60801.

Wang X, Upatham S, Panbangred W, Isarangkul D, Summpunn P, Wiyakrutta S and Meevootisomd V (2004). Purification, characterization, gene cloning and sequence analysis of a phytase from *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5. Sci Asia 30: 383-390.

Wodzinski RJ and Ullah AHJ (1996). Phytase. In Advances in Applied Microbiology. New York: Academic Press, Inc. pp. 263-302.

Wyss M, Brugger R, Kronenberger A, Remy R, Fimbel R, Oesterhelt G, Lehmann M and van Loon A (1999). Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl Environ Microbiol 65: 367-373.

Wyss M, Pasamontes L, Remy R, Kohler J, Kusznir E, Gadient M, Muller F and van Loon A (1998). Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. Appl Environ Microbiol 64: 4446-4451.

Xiang T, Liu Q, Deacon AM, Koshy M, Kriksunov IA, Lei XG, Hao Q and Thiel DJ (2004). Crystal structure of a heat-resilient phytase from *Aspergillus fumigatus*, carrying a phosphorylated histidine. J Mol Biol 339: 437-445.

Xiong AS, Yao QH, Peng RH, Li X, Fan HQ, Guo MJ and Zhang SL (2004). Isolation, characterization, and molecular cloning of the cDNA encoding a novel phytase from *Aspergillus niger* 113 and high expression in *Pichia pastoris*. J Biochem Mol Biol 37: 282-291.

Yadav RS and Tarafdar JC (2003). Phytase and phosphatase producing fungi in arid and semi-arid soils and their efficiency in hydrolyzing different organic phosphorus compounds. Soil Biol Biochem 35: 745-751.

Yao MZ, Wang X, Wang W, Fu YJ, Liang AH (2013). Improving the thermostability of *Escherichia coli* phytase, appA, by enhancement of glycosylation. Biotechnol Lett 35: 1669-1676.

Yao MZ, Zhang YH, Lu WL, Hu MQ, Wang W and Liang AH (2011). Phytases: crystal structures, protein engineering and potential biotechnological applications. J Appl Microbiol 112: 1-14.

Yoon SJ, Choi YJ, Min, HK, Cho KK, Kim JW, Zee SC and Jung YH (1996). Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4 and enzymatic properties of phytase enzyme. Enz Microb Technol 18: 449-454.

Yoon SM, Kim SY, Li KF, Yoon BH, Choe S and Kuo MMC (2011). Transgenic microalgae expressing *Escherichia coli* AppA phytase as feed additive to reduce phytate excretion in the manure of young broiler chicks. Appl Microbiol Biotechnol 91: 553-563.

Zhang W, Gruszewski HA, Chevone BI and Nessler CL (2008). An *arabidopsis* purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol 146: 431-440.

Zhu HJ, Sun LF, Zhang YF, Zhang XL, Qiao JJ (2012). Conversion of spent mushroom substrate to biofertilizer using a stress-tolerant phosphate-solubilizing *Pichia farinose* FL7. Biores Technol 111: 410-416.

Zinin NV, Serkina AV, Gelfand MS, Shevelev AB and Sineoky SP (2004). Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*. FEMS Microbiol Lett 236: 283-290.