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REVIEW ARTICLE

Molecular characterization, physicochemical properties, known and potential applications of phytases: An overview

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Abstract

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) hydrolyze the phosphate ester bonds of phytate-releasing phosphate and lower *myo*-inositol phosphates and/or *myo*-inositol. Phytases, in general, are known to enhance phosphate and mineral uptake in monogastric animals such as poultry, swine, and fish, which cannot metabolize phytate besides reducing environmental pollution significantly. In this study, the molecular, biophysical, and biochemical properties of phytases are reviewed in detail. Alterations in the molecular and catalytic properties of phytases, upon expression in heterologous hosts, are discussed. Diverse applications of phytases as feed additives, as soil amendment, in aquaculture, development of transgenic organisms, and as nutraceuticals in the human diet also are dealt with. Furthermore, phytases are envisaged to serve as potential enzymes that can produce versatile lower *myo*-inositol phosphates of pharmaceutical importance. Development of phytases with improved attributes is an important area being explored through genetic and protein engineering approaches, as no known phytase can fulfill all the properties of an ideal feed additive.

Keywords: *Eutrophication; glycosylation; phytase; phytate; thermostability; transgenics*

Introduction

Phytate/phytic acid [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate (IUPAC–IUB, 1968)] is a common constituent of plant-derived foods. Phytic acid is the principal storage form of phosphorus (P) and inositol in cereals, legumes, and oilseeds representing ~60–90% of the total phosphorus content in plants. Phytic acid was found for the first time by Pfeffer (1872) in the aleurone layers of rice grains. A calcium–magnesium salt of phytic acid is named as phytin, while the mono to dodeca anion of phytic acid is known as phytate. Phytate amounts to ~50% of the total P in nuts, peanuts, cobnuts, almonds, and coconuts (McCance and Widdowson, 1935). Cocoa and chocolate were also estimated to contain 24% of the total P as phytin. Both legume and cereal grains were found to contain ~5% phytate by weight (de Boland, Garner, and O'Dell, 1975). Phytates are known to occur in the form of a polyanion at pH 1–6 with 3–6 negative charges, in the crop, proventriculus, and gizzard of poultry, as well as the stomach of humans and swine (Bebot-Brigaud *et al.*, 1999). Phytic acid—a polyanionic chelating agent—forms complexes with several divalent cations of major nutritional significance, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} (Harland and Oberleas, 1999). Phytates are known to form complexes with proteins under both acidic and alkaline pH conditions. These interactions were found to affect the protein structure, thus decreasing the enzymatic activity, protein solubility, and proteolytic digestibility (Kies *et al.*, 2006).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) catalyze the hydrolysis of phytates to *myo*-inositol pentakisphosphate (IP_5) or to less phosphorylated *myo*-inositol phosphates IP_3 (Hara *et al.*, 1985; Kerovuo, Rouvinen, and Hatzack, 2000a; Quan *et al.*, 2004) or IP (Wyss *et al.*, 1999a). The complete hydrolysis of phytate produces one molecule of inositol and six molecules of inorganic phosphate, while partial hydrolysis results in *myo*-inositol intermediates, namely, mono-, di-, tri-, tetra-, and pentaphosphates besides inorganic phosphate (Figure 1). Phytases are broadly classified into three types depending on the initiation site of dephosphorylation of the phytate, namely, 3-phytases, 6-phytases, and 5-phytases (Cosgrove,

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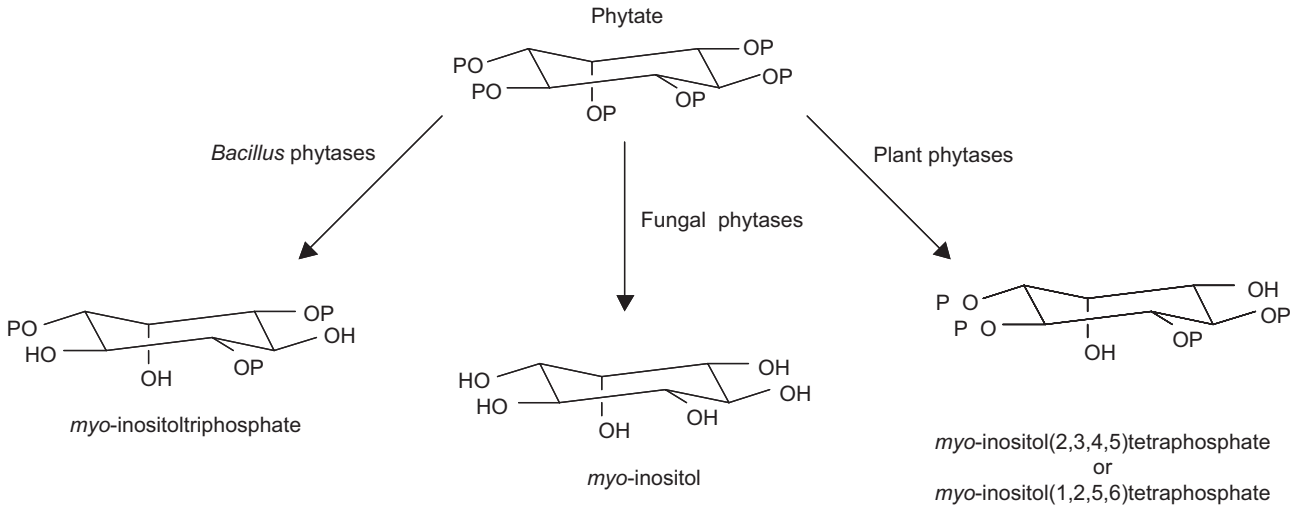

Figure 1. Schematic diagram showing end products resulting from phytate hydrolysis mediated by different phytases.

1970). *3-Phytases* (EC 3.1.3.8) isolated from *Aspergillus niger*, *Neurospora crassa*, *Pseudomonas*, *Klebsiella* sp. ASR1, and so on initiate the hydrolysis of phytate at the third phosphate group (Sajidan *et al*., 2004). However, *6-phytases* (EC 3.1.3.26) extracted from *Escherichia coli*, *Paramecium*, and so on initiate the hydrolysis of phytate at the sixth phosphate group (Greiner, Kontietzny, and Jany, 1993; Van der Kaay and Van Haastert, 1995), whereas *5-phytases* (EC 3.1.3.72) from *Medicago sativa*, *Phaseolus vulgaris*, and *Pisum sativum* initiate phytate hydrolysis at the fifth phosphate group.

On the basis of the catalytic mechanism, phytases can be classified into three groups (Tye *et al*., 2002). Group 1 includes histidine acid phosphatases or acid phosphatases (EC 3.1.3.2), Group 2 includes β-propeller phytases (EC 3.1.3.8), and Group 3 includes purple acid phosphatases or cysteine phytases (EC 3.1.3.2). Phytases from fungi and *E. coli* belong to the histidine acid phosphatases. These enzymes share the same active site sequence (RHGXRXP), the catalytic dipeptide, and 10 cysteine residues. The enzymes in Group 2 are mainly *Bacillus* phytases. These enzymes have a six-bladed propeller folding architecture. There are six calcium-binding sites in each protein molecule and the enzyme activity is dependent on calcium. Purple acid phosphatase is found as a homodimeric glycoprotein in mainly plant species, with a Fe(III)–Zn(II) active site (Dionisio, Holm, and Brinch-Pendersen, 2007). Cysteine phytases represent a new class of phytases described in the anaerobic ruminal bacterium *Selenomonas ruminantium* (Chu *et al*., 2004). According to the conserved domain database (CDD) of the NCBI, the conserved domains in acid phosphatases (Group 1), phytases (Group 2), and purple acid phosphatases (Group 3) are designated as pfam00328, pfam02333, and pfam02227, respectively.

Phytases are varied and widespread in nature, occurring in plants, microorganisms, and animal tissues (Table 1). Phytase was identified for the first time in the rice bran, which could mediate the production of various phosphatidyl inositols as intermediates or as end products (Suzuki, Yoshimura, and Takaishi, 1907). The occurrence of phytases was reported in the calf liver and blood by McCollum and Hart (1908). Phytates when fed to rabbits were hydrolyzed by phytases secreted by the microflora present in the intestine (Plimmer, 1913). In addition, different phytases were identified in negligible quantities in the mucosal extracts of small intestine of rats, humans, rabbits, and guinea pigs (Iqbal, Lewis, and Cooper, 1994; Marounek, Duskova, and Skrivanova, 2003).

The first commercial product containing phytase as animal feed additive was released ~9 decades later (1994) under the trade name Natuphos in Europe by Gist-Brocades (now DSM) and was sold by BASF. In recent years, the market volume of phytases is estimated to be 150 million Euros (Haefner *et al*., 2005).

Previous reviews dealt with parameters involved in phytase production and purification, its biochemical properties, substrate specificities, and applications (Lei and Stahl, 2001; Vohra and Satyanarayana, 2003; Oh et al., 2004). A recent review describes about the production, characterization, and applications of yeast phytases (Kaur, Kunze, and Satyanarayana, 2007). The present communication focuses on recent developments and gives a comprehensive account on diverse sources of phytases, their expression in heterologous hosts, molecular characteristics, physicochemical properties, besides various applications of phytases in animal and human nutrition, aquaculture, transgenic animals and plants, as a soil amendment, and other uses.

Molecular and biophysical characteristics of phytases

Phytases have been isolated from diverse sources, expressed in a wide range of hosts, purified, and refolded using various biochemical methods. Depending on the source and/or expression host, phytases are known to show distinctive biophysical and biochemical properties. The molecular and biophysical characteristics of phytases are summarized in Table 1.

Table 1. Source and properties of phytase.

Phytase source	Production strain	Specific activity	Molecular weight (kDa)	Temperature optimum (°C)	pH optimum	K_m (μM)	Reference
Agrocybe pediades	*Aspergillus oryzae*	400 U mg^{-1}	59	50	5.0–6.0	–	Lassen *et al.* (2001)
Aspergillus niger	*Escherichia coli*	1.5 μmol $min^{-1}mg^{-1}$	56	43–50	5.1	96	Phillippy and Mullaney (1997)
A. niger	*Saccharomyces cerevisiae*	4.0 U mg^{-1}	120	55–60	2–2.5;5–5.5	–	Han *et al.* (1999)
A. niger van Teighem	–	22,592 U mg^{-1}	66	52–55	2.5	606	Vats and Banerjee (2005)
A. niger	Pichia pastoris	25–65 U mL^{-1}	95	60	2.5, 5.5	–	Han and Lei (1999)
Aspergillus fumigatus	P. pastoris	43 U mg^{-1}	–	60	6.0	–	Rodriguez *et al.* (2000a)
Aspergillus ficuum	*Potato*	–	67.5–81.6	58	5.0	124	Ullah *et al.* (2003)
A. niger	*Soybean*	–	69–71	63	5.0	–	Li *et al.* (1997a)
A. ficuum	*Alfalfa*	3761 nKat mg^{-1}	73–100	58	5.0	50	Ullah *et al.* (2002)
A. ficuum	*Tobacco*	–	76	50	2.0, 5.5	730	Zhang *et al.* (2004)
A. fumigatus	–	–	60	–	4.0, 6.0–6.5	–	Pasamontes *et al.* (1997)
A. fumigatus	–	28 U mg^{-1}	60–76	60	5.0–6.0	–	Wyss *et al.* (1999a)
A. niger	–	102.5 U mg^{-1}	66–100	–	–	–	Wyss *et al.* (1999a)
Aspergillus terreus	–	142–196	60	70	5.0–5.5	–	Wyss *et al.* (1999a)
A. oryzae	*A. oryzae* RIB40 *niaD*$^-$	2 U mL^{-1}	74	50	5.5–6.0	–	Uchida *et al.* (2006)
Rhizomucor pusillus	–	–	–	70	5.4	–	Chadha *et al.* (2004)
Aspergillus	Canola	–	70	–	–	–	Peng *et al.* (2006)
Peniophora lycii	*A. oryzae*	1080 ± 110 U mg^{-1}	72	50–55	4.0–5.0	–	Lassen *et al.* (2001)
Ceriporia sp.	*A. oryzae*	700 ± 80 U mg^{-1}	59	55–60	5.5–6.0	–	Lassen *et al.* (2001)
Peniophora lycii	*P. pastoris*	10,540 U mL^{-1}	70–110	50	4.5	–	Xiong *et al.* (2006)
Schwanniomyces occidentalis	Rice	–	70	70	4.5	–	Hamada *et al.* (2005)
Trametes pubescens	*A. oryzae*	1210 ± 30 U mg^{-1}	62	50	5.0–5.5	–	Lassen *et al.* (2001)
Bacillus sp.		16 U mg^{-1}	40	55	7.0	392	Rao *et al.* (2008)
Bacillus subtilis	–	8.5 U mg^{-1}	36.5	60	7–.5	350	Powar and Jagannathan (1982)
B. subtilis	–	35 U mL^{-1}	44	55	7.0	–	Tye *et al.* (2002)
B. subtilis	–	9–15 U mg^{-1}	43	55	7.0	–	Kerovuo *et al.* (1998)
Bacillus laevolacticus	–	12.69 U mg^{-1}	41–46	70	7.0–8.0	526	Gulati *et al.* (2007)
Bacillus sp. KHU-10	–	36 U mg^{-1}	44	40	6.5–8.5	50	Choi *et al.* (2001)
Bacillus sp. DS11	–	–	44	70	–	–	Kim *et al.* (1998b)
Bacillus licheniformes	–	–	47	65	7.0	–	Tye *et al.* (2002)

Table 1. Continued on next page

Table 1. Continued.

Phytase source	Production strain	Specific activity	Molecular weight (kDa)	Temperature optimum (°C)	pH optimum	K_m (μM)	Reference
Lactobacillus pentosus	–	–	69	50	5.0	–	Palacios *et al.* (2005)
E. coli	–	–	42	55	4.5	–	Greiner *et al.* (1993)
E. coli	*E. coli*	–	44	60	4.5	–	Golovan *et al.* (2000)
Lactobacillus plantarum	–	0.463 U mg^{-1}	52	65	5.5	–	Zamudio, Gonzalez, and Medina (2001)
Pantoea agglomerans	–	–	42	60	4.5	340	Greiner (2004)
Emericella nidulans	–	29–33	66	–	6.5	–	Wyss *et al.* (1999a)
Myceliophthora thermophila	–	–	62	–	5.5	–	Wyss *et al.* (1999b)
Talaromyces thermophilus	–	–	128	–	–	–	Wyss *et al.* (1999b)
Thermomyces lanuginosus	–	110 U mg^{-1}	60	65	6.0	–	Berka *et al.* (1998)
Yersinia intermedia	P. pastoris	3960 U mg^{-1}	45	55	4.5	–	Huang *et al.* (2006)
Klebsiella sp. ASR1	*E. coli*	99 U mg^{-1}	–	45	5.0	280	Sajidan *et al.* (2004)
Selenomonas ruminantium	–	–	46	50–55	4.0–5.5	–	Yanke *et al.* (1999)
Enterobacter sp. 4	–	–	–	50	7.0–7.5	–	Yoon *et al.* (1996)
Obesumbacterium proteus	*E. coli*	310 U mg^{-1}	45	40–45	4.9	340	Zinin *et al.* (2004)
Citrobacter braakii	*E. coli*	1122 U mg^{-1}	49	–	–	–	Kim *et al.* (2006)
C. braakii	*S. cerevisiae*	658 U mg^{-1}	110–160	–	–	–	Kim *et al.* (2006)
Penicillium oxalicum PJ3	*P. pastoris*	306.6 U mg^{-1}	62.5	55	4.5	370	Lee *et al.* (2007)
Pseudomonas syringae MOK1	*E. coli*	2.514 U mg^{-1}	–	–	–	–	Cho *et al.* (2005)
Candida krusei	–	–	–	40	2.5, 5.5	30	Quan *et al.* (2001)
C. krusei *WZ-001*	–	–	330	40	4.6	30	Quan *et al.* (2002)
Penicillium simplicissimum	–	3.8 U mL^{-1}	65	55	4.0	–	Tseng YH, Fang, and Tseng SM (2000)
Cladosporium sp. FP-1	–	909.5 U mg^{-1}	32.6	40	3.5	15.2	Quan *et al.* (2004)
Lactobacillus sanfranciscensis	–	–	–	45	4.0	–	De Angelis *et al.* (2003)
Pichia anomala	–	–	64	60	4.0	200	Vohra and Satyanarayana (2002)
Rye	–	–	67	45	6.0	300	Greiner *et al.* (1997)
Spelt	–	262 μmol $min^{-1} mg^{-1}$	68	45	6.0	400	Konietzny *et al.* (1995)
Lilium longiflorum	–	0.066 U mg^{-1}	88	55–60	8.0	7.2	Scott and Loewus (1986)
Triticum aestivum	*E. coli*	–	54	65	4.5	246 ± 38	Dionisio *et al.* (2007)

Table 1. Continued on next page

Table 1. Continued.

Phytase source	Production strain	Specific activity	Molecular weight (kDa)	Temperature optimum (°C)	pH optimum	K_m (μM)	Reference
Hordeum vulgare	*E. coli*	–	54	65	4.5	334 ± 31	Dionisio *et al.* (2007)
Soybean	–	–	–	40–60	3.0–5.8	–	Hamada (1996)
Soybean	–	–	70–72	58	4.5–5.0	61	Hegeman and Grabau (2001)
Soybean	–	0.5 U mg^{-1}	–	60	4.8	2400	Sutardi and Buckle (1986)
Maize root	–	5.7 U mg^{-1}	71	40	5.0	–	Hubel and Beck (1996)
Maize seedlings			76	55	4.8	117	Laboure *et al.* (1993)
Tomato root	–	205 U mg^{-1}	–	50	4.3	–	Li *et al.* (1997b)
Allium fistulosum L.	–	500 μmol $min^{-1}mg^{-1}$	72	51	5.5	200	Phillippy (1998)
Phaseolus vulgaris	–	–	–	–	8.0	–	Scott (1991)
Pisum sativum	–	–	–	–	8.0	–	Scott (1991)
Medicago sativa	–	–	–	–	8.0	–	Scott (1991)
Rat intestine	–	–	70, 90	–	7.5	–	Yang *et al.* (1991)
Hybrid striped bass	–	4.8 U mg^{-1}	–	–	3.5–4.5	2500	Ellestad, Angel, and Soares (2002)
E. coli	Mice	–	55	–	–	–	Golovan *et al.* (2001a)
E. coli	Pig	–	55	–	–	–	Golovan *et al.* (2001b)

Gram-negative bacteria are known to produce phytase intracellularly while gram-positive bacteria and fungi produce it extracellularly (Greiner *et al.*, 1993; Jareonkitmongkol *et al.*, 1997; Pasamontes *et al.*, 1997; Kaur *et al.*, 2007). The fungal and *E. coli* phytases exhibit no apparent sequence similarity to each other and other known phosphatases, except for the septapeptide, RHGXRXP motif, conserved in the high molecular weight acid phosphatases (Ullah and Dischinger, 1993; Ullah and Sethumadhavan, 1998; Lim *et al.*, 2000). The histidine residue (His) of fungal phytases has been proposed to serve as a nucleophile in the formation of phosphohistidine intermediate. The other hallmark of fungal phytases is the C-terminal HD motif (His361 and Asp362), which was recognized by Van Etten *et al.* (1991). Ostanin and Van Etten (1993) reported an equivalent His residue in *E. coli* acid phosphatase, which causes the nucleophilic attack on the scissile phosphoester bond, and an equivalent Asp residue, which might protonate the substrate leaving group. Whereas, *Bacillus* phytases lack the conserved RHGXRXP active site motif of histidine acid phosphatases and do not show homology to any of the known phytases. Metal dependency for structural integrity is another unique feature of this group of phytases. These enzymes have a six-bladed β-propeller folding architecture (Figure 2A and 2B) (Ha *et al.*, 2000; Rao DECS, Rao KV, and Reddy, 2008). The TS-phytase of *Bacillus amyloliquefaciens* is a β-propeller, comprising five 4-stranded and one 5-stranded antiparallel β-sheets, aligned around a pseudo sixfold symmetry axis lying on the shaft of the propeller, which serves as a distinct central channel filled with

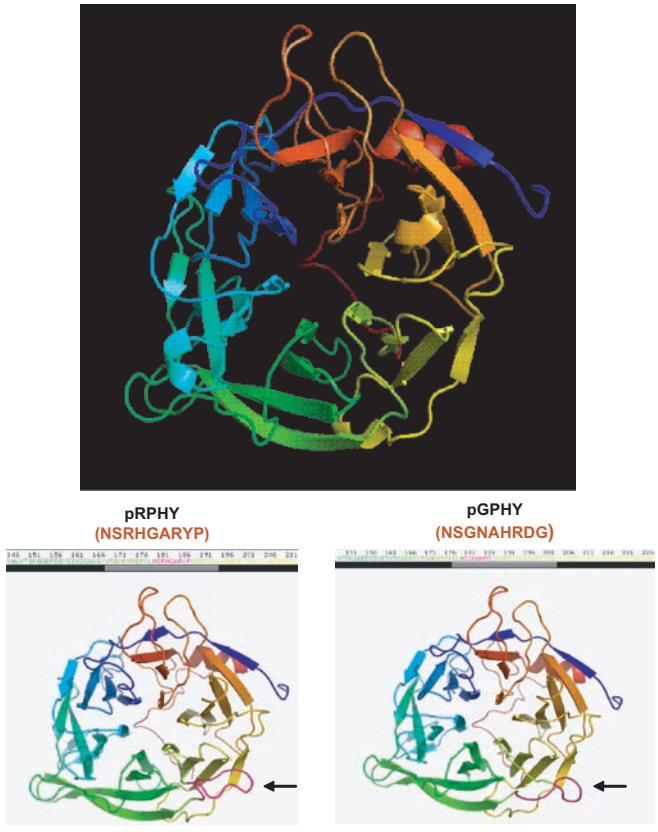

Figure 2. (A) β-Propeller structure of *Bacillus* phytase. (B) *Bacillus* phytase proteins with domain insertion between Leu179 and Asp180.

many ordered water molecules. This enzyme binds seven calcium ions, two of which are located at the periphery, one in the middle of the central channel, while the other four are bound at the top of the molecule. Unlike other β-propeller structures, it does not show any conserved sequence repeat in the β-strands (Shin *et al.*, 2001). *S. ruminantium* phytase was distinct and showed no structural similarity to that of *E. coli* and *B. amyloliquefaciens* phytases. The *S. ruminantium* phytase assumes a football-like shape and its crystal structure revealed a phytase-fold of the dual specificity phosphatase type (Chu *et al.*, 2004). Soybean phytase was found to contain motifs characteristic of a large group of phosphoesterases including purple acid phosphatases and lacks the RHGXRXP-motif (Hegeman and Grabau, 2001). Different purple acid phosphatases of plants are known to contain conserved metal-ligating residues required for enzyme function. Maize phytase, except for a homologous region of 33 amino acids including RxGxRxP-motif, showed no apparent sequence similarity to that of *A. niger* phytase (Maugenest *et al.*, 1999). Phytase from *Arabidopsis thaliana* contains a septapeptide active sequence RHGXRXP as well as the dipeptide HD region required for phosphatase activity (Mullaney and Ullah, 1998).

Most of the phytase enzymes consist of monomeric protein, while phytases from maize roots (Hubel and Beck, 1996) and maize seedlings (Laboure, Gagnon, and Lescure, 1993) are homodimeric in nature. Molecular masses of phytases are highly variable ranging from ~32 to ~330 kDa (Table 1). The higher molecular weight of fungal and yeast phytases is attributed to the glycosylation of these enzymes in the host system. Phytase from *Citrobacter braakii*, expressed in *Saccharomyces cerevisiae*, showed higher molecular weight as compared to the recombinant phytase expressed in *E. coli*, owing to N-glycosylation of the protein in the yeast system. The hyperglycosylated phytase also showed enhanced thermostability in comparison with the unglycosylated counterpart (Kim *et al.*, 2006). Similarly, phytase from *A. niger*, when expressed in *S. cerevisiae*, exhibited an increased protein mass and high thermotolerance, as compared to the phytase expressed in the native *A. niger*, because of N-glycosylation of the recombinant phytase. *Penicillium oxalicum* phytase when expressed in *Pichia pastoris* produced ~62.5 kDa protein with 25% increase in mass, caused by N-glycosylation in the host system (Lee *et al.*, 2007). *A. niger phyA* gene, expressed in *P. pastoris* and *S. cerevisiae*, produced phytase enzymes of ~95 kDa and ~120 kDa, respectively, owing to lesser glycosylation of the protein in *P. pastoris* compared to *S. cerevisiae* (Han and Lei, 1999; Han, Wilson, and Lei, 1999). Phytase gene from *Peniophora lycii*, upon expression in *Aspergillus oryzae* and *P. pastoris*, produced ~72 kDa and ~110 kDa proteins, respectively, resulting from higher glycosylation in *P. pastoris*, without any change in the thermotolerance of the proteins (Lassen *et al.*, 2001; Xiong *et al.*, 2006). *In vitro* deglycosylation of the phytase from *Peniophora lycii* had a limited effect on the equilibrium heat denaturation of the enzyme. The glycans of glycosylated phytase were shown to strongly promote the kinetic stability (i.e., reduced rate of irreversible denaturation) of the protein, while leaving the equilibrium denaturation temperature largely unaltered (Rasmus *et al.*, 2006). Recombinant fungal phytases, expressed in different hosts like *A. niger*, *Hansenula polymorpha*, and *S. cerevisiae*, with varied levels of glycosylation did not show significant differences in their thermostability, protein folding, or specific activity. Thermostability of phytases cannot be attributed to glycosylation of the protein alone but needs to be studied in relation to various other factors like host–protein interaction, buffer systems used for thermostability assays, and the relative purity of the enzymes (Wyss *et al.*, 1998, 1999b).

Expression of *Aspergillus ficuum* phytase in alfalfa and potato produced stable enzyme with lower molecular mass caused by reduced glycosylation in the plant systems. Further, the enzyme expressed in the heterologous hosts showed increased specific activity with reduced K_m value, and a shift in pH optima from 5.5 and 2.5 to 5.0 and 2.0, respectively; however, the phytase expressed in alfalfa was more sensitive to thermal denaturation than the native phytase. The variation observed in enzyme properties, despite identical primary structure, was attributed to the differential glycosylation and folding of the phytase in fungi and plants (Ullah *et al.*, 2002, 2003). Expression of *A. ficuum* (AS3.324) phytase in tobacco showed enzyme properties similar to that of native phytase; however, the difference observed in the mass of *A. ficuum* (AS3.324) phytase (~68.5 kDa) and recombinant phytase (~76 kDa) was attributed to higher glycosylation of the protein in the plant system (Zhang *et al.*, 2004). Conversely, *A. niger* phytase expressed in soybean disclosed a lesser molecular mass (~71 kDa) when compared to the native (~85 kDa) enzyme (Li *et al.*, 1997a).

In our laboratory, full-length (*sphy*) and truncated (*phy*) phytase genes of *Bacillus* were cloned independently into the bacterial expression vector pET21a(+) downstream to the T7 promoter at *Nde*I and *Bam*HI sites, which were introduced into *E. coli* BL21(DE3) host cells. The complete *sphy* sequence has been deposited in GenBank with the accession number EF536824. Molecular weights of the proteins encoded by the full-length (*pSPHY*) and truncated phytase (*pPHY*), as estimated by SDS–PAGE, were ~43 kDa and ~40 kDa, respectively. Both the expressed proteins were found in the insoluble cytoplasmic fraction as inclusion bodies. The proteins were purified and solubilized in 8.0 M urea. Among the various cosolutes used for refolding, only proline proved effective both in aggregation suppression and in enhancing refolding of the denatured protein. Nonfunctionality of the refolded full-length protein and activity exhibited by the refolded truncated protein indicate the probable interference of the first 27 amino acids of full-length protein in attaining the conformation of active phytase. EDTA inhibited enzyme activity suggesting metal dependency of phytase. A requirement of Ca^{2+} ions was found essential for both refolding and activity of the enzyme. *Bacillus* phytase exhibited the specific activity of 16 U mg^{-1} protein. The K_m value of phytase for the hydrolysis of sodium phytate has been determined as 0.392 mM. *Bacillus* phytase also revealed broad pH and temperature ranges of 5.0–8.0

and 25–70°C, respectively. The pH and temperature optima of phytase activity were 7.0 and 55°C, respectively. The enzyme exhibited ample thermostability, in the presence of calcium ions, upon exposure to high temperatures ranging from 75°C to 95°C, indicating that its heat denaturation is a reversible process. After 9 h of cultivation of transformed *E. coli* in the bioreactor, cell biomass reached 26.81 g wet weight (gww) L^{-1} accounting for 4289 U enzyme activity compared to 1.978 gww L^{-1} producing 256 U activity in shake flask cultures. *In silico* analysis revealed the β-propeller structure of phytase. The site between the 179th amino acid leucine and the 180th amino acid asparagine has been identified as the favorable region that offers scope for insertion of small peptides/domains for the production of chimeric genes without altering enzyme activity (Rao *et al.*, 2008).

Escherichia coli Appa2 phytase, when expressed in *S. cerevisiae*, *Schizosaccharomyces pombe*, and *P. pastoris*, revealed similar molecular mass, glycosylation, pH profile, temperature profile, and phytate hydrolysis efficiency (Lee *et al.*, 2005). The *E. coli* appA phytase expressed in *P. pastoris* showed an increase in the molecular mass by 5–10 kDa in comparison with the unglycosylated native form (Greiner *et al.*, 1993; Rodriguez, Mullaney, and Lei, 2000a; Rodriguez *et al.*, 2000b). Expression of the fungal phytases in the *E. coli* in active form proved futile owing to the absence of glycosylation precluding active conformation of the enzyme (Phillippy and Mullaney, 1997). *E. coli* phytase expressed in transgenic mice and pigs showed higher mass (~55 kDa) compared with the native phytase (45 kDa), resulting from N-glycosylation of the protein in mammalian systems. Furthermore, glycosylated phytase showed altered kinetic properties ($K_m = 0.37$ mM, $k_{cat} = 1401\ s^{-1}$, $k_{cat}/K_m = 3787\ s^{-1}\ mM^{-1}$) when compared to the unglycosylated native enzyme ($K_m = 0.79$ mM, $k_{cat} = 2378\ s^{-1}$, $k_{cat}/K_m = 3010\ s^{-1}\ mM^{-1}$) (Golovan *et al.*, 2001a, 2001b).

Phytases from diverse plants (Table 1), exhibiting pH optima ranging from 3.0 to 6.0, were classified as acidic phytases. Whereas phytases from *Lilium longiflorum* (Scott and Loewus, 1986), Lupin seed (Silva and Trugo, 1996) *Phaseolus vulgaris*, *P. sativum*, and *M. sativa* (Scott, 1991) exhibited a pH optima of 8.0, and were designated as alkaline phytases. Fungal, bacterial, and plant phytases, in general, have acidic pI values with the exception of *Aspergillus fumigatus* phytase with a pI of ~8.6.

Nutritional significance of the phytases led to investigations dealing with their resistance to the action of proteolytic enzymes. Recombinant phytases from *A. niger* and *E. coli* exhibited differential sensitivity to trypsin and pepsin *in vitro*. r-PhyA from *A. niger* proved resistant to trypsin while r-AppA from *E. coli* showed resistance to pepsin (Rodriguez *et al.*, 1999). High stability of the *E. coli* phytase against inactivation caused by pepsin was also confirmed by Golovan *et al.* (2000). *A. niger* phytase, compared to wheat phytase, was found to be more stable in the presence of pepsin or pancreatin (Phillippy, 1999). *Bacillus* phytase was extremely resistant to papain, pancreatin, and trypsin, but was found susceptible to pepsin (Kerovuo *et al.*, 2000a; Kerovuo, Lappalainen, and Reinikainen, 2000b). Gene site saturation mutagenesis of the *E. coli* phytase gene resulted in a mutant version of the enzyme with a 3.5-fold increase in the gastric stability and a better thermal tolerance as compared to the wild-type enzyme (Garrett *et al.*, 2004).

Effectors of phytase activity

Phytases exhibit differences in their requirement of metal ions for enzyme activity. Phytase activities of *Bacillus* sp. (Rao *et al.*, 2008), *Bacillus subtilis* (Shimizu, 1992; Kerovuo *et al.*, 2000b), *Bacillus* sp. DS11 (Kim *et al.*, 1998a), and *Bacillus* sp. KHU-10 (Choi, Suh, and Kim, 2001) were found to be Ca^{2+}-dependent. Metal depletion caused by EDTA in *B. subtilis* phytase resulted in complete enzyme inactivation owing to a conformational change, as evidenced by the differences observed in the circular dichroism spectra of the holozyme versus metal-depleted enzyme (Kerovuo *et al.*, 2000b). An aliquot of 2 mM each of EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+}, and Al^{3+} inhibited the phytase activity of *B. subtilis* natto-77 (Shimizu, 1992). Similarly, the phytase activity of *Bacillus* sp. KHU-10 was inhibited by EDTA and Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Hg^{2+}, and Mn^{2+} ions (Choi *et al.*, 2001). *Enterobacter* sp. 4 phytase was moderately inhibited by Mg^{2+} and Mn^{2+} ions and completely inhibited by Zn^{2+}, Cd^{2+}, Al^{3+}, EDTA at 1 mM (Yoon *et al.*, 1996); while the phytase activity of *Yersinia* was inhibited by Fe^{2+} and Zn^{2+} ions (Huang *et al.*, 2006). The phytase activity of *S. ruminantium* was completely inhibited by Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Hg^{2+} ions, and was significantly reduced by Zn^{2+} ions at 5 mM; conversely, 5 mM of Pb^{2+} ions caused high stimulatory effect on the phytase activity (Yanke, Selinger, and Cheng, 1999). In the presence of Cu^{2+}, Zn^{2+} ions, or EDTA (5 mM), the phytase activity of *Bacillus laevolacticus* was moderately inhibited (Gulati, Chadha, and Saini, 2007). In addition, phytase activities of *Emericella nidulans* and *Aspergillus terreus* CBS were depressed by 1 mM Cu^{2+} ions (Wyss *et al.*, 1999a). Phytase from *A. fumigatus* was inhibited by Cu^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, or Fe^{3+} ions (1 mM), while EDTA (1 mM and 10 mM) stimulated (~50%) enzyme activity (Wyss *et al.*, 1999a).

Likewise, activities of wheat and barley phytases were inhibited by various metal ions, namely, Cu^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Na^{+}, or Li^{+}(1 mM) (Dionisio *et al.*, 2007); while soybean phytase activity was inhibited by Zn^{2+}, Cu^{2+}, and Hg^{2+} ions (Sutardi and Buckle, 1986). Whereas, activity of maize-seedling phytase was stimulated by Ca^{2+} and inhibited by Zn^{2+} and Fe^{2+} ions (Laboure *et al.*, 1993). Mg^{2+} and Ca^{2+} ions had no significant effect on the phytase activity of rye, while Hg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+} could inhibit the enzyme activity (Greiner, Kontietzny, and Jany, 1997). Analogous to *Bacillus* phytases, enzymes from the pollen grains of *L. longiflorum* (Scott and Loewus, 1986) and *Typha latifolia* (Hara *et al.*, 1985) were activated by Ca^{2+} (2 mM) ions.

Substrate specificity of phytases

Phytases with acidic pH optima, in general, are known to show broad substrate specificity. Enzymes from

A. niger (Casey and Walsh, 2003), *A. fumigatus*, *E. nidulans*, *Myceliophthora thermophila* (Wyss *et al.*, 1999a), rye, wheat, spelt, and *Enterobacter* (Herter *et al.*, 2006) were found to show broad substrate specificity for $InsP_6$, glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate, *p*-nitrophenyl phosphate, AMP, ADP, ATP, 1-naphthylphosphate, 2-naphthylphosphate, and phenyl phosphate.

Phytases from *Bacillus* sp., *Bacillus* KHU-10, *Bacillus* DS11, *B. subtilis*, *Klebsiella pneumoniae* sub sp., *Pneumoniae XY*-5, and *E. coli* exhibited high substrate specificity for phytates (Powar and Jagannathan, 1982; Greiner *et al.*, 1993; Jareonkitmongkol *et al.*, 1997; Kim *et al.*, 1998a; Choi *et al.*, 2001; Wang *et al.*, 2004; Rao *et al.*, 2008). Phytases from lily pollen (Baldi *et al.*, 1988), cattail pollen (Hara *et al.*, 1985), and *A. terreus* CBS and *A. terreus* 9A1 (Wyss *et al.*, 1999a) revealed similar substrate specificity for phytates. Using site-directed mutagenesis approach, an increase in the specific activity of *A. terreus* phytase was achieved without affecting the broad substrate specificity and activity over a broad pH range of the enzyme (Tomschy *et al.*, 2000).

Thermostability of phytases

Thermostability of phytases has a crucial role in animal feed applications, where the enzyme is normally incorporated into the grains before pelletization, and the feed briefly reaches processing temperatures of 85–90°C. The requirement of higher enzyme thermostability led to the cloning of phytase genes from thermophilic fungi. Phytase isolated from the thermophilic *Rhizomucor pusillus* showed optimum enzyme activity at 70°C (Chadha *et al.*, 2004). *Thermomyces lanuginosus* phytase retained its activity at 75°C and demonstrated superior catalytic efficiency when compared to the activity of fungal phytases at 65°C (Berka *et al.*, 1998). A heat-stable phytase from *A. fumigatus* could withstand temperatures of up to 100°C for 20 min, with a 10% loss in the enzyme activity (Pasamontes *et al.*, 1997). The thermostability of *A. fumigatus* phytase, *A. niger* phytase, and *A. niger* acid phosphatase was investigated based on circular dichroism, fluorescence studies, and enzyme activity. *A. fumigatus* phytase alone could refold into a fully active native conformation even after heat denaturation at 90°C. In feed pelleting experiments performed at 85°C, the recovery of enzyme activity was significantly higher for *A. fumigatus* phytase (51%) than that of *A. niger* phytase (31%) or *A. niger* acid phosphatase (14%) (Wyss *et al.*, 1998). Fungal phytases from *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia* sp., and *Trametes pubescens* showed high degree of refolding after thermal unfolding, as evidenced by differential scanning calorimetric studies (Lassen *et al.*, 2001). Thermal stability of fungal phytases is often attributed to the high reversible thermal unfolding rather than to their intrinsic thermostability (Wyss *et al.*, 1998).

Aspergillus niger phytase expressed in *P. pastoris*, upon glycosylation, revealed enhanced (60%) thermostability in comparison to the unglycosylated form (Han and Lei, 1999). The higher thermal tolerance disclosed by *A. fumigatus* phytase was attributed to the differences in the post-translational modifications rather than to the primary structure of the enzyme (Mullaney *et al.*, 2000). In *Enterobacter* sp. 4 phytase, a residual activity of 60% at 60°C after 20 h was observed, while a complete loss in activity was recorded at 70–80°C after 20 h (Yoon *et al.*, 1996). A 4.6-fold increase in the activity (at 80°C) of transgenic tobacco phytase, compared to the native *A. ficuum* (AS3.324) phytase, was attributed to the enhanced glycosylation of the enzyme in the heterologous host (Zhang *et al.*, 2004). *A. niger* phytase, expressed in soybean, showed a 20% reduction in enzyme activity (at 63°C), when compared to 60% reduction in the activity of native enzyme. The enhanced thermal stability was attributed to differences in the glycosylation profiles of the protein in plant and fungal systems and/or to the addition of two amino acids at the translational fusion site (Li *et al.*, 1997a).

Phytase (*phyL*) from *Bacillus licheniformis* exhibited higher thermostability (at 95°C for 15 min) with a recovery of 61% of its activity, compared to thermostable phytase of *B. amyloliquefaciens* and phytase of *Bacillus* sp. DS11, which could recover ~50% activity after denaturation at 90°C for 10 min (Kim *et al.*, 1998b; Ha *et al.*, 2000; Tye *et al.*, 2002). The half-life of phytases from *Bacillus* sp. DS11 and *A. ficuum* (at 80°C) were 42.1 min and 0.2 min, respectively, which amply indicate the higher thermostability of the *Bacillus* phytase (Kim *et al.*, 1999). *Bacillus* sp. phytase (*phy*) upon renaturation before denaturation for 10 min at 75°C, 85°C, and 95°C exhibited the restoration of 86%, 54%, and 37% activity, respectively, in the presence of 5 mM $CaCl_2$ (Rao *et al.*, 2008). *B. subtilis* phytase (*phyC*) retained ~90% and ~95% of its activity at 60°C for 10 min in the presence of 5 mM and 10 mM Ca^{2+}, respectively, owing to the strong stabilizing effect of calcium against thermal denaturation of the enzyme (Kerovuo *et al.*, 2000b). Whereas *B. laevolacticus* phytase alone revealed ~80% activity, at 70°C for 3 h, both in the presence and in the absence of 5 mM $CaCl_2$, thus suggesting its calcium-independent nature (Gulati *et al.*, 2007). Employing 13 diverse fungal phytases, a consensus synthetic phytase was developed, which showed substantial increase in its thermostability by 15–26°C compared to the parental phytases used in its design (Lehmann *et al.*, 2000). A chimeric gene, with a secretory signal sequence for 13 amino acids—coding for bifunctional enzyme endowed with endoglucanase and phytase activities—has been constructed and expressed in heterologous host *E. coli* in soluble form. The resultant stable, functional fusion protein of 73 kDa exhibited dual enzyme activities with temperature and pH ranges of 25–75°C and 4–8, respectively (Reddy *et al.*, 2009).

Diverse applications of phytases

Phytases in animal nutrition

Monogastric animals such as swine, fish, and poultry show negligible or no phytase activity in their digestive tracts. Consequently, phytates cannot be metabolized by the animals, thus creating a need to enhance phosphate and

mineral bioavailability via phytase supplementation of animal feed. Of late, phytases are also viewed as environment-friendly products, which can reduce the level of phosphate pollution in intensive livestock management areas by avoiding the addition of exogenous phosphate (Vats et al., 2005). Undigested phytate of monogastric manure is washed off the farmland that imperils adjacent waterways by eutrophication (Common, 1989; Pen *et al.*, 1993; Woodzinski and Ullah, 1996).

During the past two decades, there has been significant increase in the use of phytases as feed additive in pig, poultry, and fish diets (Table 2). In numerous studies, the efficacy of microbial phytases to release phytate-bound P has been demonstrated in various animals (Cromwell *et al.*, 1995b; Ravindran, Bryden, and Kornegay, 1995; Camden *et al.*, 2001; Zyla *et al.*, 2004; Onyango, Bedford, and Adeola, 2005; Fritts and Waldroup, 2006; McClung *et al.*, 2006). Phytases were also found to enhance the utilization of different minerals like Zn^{2+}, Mg^{2+}, Fe^{2+}, and so on (Sebastian, Touchburn, and Chavez, 1998; Mohanna and Nys, 1999; Viveros *et al.*, 2002; Silversides, Scott, and Bedford, 2004). Besides improving mineral availability, microbial phytases were also shown to ameliorate protein digestibility often reduced by phytate (Sebastian *et al.*, 1997; Ravindran *et al.*, 1999; Selle *et al.*, 2000; Cowieson, Acamovic, and Bedford, 2006). Supplementation of poultry diets with phytase showed positive effects on growth performance, bone ash, toe ash, egg production, and egg quality (Gordon and Roland, 1998; Onyango *et al.*, 2005; Pintar *et al.*, 2005; Wu *et al.*, 2006; Ebrahimnezhad *et al.*, 2008).

Phytases from different sources have been evaluated individually and in combination for their efficacy as feed additives in poultry (Wu *et al.*, 2001, 2006; Payne, Lavergne, and Southern, 2005; Elkhalil *et al.*, 2007; Chauynarong *et al.*, 2008; Perić *et al.*, 2008). Bacterial phytases with neutral pH optima are being developed for use as feed additives. Preliminary

Table 2. Use of phytases in animal nutrition.

Phytase	Animal	Diet/feed	Response criteria	Reference
Escherichia coli	Poultry	Maize–soybean	Increased intake of apparent metabolizable energy (AME) and metabolizable N, P, and amino acids	Pirgozliev *et al.* (2008)
E. coli	Pig	Maize–wheat–soybean	Improved growth performance and energy utilization	Olukosi, Sands, and Adeola (2007)
Quantum 2500D	Poultry	Corn–soybean meal	Increased feed intake, weight gain, AME, gross energy metabolizability coefficient, and dry matter digestibility coefficient	Pirgozliev *et al.* (2007)
Natuphos *Aspergillus niger* phytase	Pig	Barley-based diet	Improved P digestibility and utilization and decreased P excretion	Htoo *et al.* (2007)
Phyzyme XP phytase	Pig	Corn–wheat–soy–canola-based diet	Improved P digestibility and P retention	Olukosi *et al.* (2007)
Natuphos	Rat	AIN-93 formulation (low-zinc diet)	Improved zinc status, increased body weight, and bone strength	McClung *et al.* (2006)
Natuphos	Poultry	–	Increased plasma levels of Ca and P and reduced activity of alkaline phosphatase	Tsokova *et al.* (2006)
Microbial phytase	Fish	Plant-based basal diet	Significant increase of bone Na, Ca, K, P, and Fe contents	Baruah *et al.* (2005)
E. coli	Poultry	Corn–soybean meal	Improved growth performance, bone characteristics, and retention of P, Ca, N, and amino acids	Onyango *et al.* (2005)
E. coli	Poultry	Corn–soybean meal	Improved growth performance, bone mineralization, P utilization	Dilger *et al.* (2004)
NovoNordisk	Poultry	Cereal–soybean meal	Increased body weight gain and feed consumption	Pintar *et al.* (2005)
E. coli	Poultry	Wheat–canola–soybean meal	Positive effect on growth, nutrient digestibility, and toe ash	Silversides *et al.* (2004)
Ronozyme	Fish	Soybean meal based diet	Enhanced protein and P utilization; decreased phytic acid in excreta	Vielma *et al.* (2004)
Allzyme	Poultry	wheat–soybean	Improved P utilization, apparent metabolizable energy, and ileal nitrogen digestibility	Wu *et al.* (2001)
Natuphos 500	Poultry	Corn–soybean meal	Improved Ca, P, Mg, Zn retentions, tibia weight, tibia ash, Mg and Zn content in tibia	Viveros *et al.* (2002)
Finnfeed	Poultry	Maize–soybean	Improved ileal digestibility of nitrogen, amino acids, starch, and lipids	Camden *et al.* (2001)
Natuphos and Phytaseed	Poultry	Corn–soybean meal	Increased body weight gain, feed intake, gain:feed, retention of dry matter, P and Ca; decreased P excretion	Zhang *et al.* (2000)
Microbial phytase	Fish	Fish meal–soybean meal	Positive effect on P digestibility and retention	Van Weerd *et al.* (1999)
Recombinant *A. niger* phytase	Pig	Corn–soybean meal	Improved bioavailability of phytate P	Cromwell *et al.* (1995a)
Allzyme	Pig	Corn–soybean meal	Improved bioavailability of P	Cromwell *et al.* (1995b)

studies have proved that they are on par with fungal phytases in their biological activity when supplemented with feed. Use of both bacterial and fungal phytases together as feed additive would be another promising alternative in improving the phosphorus utilization and alleviation of mineral deficiency, owing to their synergistic activities throughout the gastrointestinal tract of the animals (Elkhalil *et al*., 2007).

Experiments evaluating the efficacy of *Bacillus* phytase on 1-day-old chicks over a period of 28 days have been conducted using randomized block design with three replications, each with five birds (Rao, 2008). Four treatments comprising normal diet of adequate non-phytate phosphorus (positive control); deficient diet containing 50% non-phytate phosphorus (negative control); and test treatments of deficient diet supplemented with either 200 U or 400 U phytase per kg feed. Phytase supplementation at 400 U kg^{-1} feed resulted in significant increases in both body weight gain and feed utilization efficiency of birds. Birds fed on deficient diet showed impaired locomotor activity while the birds fed with phytase-supplemented diets showed normal behavior and unimpaired locomotor activity.

Increased levels of ionized calcium in the plasma of birds fed with phosphate-deficient diet depressed the release of parathyroid hormone and reduced tubular reabsorption of phosphate, resulting in the maintenance of ion homeostasis. Also, imbalances occurring in P:Ca in the plasma led to the resorption of calcium from the bone, resulting in fragility and elasticity of bones. Supplementation of phytase to deficient diet increased the levels of plasma phosphorus with a concomitant decrease in plasma calcium. Birds fed with phytase-supplemented diets also showed increased plasma magnesium and zinc levels when compared to the birds fed on deficient diet. Increase in mineral nutrient uptake and assimilation was attributed to the hydrolysis of phytate by the recombinant phytase and subsequent release of minerals chelated to phytate.

Supplementation of *Bacillus* phytase to deficient diet increased tibia ash content, indicating improved bone mineralization due to enhanced bioavailability of phosphorus and calcium. Phosphorus content of tibia ash was comparable in birds fed with normal diet (13.51%) followed by birds fed with 400 U (13.33%) and 200 U (13.01%) phytase-supplemented diets, while it was reduced (12.63%) in birds fed on deficient diet. Increased calcium content in tibia ash was observed in birds fed with 400 U (32.60%), 200 U (30.95%) phytase-supplemented diets and normal diet (30.74%) compared with birds fed with deficient diet (28.32%).

No significant difference was observed between the phytate phosphorus content in the excreta of birds fed with either normal diet or deficient diet. However, phytase supplementation at 200 U kg^{-1} and 400 U kg^{-1} reduced phytate phosphorus excretion by 44.30% and 47.50%, respectively, compared to that of normal diet. Non-phytate phosphorus in excreta was reduced by 16.27%, 25.30%, and 30.12% in birds fed with deficient diet, 200 U, and 400 U phytase-supplemented diets, respectively, compared to birds fed on normal diet. These observations amply suggest that exogenous addition of recombinant phytase resulted in the hydrolysis of phytate and subsequent utilization of the released inorganic phosphorus. Histopathological analysis of liver, spleen, and kidney showed no disease lesions and no differences were observed among various treatments. Thus, the recombinant *Bacillus* phytase proved to be nontoxic and can serve as a safe feed additive (Rao, 2008).

Broilers fed with low-phytate corn grains showed increased available P, enhanced growth performance, and decreased P excretion into the environment (Jang *et al*., 2003). Transgenic soybean with *A. niger* phytase, when fed to broilers, exhibited enhanced phytate phosphorus utilization as evidenced by increased body weight gain, toe ash, feed efficiency, and so on (Denbow *et al*., 1998).

Phytases in human nutrition

Mineral deficiency of diets, caused by radical changes in food habits, is a major concern for developing countries. Phytate present in cereal-based and legume-based complementary foods has been found to inhibit mineral absorption (Hurrell *et al*., 2003). The human small intestine has limited ability to digest undegraded phytates, resulting in adverse nutritional consequences with respect to metabolic cation imbalances (Iqbal *et al*., 1994). Phytic acid (PA)—containing 12 dissociable protons with pK_a values ranging from ~1.5 to 10—is a highly reactive and potent chelator of many mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}. PA forms insoluble salts, at normal acidity (pH 3.0–6.8), in the human digestive tract, thereby reducing the bioavailability of these critical mineral nutrients for absorption (Costello, Glonek, and Myers, 1976; Harland and Oberleas, 1999). Mucosal phytase and alkaline phosphatases, even if present in the human small intestine, do not seem to play a significant role in the phytate digestion, while dietary phytase serves as an important factor in phytate hydrolysis (Sandberg and Anderson, 1988).

Use of phytases for alleviation of mineral deficiency caused by phytate has been extensively studied. The addition of *A. niger* phytase to the phytate-containing meal before consumption was found to promote marked increase (14–26%) in iron absorption (Sandberg, Hulthen, and Turk, 1996). Low Zn^{2+} uptake from soy formula was improved by reducing the phytate level in the diets of infant rhesus monkeys and suckling rat pups, caused by decreased chelation of zinc by phytic acid (Lonnerdal *et al*., 1988). Phytate was degraded through food processing techniques, such as soaking, malting, and fermentation, by enhancing natural phytase activity or by phytase pretreatment of legume and cereal grains (Greiner and Konietzny, 1998, 1999, 2006). Release of phosphorus was significantly higher in malted oats compared to untreated oats, owing to reduction in the phytate levels (Larsson and Sandberg, 1995). Endogenous phytase of wheat bran, rather than the intestinal phytase, was found to play a key role in phytate degradation in the stomach and small intestine of humans (Sandberg and Anderson, 1988). In addition, micronutrient malnutrition could be reduced through introduction of phytase genes into rice and wheat grains, the principal staple food

of many populations in the world. Undersimulated acidic conditions of stomach, the transgenic rice grains, expressing *A. niger* phytase, retained the same enzymatic activity as before acidic treatment (Lucca, Hurrell, and Potrykus, 2001). However, further research needs to be envisaged for alleviation of mineral deficiency in humans by consumption of cereal grains expressing phytase genes.

Furthermore, *A. niger* phytase was found to serve as a potential bread-making additive (Haros, Rosell, and Benedito, 2001). Phytase used in various bread formulations containing fiber leads to accelerated bread proofing, improved crumb texture and shape besides increased specific volume. Addition of phytase reduced the phytate content in dough and fresh bread besides reducing the fermentation time without affecting the dough pH. In whole wheat bread-making process, both *lactobacilli* and bifidobacterium strains, used as starters, disclosed high phytate degrading activity (Palacios *et al.*, 2005, 2006, 2008a, 2008b).

Phytases in aquaculture

A major concern in aquaculture is the utilization of dietary phosphates which critically affects fish growth as well as the aquatic environment. An efficient utilization of feed leading to optimum fish growth serves as a benchmark of successful aquaculture worldwide. High concentration of dietary phytic acid decreased the growth of Chinook salmon, food and protein efficiency and thyroid function, increased mortality, promoted cataract formation, and induced anomalies in pyloric cecal structure (Richardson *et al.*, 1985). In trials conducted on African catfish (*Clarias gariepinus*), phytase supplementation revealed positive effects, particularly on phosphorus digestibility, retention, and phosphorus conversion efficiency (Van Weerd *et al.*, 1999). Atlantic salmon (*Salmo salar*) fed with phytase-supplemented meal exhibited enhanced growth and neutralized the negative effect of phytate on protein digestibility (Sajjadi and Carter, 2004). Addition of phytase to plant-based diets of *Labeo rohita* juveniles enhanced the bioavailability of minerals thereby leading to increased bone mineralization (Baruah *et al.*, 2005). The positive effects of phytase were further increased by adding 3% citric acid to the feed. *Pangasius pangasius* fingerlings, fed with diets containing 35% crude protein, added with phytase, disclosed improved growth, dry matter, and crude protein digestibility (Debnath *et al.*, 2005). Studies using phytase as feed additive in aquaculture amply establish that phytase supplementation could enhance the bioavailability of P, nitrogen, and other minerals, thereby decreasing phosphorus-load in the aquatic environment (Vielma *et al.*, 2004; Baruah *et al.*, 2007; Nwanna and Schwarz, 2007).

Transgenic plants expressing microbial phytases

Transgenic plants can serve as bioreactors for the production of phytases, and for harnessing the solar energy into metabolic energy. Expression of phytases in plants has enormous potential in improving plant phosphorus acquisition and phytoremediation. Transgenics expressing phytase genes can be directly used as animal feed, thereby reducing the downstream processing and formulation costs involved in the commercial production of phytases. Production of transgenics with substrate-specific phytases would be ideal, since the introduction of phytases with broad substrate specificity might disturb the metabolic pathways in plants, leading to decreased yields. Phytases have been expressed in several dicotyledonous plants like tobacco (Pen *et al.*, 1993), *A. thaliana* (Richardson, Hadobas, and Hayes, 2001a; Xiao, Harrison, and Wang, 2005), alfalfa (Ullah *et al.*, 2002), canola (Ponstein *et al.*, 2002), soybean (Li *et al.*, 1997a; Chiera, Finer, and Grabau, 2004), and so on. Stable transgenic rice and wheat plants, expressing fungal phytases, exhibited enhanced bioavailability of phosphate and iron (Lucca *et al.*, 2001; Brinch-Pedersen *et al.*, 2003; Hong *et al.*, 2004). Transgenic rice grains expressing *A. fumigatus* phytase, when boiled for 20 min, retained only 8% of the phytase activity, while rice flour added with *A. fumigatus* phytase, when incubated under same conditions, retained 59% of the enzyme activity (Lucca *et al.*, 2001). *A. fumigatus* phytase expressed in tobacco exhibited high thermostability and retained 28.7% of the initial activity even after incubation at 90°C for 15 min (Wang *et al.*, 2007). *Schwanniomyces occidentalis* phytase expressed in rice exhibited increased thermal stability as compared to the native yeast phytase. After preincubation of the crude extract from transgenic rice leaves at 66°C, 70°C, and 73°C for 15 min, 98%, 96%, and 86% of the activity remained for the heterologous phytase, whereas 91%, 84%, and 71% of the activity remained for the yeast phytase (Hamada *et al.*, 2005). Two *Aspergillus* phytase genes have been expressed independently in potato leaves (Ullah *et al.*, 2003) and tobacco seeds and leaves (Pen *et al.*, 1993; Verwoerd *et al.*, 1995), which could serve as feed additives. In wheat seeds, recombinant *A. niger* phytase along with the native phytase revealed concerted action on phytic acid degradation (Brinch-Pedersen *et al.*, 2003). Supplementation of broiler diets with transgenic tobacco (Pen *et al.*, 1993) and rice seeds (Hong *et al.*, 2004) resulted in an improved growth rate comparable to the diets added with fungal phytase or phosphorus alone. *Aspergillus* phytase expressed in maize seeds exhibited an increase in iron bioavailability, as evidenced by *in vitro* digestion and Caco-2 cell model studies (Drakakaki *et al.*, 2005). Compared to untransformed rice grains, two transgenic lines expressing *A. fumigatus* phytase gene produced seeds with doubled phytase activity, whereas a third line exhibited ~130-fold increase (from 72 to 9415 phytase units per gram of rice) in the phytase activity (Lucca *et al.*, 2001). Transgenic tobacco, expressing *B. subtilis* phytase gene, disclosed increased plant growth when compared with the untransformed control plants (Yip *et al.*, 2003). Expression of heterologous phytases in transgenic *Trifolium subterraneum* and *Nicotiana tabacum* was shown to improve the acquisition of organic phosphorus from the rhizosphere (George *et al.*, 2004, 2005a, 2005b; Xiao *et al.*, 2005). Thermotolerance of *in planta* synthesized heterologous phytase from *A. fumigatus* and an engineered consensus phytase expressed in transgenic wheat showed

low denaturation temperature (62.5°C) but a high refolding capacity. High levels of endosperm-specific expression were ensured by the wheat high molecular weight glutenin 1DX5 promoter. Immunodetection using light and electron microscopes showed that the heterologous phytase was deposited in the vacuole. Evaluation of heat stability properties and kinetic properties unraveled that, under these deposition conditions, heat stability based on high unfolding temperature is superior to high refolding capacity. The thermostable phytase expressed in transgenic wheat proved effective for enhanced phosphate and mineral bioavailability in cereal-based feed and food (Brinch-Pedersen *et al.*, 2006). Expression levels of heterologous phytase in diverse transgenic plants are presented in Table 3.

An alternative approach to address nutritional and environmental problems entails reducing phytic acid levels in cereal grains and oilseeds by the production of low-phytate crops (Shi *et al.*, 2007). However, the major concerns encountered in the development of low-phytate maize and soybean crops have been drastic reduction in grain yield, reduced seed dry weight, retarded vegetative growth, poor germination, and seedling establishment under field conditions (Meis, Fehr, and Schnebly, 2003; Bregitzer and Raboy, 2006).

Transgenic animals expressing phytases

Transgenic animals producing endogenous phytase along with other digestive enzymes are known to increase the assimilation of phosphorus and other mineral nutrients, thereby checking environmental pollution by reducing phosphate output in the manure. A transgenic mouse model has been developed with *appA* phytase gene from *E. coli*, driven by the upstream promoter of pig parotid secretory protein gene. Expression of the salivary phytase in transgenic mouse resulted in the reduction of fecal phytate by 8.5–12.5% (Yin *et al.*, 2006). The transgenic Enviropig, expressing *E. coli appA* phytase, could secrete active phytase into saliva, and showed substantial reduction (60%) in the excretion of phosphorus as compared to the nontransgenic animals (Forsberg *et al.*, 2003). Mice expressing *appA* phytase secreted active phytase into saliva, and compared to the normal mice showed 11% reduction in the fecal phosphorus (Golovan *et al.*, 2001a). Transgenic pigs, expressing bacterial phytase in salivary glands, exhibited normal growth on feed devoid of inorganic phosphorus, and showed marked reduction (75%) in the excretion of phosphorus as compared to the nontransgenic animals (Golovan *et al.*, 2001b). These findings amply indicate that the expression of phytase in monogastric animals obviates the need for phosphate supplementation of animal diets, and mitigate phosphorus pollution resulting from animal agriculture.

Table 3. Expression of phytase in transgenic plants.

Transgenic plant	Source of phytase	Tissue expressed	Enzyme activity	Reference
Tobacco	*Aspergillus niger*	Seeds	15 FTU g^{-1}	Pen *et al.* (1993)
Tobacco	*A. niger*	Leaves	2400 ng mg^{-1} DW^{-1}	Verwoerd *et al.* (1995)
Soybean	*A. niger*	Cell suspension culture	920 pKat μg^{-1} protein	Li *et al.* (1997a)
Alfalfa	*A. ficuum*	Leaves	389.3 nKat g^{-1} FW^{-1}	Ullah *et al.* (2002)
Potato	*A. ficuum*	Leaves	29.79 nKat mg^{-1} protein	Ullah *et al.* (2003)
Wheat	*Aspergillus*	Seeds	1353 FTU kg^{-1}	Brinch-Pedersen *et al.* (2003)
Tobacco	*A. ficuum*	Plant	17.6% of total protein	Zhang *et al.* (2004)
Trifolium subterraneum L.	*A. niger*	Shoots	30.5 nKat g^{-1} FW^{-1}	George *et al.* (2004)
Rice	Yeast	Leaves	10.6 U g^{-1} FW^{-1}	Hamada *et al.* (2005)
Arabidopsis	*Medicago truncatula*	Roots	>150 mU mg^{-1} protein	Xiao *et al.* (2005)
Canola	*Aspergillus*	Seeds	41 FTU g^{-1}	Peng *et al.* (2006)

Role of phytases in soil amendment

Phosphorus is an essential plant nutrient that limits agricultural production on a global scale. Approximately 30–80% of the total P in soils is bound in organic form (Harrison, 1987). Phytate constitutes ~50% of the total organic P pool in the soil and is poorly utilized by plants (Anderson, 1980). Extracellular phytase activities have been reported under phosphate stress conditions, in diverse plant species, namely, tobacco (Lung and Lim, 2006), barley (Asmar, 1997), tomato, alfalfa (Li *et al.*, 1997c), and so on. The ability of plants to use phosphorus from low phosphate or phytate containing media and/or from soil is improved when soil/media are inoculated with microorganisms that possess the ability to exude phytase, or when a purified phytase is added (Hayes, Simpson, and Richardson, 2000; Richardson, Hadobas, and Hayes, 2000; Richardson *et al.*, 2001b). Under limited phosphorus conditions, as compared to the control plants sans bacterial inoculums in the soil, plants inoculated with *B. amyloliquefaciens* FZB45 with extracellular phytase activity revealed significant growth stimulation (Idriss *et al.*, 2002). Engineering the trichoblasts of the root–soil interface with a synthetic phytase gene, and secretion of the enzyme in adequate amounts released phosphate from the phytate present in the soil (Zimmermann *et al.*, 2003).

Phytases for the production of lower myo-inositol phosphates

Saccharomyces cerevisiae phytase was used extensively in the preparation of medicinally important compounds, namely, D-*myo*-inositol-1,2,6-triphosphate, D-*myo*-inositol-1,2,5-triphosphate, L-*myo*-inositol-1,3,4-triphosphate, and *myo*-inositol-1,2,3-triphosphate (Siren *et al.*, 1986a, 1986b). Lower phosphorylated derivatives of phytate were found to play an important role as intracellular secondary messengers (Berridge and Irvine 1984; Dasgupta *et al.*, 1996). Different isomers of *myo*-inositol phosphates have shown pharmacological effects for the prevention of diabetic complications, anti-inflammatory effects (Claxon *et al.*, 1990; Carrington *et al.*, 1993), and antiangiogenic and antitumor effects (Maffucci *et al.*, 2005).

Myo-inositol phosphates are also known to ameliorate heart disease conditions by controlling hypercholesterolemia and atherosclerosis (Jariwalla *et al.*, 1990), and also prevent renal stone formation (Grases *et al.*, 2000).

Conclusions and perspectives

Use of phytases in animal nutrition has been increasing worldwide over the years. The expanding role of phytase, not only as a feed additive but also as a nutraceutical, has led to significant progress in this area. The growing demand for phytase is amply reflected by the multitude of sources screened for phytases with desired attributes. Identification of various phytases from diverse sources and their expression in heterologous systems need to be worked out not only to enhance the enzyme production but also to decrease the cost of production. Physicochemical properties of phytases, namely, broad pH range to survive under varied pH conditions in animal digestive tract, resistance to proteolytic degradation, thermal stability to resist higher temperatures during feed pelleting and substrate specificity, and so on need thorough evaluations to design versatile "second-generation" phytases with wider applicability. Modification and upgradation of enzymatic properties can be achieved through adoption of genetic and protein engineering methods. Combination of fungal and bacterial phytases as feed additives might improve the bioavailability of phosphorus and minerals owing to their synergistic activity in animal digestive system.

Development of transgenic plants expressing phytase and production of low-phytate crops to avoid the expensive and time-consuming downstream processing is garnering increased acceptance. Expression of phytases in transgenic crops not only obviates the problem of mineral malnutrition, phosphate uptake, and assimilation in animals and humans, but also mitigates the environmental pollution. Transgenic animals with tissue-specific expression of phytase have been developed which need further investigation and evaluation. Production of functional foods and food supplements with health benefits, especially for women and children for alleviation of mineral deficiency, is another aspect wherein phytases can find applications. Manipulation of phytate-hydrolysis products, to produce desired specific isomers of lower *myo*-inositol phosphates, has enormous potential for use in transmembrane signaling processes, and for calcium mobilization from the intracellular store of animal and plant tissues. Further intensive investigations, using diverse phytases, need to be undertaken for designing and producing pharmacologically important lower *myo*-inositol phosphates.

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