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Standard Review

Developments in biochemical aspects and biotechnological applications of microbial phytases

Bijender Singh[1]*, Gotthard Kunze[3] and T. Satyanarayana[2]

[1]Department of Microbiology, Maharshi Dayanand University, Rohtak- 124 001, India.
[2]Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110 021, India.
[3]Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Corrensstr. 3, D-06466 Gatersleben, Germany.

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Phytases belong to the class of phosphatases, which catalyze the hydrolysis of phytic acid to inorganic phosphate and *myo*-inositol phosphate derivatives. The enzyme has potential applications in food and feed industries for ameliorating digestibility and assimilation of nutrients of foods and feeds by mitigating the anti-nutritional effects of phytic acid. Phytases have been shown to be useful in improving growth of poultry, pigs and fishes, and they play a role in promoting growth of plants, as well as improve the nutritional quality of bread, soymilk and oil seed cakes by dephytinization. The crystal structures of some phytases have been analyzed for understanding the reaction mechanism. The phytases with desirable properties have been generated through protein engineering approaches, since native phytases do not possess all the properties of an ideal additive feed/food. Recent developments on the characteristics of an ideal phytase, crystal structure, protein engineering, and the potential biotechnological applications of microbial phytases with special reference to their utility in improving growth performance of monogastrics, dephytinization of foods and feeds, plant growth promotion, and combating environmental phosphorus pollution will be discussed in this review.

Key words: Phytic acid, microbial phytase, crystal structure, monogastrics, protein engineering, dephytinization, plant growth promotion.

INTRODUCTION

The hydrolysis of phytic acid to *myo*-inositol and inorganic phosphate by phytases (*myo*-inositol hexakisphosphate phosphohydrolase) is an important reaction for energy metabolism, metabolic regulation and signal transduction pathways in biological systems. Although phytic acid (*myo*-inositolhexakis phosphate), which is an organic form of phosphorus (P), is abundantly present in plants' materials (1 to 5% by weight) such as edible legumes, cereals, oilseeds, pollen and nuts, it is largely unavailable to monogastrics like poultry birds, pigs, fishes and humans, due to the lack of adequate levels of phytases (Wodzinski and Ullah, 1996; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Greiner and Konietzny, 2006; Rao et al., 2009). The phytic acid present in the plant derived foods acts as an anti-nutritional factor, since it causes mineral deficiency due to efficient chelation of metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}, which form complexes with proteins, and thus affect their digestion and also inhibit certain digestive enzymes like α-amylase, trypsin, acid phosphatase and tyrosinase (Harland and Morris, 1995). Due to the lack of adequate levels of phytases in monogastric animals, phytic acid is excreted in faeces, which on degradation by soil microorganisms, release phosphorus in the soil. The phosphorus reaches aquatic bodies that cause eutrophication (Mullaney et al., 2000). Phytic acid can be removed by some physical (autoclaving, cooking and steeping) and chemical (ion exchange and acid hydrolysis) methods, but these methods decrease the nutritional value of foods. The reduction of phytic acid content in foods and feeds by enzymatic hydrolysis using phytase is desirable since it improves their nutritional value. Besides its immense

*Corresponding author. E-mail: ohlanbs@gmail.com. Fax: 091-11-24115270.

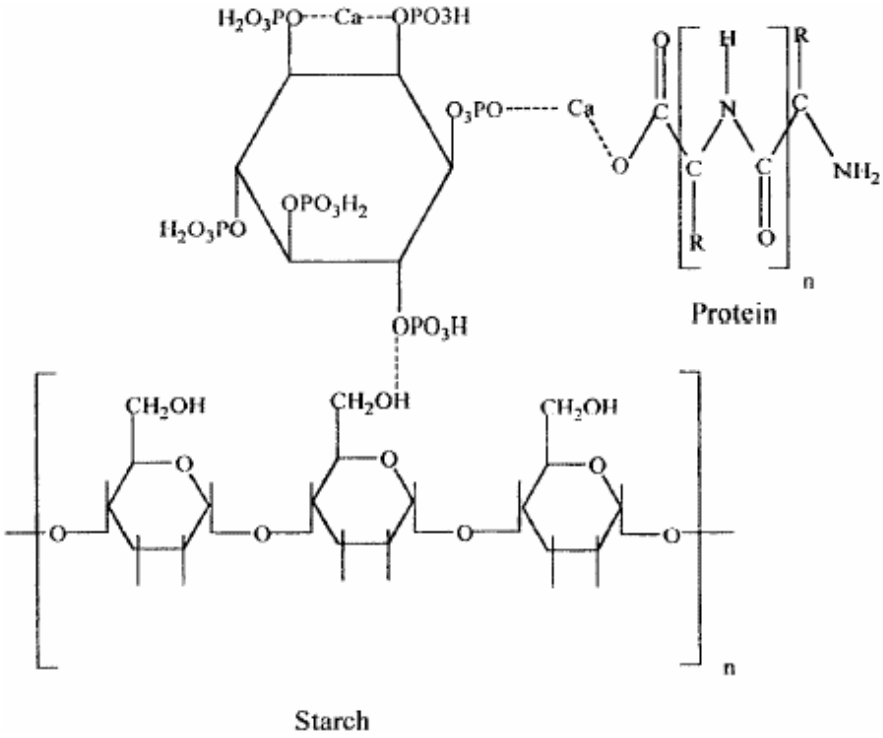

Figure 1. Interaction of phytic acid with metals, proteins and carbohydrate.

commercial value in food and feed industries, the enzyme has potential applications in other fields too. The annual sale of commercial supplemental phytase is estimated at US$ 50 million, which is one-third of the entire feed enzyme market (Sheppy, 2001) and recently increased to 150 million euro (Greiner and Konietzny, 2006). The term phytase has been used in this article to mean microbial phytase.

During the last 15 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology. Undoubtedly, increasing public concern regarding the environmental impact of high phosphorus levels in animal excreta has driven the biotechnological development of phytase and its application in animal nutrition. The feeding trials have shown the effectiveness of supplemental microbial phytases in improving utilization of phytate-P and the phytate-bound minerals by swine, poultry and fishes (Lei and Stahl, 2001; Singh et al., 2006; Cao et al., 2007; Selle and Ravindran, 2007, 2008; Rao et al., 2009). Inorganic P supplementation of the diets for swine and poultry can be obviated by including adequate amounts of phytase along with an appropriate manipulation of other dietary factors (Han et al., 1997). As a result, the P excretion of these animals may be reduced by about 50% (Lei et al., 1993a, b; Satyanarayana and Vohra, 2003; Vohra et al., 2006). The cost and thermotolerance constraints of the current commercial phytases have, however, precluded the widespread use of these enzymes in animal feeds.

Several reviews have been published recently on the phytases, which mainly focused on the production, characteristics and their basic applications (Pandey et al., 2001; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Greiner and Konietzny, 2006; Kaur et al., 2007; Fu et al., 2008; Rao et al., 2009). None of these dealt with the ideal and designer phytase, crystal structure and the directed evolution and protein engineering of phytases; and therefore, a comprehensive account is given in this review on the recent developments on all these aspects of microbial phytases and their potential biotechnological applications.

PHYTIC ACID: A FRIEND OR A FOE

Phytic acid is the major storage form of phosphorus in cereals, legumes and oilseeds (Maga, 1982; Tyagi et al., 1998). It has several physiological roles and also affects the functional and nutritional properties of food ingredients. The correct chemical description of phytic acid is *myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate (IUPAC-IUB, 1977). Phytic acid occurs primarily as salts of mono- and divalent cations (for example, potassium-magnesium salt in rice and calcium-magnesium-potassium salt in soybeans) in discrete regions of cereal grains and legumes (Figure 1). It

accumulates in seeds and grains during ripening along with other storage substances such as starch and lipids. In cereals and legumes, phytic acid accumulates in the aleurone particles and globoid crystals, respectively (Reddy et al., 1982; Tyagi et al., 1998).

Besides phosphate storage, phytate acts as a strong chelator for divalent metal cations and exists as a stable metal-phytate complex with metal ions in plants (Asada et al., 1969; Reddy et al., 1982). Phytic acid in seeds and grains serves as a phosphorus store, an energy store, a source of cations, a source of *myo*-inositol, and also helps in initiating dormancy. Phytic acid may also serve several other unknown functions in seeds (Reddy et al., 1982). Graf et al. (1987) suggested that the role of phytic acid in seeds is a natural antioxidant during dormancy. Phytic acid has been shown to exert an antineoplastic effect in animal models of both colon and breast carcinomas. The presence of undigested phytic acid in the colon may protect against the development of colonic carcinoma (Iqbal et al., 1994). The inositol phosphate intermediates play an important role in the transport of materials into the cell, and the role of inositol triphosphates, especially in signal transduction and regulation of cell functions in plant and animal cells, is a very active area of research in order to understand signaling pathways (Wodzinski and Ullah, 1996; Vohra and Satyanarayana, 2003; Greiner and Konietzny, 2006; Rao et al., 2009). Besides these functions, phytic acid also acts as an anti-nutritional factor in several ways due to the interactions with metal ions, proteins and enzymes.

PHYTASES

Phytases *myo*-inositolhexaphosphate phosphohydrolase) hydrolyze phytic acid to *myo*-inositol and inorganic phosphates through a series of *myo*-inositol phosphate intermediates, and eliminate its anti-nutritional characteristics. Phytase is widespread in nature, and it occurs in microorganisms, plants and some animals (Wodzinski and Ullah, 1996; Vohra and Satyanarayana, 2003; Angelis et al., 2003; Vats and Banerjee, 2004; Kaur et al., 2007; Fu et al., 2008; Rao et al., 2009; Raghavendra and Halami, 2009). A large number of bacteria, filamentous fungi and yeasts have been reported to produce phytase extra- and intra-cellularly as well as in the cell-bound form (Shieh and Ware, 1968; Wodzinski and Ullah, 1996; Pandey et al., 2001; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Kaur et al., 2007; Fu et al., 2008; Rao et al., 2009). A list of phytase producing organisms is given in Table 1. There are two types of phytases as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN): 3-phytase (EC 3.1.3.8) that first hydrolyses the ester bond at the 3 position of *myo*-inositol hexakisphosphate, and is mainly reported in microorganisms; and the 6-phytase (EC 3.1.3.26) that first hydrolyses the ester bond at the 6 position of *myo*-inositol hexakisphosphate, and is mostly reported in plants. This had also been reported in some basidiomycetous fungi (Lassen et al., 2001). An alkaline 5-phytase from lily pollen was found to start phytate hydrolysis at the D-5 position (Barrientos et al., 1994). Phytases can be broadly categorized into two major classes based on the pH for activity: acid phytases and alkaline phytases (Figure 2). More focus has been on acidic phytases because of their applicability in animal feeds and broader substrate specificity than those of alkaline phytases. Recently, phytases have also been classified as HAP (Histidine acid phosphatase), BPP (β-Propeller phytase), CP (cysteine phosphatase) and PAP (purple acid phosphatase) based on their catalytic properties (Mullaney and Ullah, 2003).

AN IDEAL PHYTASE AND ITS DESIGNING

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

Phytase should not be detected at the end of the small intestine. This is necessary because in this way the phytase produced by genetically modified organisms should not enter the environment (Jongbloed et al., 1992). Furthermore, it should be effective in releasing phytate-P in the digestive tract and stable to resist proteases (trypsin and pepsin) and inactivation by heat during feed pelleting and storage with low cost of production. The ability of any given phytase to hydrolyze phytate-P in the digestive tract is determined by its properties, such as catalytic efficiency, substrate specificity, temperature and pH optima, which are resistance to proteases. As the stomach is the main functional site of supplemental phytase, a phytase with pH optimum in the acidic range is desirable for improving nutrition. Also, phytase must exhibit resistance to pepsin and trypsin, which are encountered in the intestine. Since the food and feeds are often processed through a pelleting machine at 65 to 80°C with steam to eliminate salmonellae, an ideal phytase must be able to withstand the high temperature and steam encountered during the pelleting process. Similarly, an enzyme that can tolerate long-term storage or transport at ambient temperature is generally preferred for food and feed industry. Finally, a phytase produced in high yield and purity by a relatively inexpensive system is attracting for food industries worldwide. It is now realized that any single phytase may never be 'ideal' for all feeds and foods. For example, the stomach pH in finishing pigs is much more acidic than

Table 1. Optimized culture conditions for the production of phytase by various microorganisms.

	Culture conditions					
Microbial strain	**pH_{opt}**	**T_{opt}**	**Fermentation**	**Carbon source**	**Nitrogen source**	**Reference**
Filamentous fungi						
A. fumigatus SRRC 322	5.0	37	SmF*	Hylon starch	$NaNO_3$	Mullaney et al. (2000)
A. niger	5.5	30	SmF	Glucose starch	--	Vats and Banerjee (2005)
A. ficuum	5.0	30	SmF	Corn starch, glucose	$NaNO_3$	Shieh and Ware (1968)
A. oryzae	6.4	37	SmF	Glucose	$(NH_4)_2SO_4$	Shimizu (1993)
Rhizopus oligosporus	5.5	27	SmF	Corn starch, glucose	$NaNO_3$	Casey and Walsh (2004)
R. oryzae	5.5	30	SSF#	Glucose	NH_4NO_3	Ramachandaran et al. (2005)
Mucor racemosus	5.5	30	SSF	Starch	$NaNO_3$	Roopesh et al. (2005)
Peniophora lycii	5.5	26	SmF	Maltodextrin, soya flour	Peptone	Lassen et al. (2001)
Thermoascus aurantiacus	5.5	45	SmF	Starch, glucose, wheat bran	Peptone	Nampoothiri et al. (2004)
Rhizomucor pusillus	8.0	50	SSF	Wheat bran	Asparagine	Chadha et al. (2004)
Myceliopthora thermophila	5.5	45	SmF	Glucose	$NaNO_3$	Mitchell et al. (1997)
Sporotrichum thermophile	5.0	45	SmF	Starch, glucose	Peptone	Singh and Satyanarayana (2008a)
S. thermophile	5.0	45	SSF	Sesame oil cake, glucose	$(NH_4)_2SO_4$	Singh and Satyanarayana (2006a)
Yeasts						
Pichia anomala	6.0	25	SmF	Glucose	Beef extract	Vohra and Satyanarayana (2001)
Schwanniomyces castellii	4.4	77	SmF	Galactose	$(NH_4)_2SO_4$	Segueilha et al. (1992)
Arxula adeninivorans	5.5	28	SmF	Galactose	Yeast extract	Sano et al. (1999)
P. rhodanensis	4.5	70	SmF	Glucose	-	Nakamura et al. (2000)
P. spartinae	4.5	75	SmF	Glucose	-	Nakamura et al. (2000)
Candida krusei	4.6	40	SmF	Glucose	Polypeptone	Quan et al. (2001)
Bacteria						
B. subtilis	7.0	37	SmF	Glucose	NH_4NO_3	Kerovuo et al. (1998)
B. amyloliquefaciens	6.8	37	SmF	Glucose	Casein, peptone	Idriss et al. (2002)
Escherichia coli	7.0	37	SmF	--	Tryptone	Sunita et al. (2000)
Klebsiella aerogenes	7.0	30	SmF	Sodium phytate	Yeast extract	Tambe et al. (1994)
*Lactobacillus sanfranciscensis**	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
*L. fructivorans**	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
L. lactis subsp *lactis**	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
*L. rhamnosus**	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)
*L. amylovorus**	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)
*Pediococcus pentosaceus**	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)

*SmF = Submerged fermentation, #SSF = Solid state fermentation.

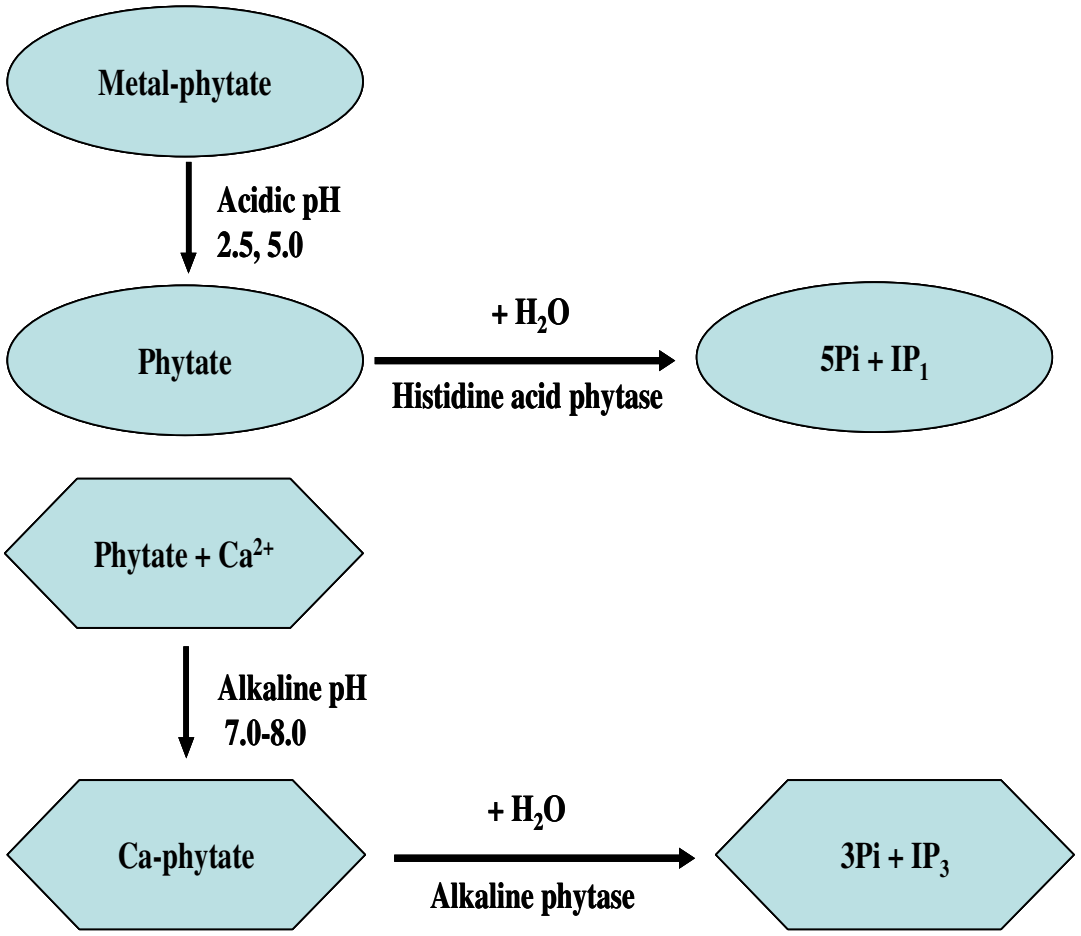

Figure 2. Schematic representation of the hydrolysis of substrate by histidine acid phytase and β-propeller phytase.

that of weanling pigs (Radcliffe et al., 1998). Thus, phytase with optimum pH close to 3.0 will perform better in the former than in the latter. For poultry, an enzyme would be beneficial if it is active over broad pH range, that is, acidic (stomach) to neutral pH (crop) (Riley and Austic, 1984). Phytases used for aquaculture application require a lower temperature that is optimum than the swine or poultry (Ramseyer et al., 1999). The choice of an organism for phytase production is, therefore, dependent upon the target application. Nowadays, there is a great demand for the development of an ideal phytase using directed evolution and protein engineering.

Based on this, the desirable and ideal phytase could be designed as per target application. All these features are not present within a single phytase, and therefore, based on the sequence of the available phytases, a consensus phytase could be designed (Lehman et al., 2000a, b, c). Genetic engineering techniques such as site directed mutagenesis could be employed for further ameliorating the properties. The strategies used for the designing and developing of an ideal phytase are presented in Figure 3.

BIOCHEMICAL AND MOLECULAR CHARACTERISTICS OF PHYTASES

The major properties of enzymes are useful in determining their potential in different industries. The biochemical and molecular properties of some phytases are presented in Table 2.

Phytases with high temperature optima are desirable in the animal feed industry because feed pelleting involves a step of 80 to 85°C for few seconds (Wyss et al., 1999a). Phytase of *A. fumigatus* (Pasamontes et al., 1997b) and *A. niger* NRRL 3135 (Howson and Davis, 1983) exhibited optimum activity at 37°C and at 55°C, respectively. Phytase of *S. castellii* was optimally active at 77°C (Segueilha et al., 1992) and that of *Arxula adeninivorans* at 75°C (Sano et al., 1999). The phytases from *Pichia rhodanensis* and *P. spartinae* showed optimal reaction temperature at 70 to 75°C and 75 to 80°C, respectively (Nakamura et al., 2000), while that of *Pichia anomala* showed optimal activity at 60°C (Vohra and Satyanarayana, 2002). Among the thermophilic fungi,

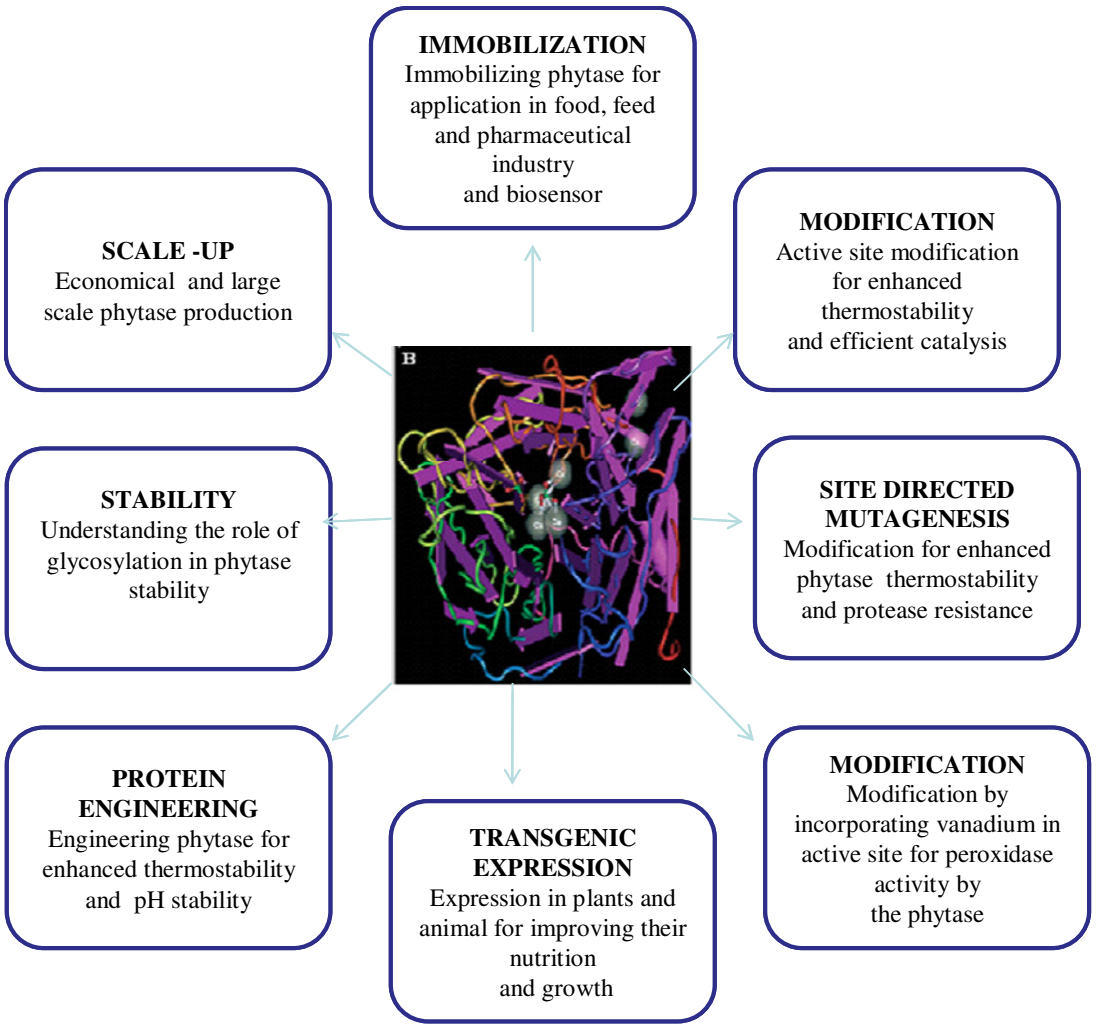

Figure 3. Designing an ideal phytase for biotechnological applications.

Thermomyces lanuginosus phytase exhibited optimum activity at 65°C (Berka et al., 1998), and that of *Rhizomucor pusillus* at 70°C (Chadha et al., 2004). Phytases of *Thermoascus aurantiacus* (Nampoothiri et al., 2004) and *S. thermophile* (Singh and Satyanarayana, 2009) were optimally active at 55°C and 60°C, respectively (Table 3). Phytase from *B. subtilis* (Powar and Jagannathan, 1982), *E. coli* (Greiner et al., 1993), *Klebsiella aerogenes* (Tambe et al., 1994), *Enterobacter* sp.4 (Yoon et al., 1996), *K. oxytoca* MO-3 (Jareonkitmongkol et al., 1997), *Selenomonas ruminantium* (Yanke et al., 1998) were optimally active in the temperature range between 50 and 60°C, while phytase of *Aerobacter aerogenes* had an optima at 25°C (Greaves et al., 1967), and that of *Bacillus* sp. DS11 at 70°C (Kim et al., 1998).

Most microbial phytases studied so far show their optimum activity in the acidic pH range (Pandey et al., 2001; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Singh and Satyanarayana, 2009; Rao et al., 2009). Phytases from fungal origin exhibit optimal activity at pH 4.5 to 5.5, while some bacterial enzymes at pH 6.5 to 7.5. For the phytase of *Aerobacter aerogenes* (Greaves et al., 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *E. coli* (Greiner et al., 1993), *Selenomonas ruminantium* (Yanke et al., 1998) and *Lactobacillus amylovorus* (Sreeramulu et al., 1996), the pH optimum was between 4.0 and 5.5. The pH optimum for the phytase of *Enterobacter* sp.4 (Yoon et al., 1996) and *Bacillus* sp. DS11 (Kim et al., 1998) was at 7 to 7.5. *A. niger* NRRL 3135 secreted two different phytases, one with pH optima at 5.5 and 2.5, and the other at 2.0; as such, these enzymes were designated as phyA and phyB, respectively (Howson and Davis, 1983). Phytases of *T. lanuginosus* (Berka et al., 1998) and *A. fumigatus* (Pasamontes et al., 1997b) were optimally active at pH 6.0 to 6.5. The yeast phytases showed optimal activity in the pH range of 4.0 to 5.0 (Nakamura et al., 2000). The

Table 2. The biochemical properties of phytases from various microbes.

Source	MW(kDa)	T_{opt}	pH_{opt}	K_m(mM)	pI	Specificity	Reference
Fungi							
A. fumigatus	75	58	5.0	-	-	-	Mullaney et al. (2000)
A. niger	85	58	2.5 5.0	0.04	4.5	P	Ullah and Gibson (1987)
A. niger SK-57	60	50	5.5, 2.5	0.0187	-	P	Nagashima et al. (1999)
A. niger	-	55	5.5	0.20	4.9	-	Berka et al. (1998)
A. niger	353	55	2.5	0.606	-	P	Vats and Banerjee (2005)
A. oryzae	120–140	50	5.5	0.33	4.15	B	Shimizu (1993)
A. nidulans	77.8	55	5.5	-	-	-	Wyss et al. (1999b)
R. oligosporus	-	55	4.5	0.15	-	-	Sutardi and Buckle (1988)
A. niger ATCC9142	84	65	5.0	0.10	-	B	Casey and Walsh (2003)
R. oligosporus	124	65	5.00	0.010	-	B	Casey and Walsh (2004)
Peniophora lycii	72	50-55	4-4.5	-	3.61	-	Lassen et al. (2001)
Ceriporia sp.	59	55-60	5.5-6.0	-	7.36-8.01	-	Lassen et al. (2001)
Agrocybe pediades	59	50	5.0-6.0	-	4.15-4.86	-	Lassen et al. (2001)
Trametes pubescens	62	50	5.0-5.5	-	3.58	-	Lassen et al. (2001)
Thermomyces lanuginosus	60	65	7.0	0.11	4.7-5.2	B	Berka et al. (1998)
Thermoascus aurantiacus	-	55	-	-	-	-	Nampoothiri et al. (2004)
Rhizomucor pusillus	-	70	5.4	-	-	B	Chadha et al. (2004)
Myceliopthora thermophila	-	37	6.0	-	-	B	Mitchell et al. (1997)
Sporotrichum termophile	456	60	5.5	0.15	4.9	B	Singh and Satyanarayana (2009)
Yeasts							
Saccharomyces cerevisiae	-	45	4.6	-	-	-	Nayini and Markakis (1984)
Schwanomyces castellii	490	77	4.4	0.038	-	B	Segueilha et al. (1992)
Arxula adeninivorans	-	75	4.5	0.25	-	P	Sano et al. (1999)
Candida krusei WZ001[#]	330	40	4.6	-	-	-	Nakamura et al. (2000)
Pichia anomala[#]	64	60	4.0	0.20	-	B	Vohra and Satyanarayana (2002)
P. rhodanensis	-	70-75	4.0-4.5	0.25	-	-	Nakamura et al. (2000)
P. spartinae	-	75-80	4.5-5.0	0.33	-	-	Nakamura et al. (2000)
Bacteria							
Aerobactor aerogens[*]	-	25	4.0-5.0	0.135	-	-	Greaves et al. (1967)
Bacillus sp. DS 11	-	70	7.0	0.55	5.3	P	Kim et al. (1998)
Bacillus subtilis	37	60	7.5	0.04	-	-	Powar and Jagannathan (1982)
B. subtilis (natto)	38	60	6.0–6.5	-	-	-	Shimizu (1992)
B. subtilis	43	55	7.0–7.5	-	6.5	P	Kerovuo et al. (1998)

Table 2. Contd.

B. subtilis	44	55	6.0-7.0	-	5.0	P	Tye et al. (2002)
B. icheniformis	47	65	6.0-7.0	-	5.1	-	Tye et al. (2002)
B. amyloliquefaciens	44	70	7.0–7.5	-	-	-	Kim et al. (1998)
Escherichia coli[*]	42	55	4.5	0.13	6.3-6.5	P	Greiner et al. (1993)
Klebsiella oxytoca	40	55	5.0–6.0	-	-	-	Jareonkitmongkol et al. (1997)
K. aerogenes	700	65	4.5	-	3.7	P	Tambe et al. (1994)
Pseudomonas syringe[*]	47	40	5.5	0.38	-	P	Cho et al. (2003)
Lactobacillus sanfranciscensis[*]	50	45	4.0	-	5.0	B	Angelis et al. (2003)

Phytase location is *intracellular, #Cell bound and in all other cases it is extracellular; B = Broad spectrum, P = Phytate specific.

cell-bound phytase of *Pichia anomala* was maximally activated at pH 4.0 (Vohra and Satyanarayana, 2002), while that for *S. castellii* phytase was at pH 4.4 (Segueilha et al., 1992) and *Arxula adeninivorans* was at pH 4.5 (Sano et al., 1999). Phytases of plant origin have pH optima in the range between 4.0 and 5.6. Recently, alkaline phytase having maximum activity at pH 8.0 was reported from legume seeds (Scott, 1986). Another alkaline phytase was detected in the mature lily pollen that exhibited optimal activity at pH 8.0 (Hara et al., 1985).

Phytases usually show broad substrate spectrum with the highest affinity for phytate. The *A. fumigatus*, *Emericella nidulans* and *M. thermophila* phytases exhibited broad substrate specificity, while phytases of *A. niger, A. terreus* CBS and *E. coli* were rather specific for phytic acid (Wyss et al., 1999b). Broad substrate specificity was reported for phytases of *S. castellii* (Segueilha et al., 1992) and *S. thermophile* (Singh and Satyanarayana, 2009), while cell-bound phytase from *P. anomala* exhibited broad substrate specificity (Vohra and Satyanarayana, 2002). Only a few phytases have been described as highly specific for phytate such as the alkaline phytases from *B. subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992), *B. amyloliquefaciens* (Kim et al., 1998), lily pollen and cattail pollen (Hara et al., 1985). The acid phytases from *E. coli* (Greiner et al., 1993), *A. niger* and *A. terreus* (Wyss et al., 1999a) had also been reported to be rather specific for phytate.

With the exception of the phytases from *Emericella nidulans* and *Myceliophthora thermophila* (Mitchell et al., 1997), all phytases hitherto studied follow Michaelis-Menten kinetics. In general, phytases from microbial sources exhibit the highest turnover number with phytate, whereas their plant counterparts yield the highest relative rates of hydrolysis with pyrophosphate and ATP (Greiner and Konietzny, 2006). Most of the phytases characterized so far displayed the highest affinity to phytate among all phosphorylated compounds tested. The K_m values of the phytases ranged between 10 and 650 µM (Table 3). Relatively low K_m values have been reported for the phytases from *A. niger* (10 to 40 µM), *A. terreus* (11 to 23 µM), *A. fumigatus* (<10 µM), *Schwanniomyces castellii* (38 µM), *K. aerogenes* (62 µM) and some plant phytaes (Greiner and Konietzny, 2006). The K_m and V_{max} values of *S. thermophile* phytase were 0.156 mM and 83.4 U mg^{-1} protein s^{-1} for phytic acid, respectively (Singh and Satyanarayana, 2009). The catalytic constants for the degradation of phytate by phytases reported so far ranged between <10 (soybean and maize) and 1744 s^{-1} (*E. coli*) [Greiner and Konietzny, 2006]. The kinetic efficiency of an enzyme is validated by means of the k_{cat}/K_m values for a given substrate. The phytase of *E. coli* had a k_{cat}/K_m value of 1.34 x 10^7 M^{-1} s^{-1} (Golovan et al., 2001), which is the highest value reported for any phytase. The turnover number of 6209 s^{-1} and of 4.78 x 10^7 $m^{-1}s^{-1}$ was reported for *E. coli* phytase (Greiner et al., 1993). The k_{cat}/K_m value of the recombinant phytase of *P. anomala* expressed in *Hansenula polymorpha* is 72.5 (μM^{-1} s^{-1}) (Kaur et al., 2010).

Phytases are high molecular weight proteins ranging between 40 and 700 kDa (Table 3). The majority of phytases characterized so far acted like monomeric proteins with molecular masses between 40 and 70 kDa. However, some phytate-degrading enzymes appear to be made up of multiple subunits. Phytase of *S. castellii* has a molecular weight of 490 kDa with a glycosylation of around 31% (Seguilha et al., 1992). The glycosylated protein was tetrameric, with one large subunit (MW 125 kDa) and three identical small subunits (MW 70 kDa). Purified phytase

Table 3. List of commercially available microbial phytases (Modified from Cao et al., 2007).

Company	Phytase source	Production strain	Trademark
AB Enzymes	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	*A. oryzae*	*A. oryzae*	SP, TP and SF
Alltech	*A. niger*	*A. niger*	Allzyme phytase
BASF	*A. niger*	*A. niger*	Natuphos
Biozyme	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	*P. lycii*	*A. oryzae*	Bio-Feed phytase
Fermic	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	*A. awamori*	*T. reesei*	Avizyme
Roal	*A. awamori*	*T. reesei*	Finase
Novozyme	*Peniophora lycii*	*A. oryzae*	Ronozyme® Roxazyme®

from *A. fumigatus* revealed a protein with a molecular mass of 60 kDa by SDS-PAGE (Pasamontes et al., 1997a). The molecular masses of the monomeric form of phyA, phyB and acid phosphatase were estimated by SDS-PAGE as 85, 65 and 85 kDa, respectively. An extracellular phytase and an extracellular acid phosphatase were purified from *A. oryzae* K1 and their molecular masses were 60 and 70 kDa, respectively (Shimizu, 1993). The phytase of *A. niger* van Teighem was a 353 kDa homopentameric protein with a monomeric molecular mass of 66 kDa (Vats and Banerjee, 2005), while the phytase of *S. thermophile* is a homopentameric 456 kDa glycosylated protein with a monomeric mass of 90 kDa (Singh and Satyanarayana, 2009), and that of *P. anomala* is a homohexamer with a molecular mass of 390 kDa (Kaur et al., 2010). The rat intestine phytase was reported to be a heterodimer comprising 70- and 90-kDa subunits (Yang et al., 1991). However, the phytases isolated from maize roots (Hubel and Beck, 1996), germinating maize seeds (Laboure et al., 1993), tomato roots (Li et al., 1997), soybean seeds (Hegeman and Grabau, 2001) and *A. oryzae* (Shimizu, 1993) were homodimeric proteins, while a homohexameric structure was proposed for the *A. terreus* enzyme (Yamamoto et al., 1972). Two different forms of phytases have been reported in *K. aerogenes* (Tambe et al., 1994). One, possibly the native enzyme, has an exceptionally large size (700 kDa), and the other, may be a fraction of the native enzyme, exhibits an exceedingly small molecular mass (10 to 13 kDa) with full complement of the activity. Fungal and several plant phytases have been found to be glycosylated with a carbohydrate content of 27.3% (Ullah, 1988). Glycosylation may have an effect on the catalytic properties, the stability or the isoelectric point of an enzyme. The molecular mass and the homogeneity of the purified enzyme from *Bacillus* sp. DS11 were estimated by gel filtration and SDS-PAGE. PAGE under denaturation conditions revealed a single protein band of 44 kDa whose size corresponded well with the molecular mass of 40 kDa obtained by superose-12 column chromatography (Kim et al., 1998). An extracellular phytase of *B. subtilis* (natto) N-77, purified 322-fold by gel filtration and DEAE chromatography had a molecular mass of 36 kDa (Shimizu, 1992), whereas two periplasmic phytases (P_1 and P_2) purified from *E. coli* close to homogeneity, were monomers with a molecular mass of 42 kDa (Greiner et al., 1993).

CRYSTAL STRUCTURE OF PHYTASES

For designing an ideal phytase and its genetic engineering, it is important to have an idea about its structure. Therefore, scientists all over the world are working on this aspect. Recently, the crystal structure of phytase from *Klebsiella* sp. ASR1 has been determined to 1.7 Å resolution using single-wavelength anomalous-diffraction phasing (Bohm et al., 2010). The phytase is different from the *E. coli* phytase in its sequence and phytate degradation pathway, but the overall structure of *Klebsiella* phytase is similar to other histidine-acid phosphatases, such as *E. coli* phytase and human prostatic-acid phosphatase. The stucture of this phytase consisted of two domains (one α and one α/β domain) in which the active site is present in a positively charged cleft between these domains.

The crystal structures of the phytases from *A. niger* (Kostrewa et al., 1997), *E. coli* (Lim et al., 2000) and *B. amyloliquefaciens* (Ha et al., 2000) have been determined. The structures of the *A. niger* and *E. coli* enzyme closely resembled the overall fold of other histidine acid phosphatases. These structures contained a conserved α/β-domain and a variable α-domain and the active site is present at the interface between these domains. This structure also provides the information

about substrate binding and the catalytic mechanism. In case of *E. coli* phytase, it was shown that the phosphate is co-ordinated by the two arginine residues of the RHGXRXP-motif, as well as by conserved residues downstream, a further arginine residue and the histidine and aspartate residue of the HD-motif. Furthermore, the histidine residue of the RHGXRXP-motif was shown to be oriented for nucleophilic attack. The phytase from *S. ruminantium* shared no sequence identity with other microbial phytases (Chu et al., 2004). The active site of this phytase is located close to a conserved cysteine-containing (Cys241) P loop. The co-crystallization of *myo*-inositol hexasulfate, with the enzyme revealed that the inhibitor was bound in a pocket slightly away from Cys241 and at the substrate binding site where the phosphate group to be hydrolyzed is held close to the -SH group of Cys241. Crystal structure of *Aspergillus fumigatus* phytase was determined at 1.5 Å resolution to understand the structural basis for its high thermostability (Xiang et al., 2004). However, the overall folding has a resemblance with the structure of other phytases.

Crystal forms I and II were obtained with $CdCl_2$ and $HgCl_2$ and diffracted to 1.5 Å and 2.25 Å resolution, respectively (Lim and Jia, 2002). Hg^{2+} and Cd^{2+} both acted as molecular bridge(s) and played a crucial role in the crystallization of phytase by bridging neighbouring molecules. Despite a lack of sequence similarity, the structure closely resembled the overall folds of other histidine acid phosphatases (Lim et al., 2000). The crystal structure of a thermostable, calcium-dependent and beta propeller type *Bacillus* phytase, complexed with inorganic phosphate, revealed that two phosphates and four calcium ions are tightly bound at the active site (Shin et al., 2001). Mutation of the residues involved in the calcium chelation resulted in severe defects in the enzyme activity. One phosphate ion, chelating all of the four calcium ions, is close to a water molecule bridging two of the bound calcium ions. The enzyme has two phosphate binding sites, the 'cleavage site', which is responsible for the hydrolysis of a substrate, and the 'affinity site', that increases the binding affinity for substrates containing adjacent phosphate groups.

The crystal structure of *A. niger* NRRL3135 phytase determined at 2.5 Å resolution served to specify all active site residues (Tomschy et al., 2000a, b). Using multiple amino acid sequence alignment approach, Gln27 of *A. fumigatus* phytase was identified as likely to be involved in substrate binding and/or release and, possibly, to be responsible for the considerably lower specific activity of *A. fumigatus* phytase as compared to that of *A. terreus* phytase, which has a 'leu' at an equivalent position. Site-directed mutagenesis of Gln27 of *A. fumigatus* phytase to leu, in fact increased the specific activity, and this and other mutations at position 27 yielded an interesting array of pH activity profiles and substrate specificities. A novel bacterial phytase from a *B. amyloliquefaciens* strain was crystallized using the hanging-drop vapour-diffusion method (Ha et al., 1999). High-quality single crystals of the enzyme in the absence of calcium ions were obtained using a precipitant solution containing 20% 2-methyl-2, 4-pentanediol and 0.1 M MES (pH 6.5). The crystals contain one monomer per asymmetric unit. Phytase has a α/β-domain similar to that of rat acid phosphatase and α-domain with a new fold (Kostrewa et al., 1997).

DIRECTED EVOLUTION AND PROTEIN ENGINEERING OF PHYTASES

The natural enzymes are adapted in a living cell to perform a particular function, but in most cases, they are poorly suited for industrial applications. Protein engineering is a very active area of research for understanding the structure-function relationships of a particular protein (Lehman et al., 2000a, b, c; Tomschy et al., 2000a, b). In recent years, there has been a widespread enthusiasm for 'directed evolution' as a new tool to optimize the properties of an enzyme of interest (Dalboge and Borchert, 2000; Arnold, 2001). Mostly, enzymes are stabilized by the cumulative effects of small improvements at many locations within the protein molecule (Lehman et al., 2000a, b, c; Tomschy et al., 2000a, b; Coco et al., 2001). The engineering of proteins for improved thermostability is an exciting and challenging field because of its applicability for the industrial use of recombinant proteins (Lehman et al., 2000a, b, c; Tomschy et al., 2000a, b).

Rational design principles and directed evolution

The stability of a protein is determined by both local and long-range interactions between the residues (Tomschy et al., 2000a, b). The thermostability of an enzyme can be enhanced by multiple amino acid exchanges, each of which slightly increases the unfolding temperature of the protein. The rational approaches for thermostability engineering involve the comparison of the amino acid sequence of the protein of interest with a more thermostable, homologous counterpart, followed by replacement of selected amino acids (Tomschy et al., 2000a, b). Three-dimensional structure of the protein of interest could be helpful in this regard. The thermostabilization concepts include the introduction of additional disulfide bridges, improvements in the packing of the hydrophobic core, engineering of surface salt bridge networks or α-helix dipole interactions, changes in α-helix propensity and changes in entropy (Haney et al., 1999; Tomschy et al., 2000a, b). All these rational approaches have been used successfully in the engineering of phytases for improved catalytic activity. Site directed mutagenesis of amino acid residue 300 was resulted in a high phytase activity by *A. niger* NRRL 3135

at pH 3.0 to 5.0, while a single mutation (K300E) resulted in an enhanced hydrolysis of phytic acid at pH 4.0 and 5.0. In this study, the basic amino acid residue lysine (K) was replaced by acidic residue. However, this replacement with another basic residue, or an uncharged but polar residue, did not significantly alter the activity at pH 4.0; but a replacement with basic residue arginine (R) lowered the activity over the pH range from 2.0 to 6.0 (Mullaney et al., 2002).

In *A. fumigatus,* a 3D structure of the native *A. niger* NRRL 3135 phytase was used to identify non-conserved amino acids that were not associated with increased catalytic activity (Tomschy et al., 2000a). Consequently, they changed the single amino acid residue (Q27), and this displayed a significant effect on specific activity, pH profile and substrate specificity. *A. niger* NRRL 3135 and *A. niger* T213 wild phytases displayed a 3-fold difference in specific activity, despite only 12 amino acid residues difference (Tomschy et al., 2000b). Out of these 12 amino acid residues, nine were distantly placed from active site, and therefore, are not responsible for catalytic activity. In the remaining 3 residues, R297Q mutation was found to fully account for this difference in catalytic activity, because out of the 3 single mutants (E89D, H292N and R297Q), 2 double mutants (E89D R297Q and H292N R297Q) and a triple mutant (E89D H292N R297Q) revealed a 3-fold increase in specific activity. This specific activity is close to the wild type. Molecular modeling revealed that R297Q may directly interact with the phosphate group of phytic acid. This presumed ionic interactions caused strong binding of the substrate and product indicating the product release as the rate-limiting step of the reaction, which is responsible for lower specific activity.

When expressed in *A. niger*, several fungal phytases were susceptible to proteases (Wyss et al., 1999b). N-terminal sequences of the fragments revealed that cleavage invariably occurred at exposed loops on the surfaces of the molecules. Site directed mutagenesis at the protease-sensitive sites of *Aspergillus fumigatus* (S151N and R151L/ R152N) and *Emericella nidulans* phytase (K186G and R187R) yielded mutants with reduced susceptibility to proteases, without affecting the specific activity. Based on *E. coli* phytase crystal structure, substitution of C200N in a mutant seems to eliminate the disulfide bond between the G helix and the GH loop in the α-domain of the protein which might be modulating the domain flexibility, and thereby the catalytic efficiency and thermostability of the enzyme (Rodriguez et al., 2000).

The consensus approach

The consensus approach is based on the hypothesis that at a given position in an amino acid sequence alignment of homologous proteins, the respective consensus amino acid contributes more than average to the stability of the protein than the non-consensus amino acids (Lehman et al., 2000a, b, c). Consequently, substitution of non-consensus by consensus amino acids may be a possible approach for improving the thermostability of a protein.

Each amino acid of a protein contributes towards its stability. The mutations responsible for thermostability of a protein with a small effect on the protein stability were combined to generate a consensus protein variant that showed enhanced thermostability (Lehman et al., 2000 a, b, c).

Lehman et al. (2000a) used a computer program to calculate an entire consensus sequence from 13 homologous amino acid sequences of wild-type phytases from mesophilic fungi. This phytase showed an identity of 58.3 to 80% with the parent phytases. The recombinant expression of a synthetic gene gave rise to a consensus phytase (consensus phytase-1) that was 15 to 26°C more thermostable and showing 15 to 22°C more denaturing temperature than the wild-type. The backbone of this consensus phytase was modified by Lehman et al. (2000b). They modified the catalytic property by replacing a part of the active site with the corresponding residue of *A. niger* NRRL3135 phytase, which displayed a pronounced difference in specific activity, substrate specificity and pH profile. This exchange of active site resulted in a decrease in denaturing temperature, but the consensus phytase was still more thermostable than its parents. Further addition of wild-type sequences in the alignment resulted in consensus phytase-10, which displayed a further 7.4°C increase in denaturing temperature. In another approach, the consensus approach was refined by including six more sequences that yielded consensus phytases-10 and -11 with an increase of 7.4°C in denaturing temperature. Site directed mutagenesis identified some residues showing their effect on protein thermostability. Nonetheless, the combination of these residues resulted in an increase in the denaturing temperature from 88.0 to 90.4°C.

MULTIFARIOUS APPLICATIONS OF PHYTASES

Amelioration of the nutritional status of foods and feeds

Phytases are useful in food and feed industries, preparation of *myo*-inositol phosphate intermediates, combating phosphorus pollution and in plant growth promotion (Idriss et al., 2002; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Greiner and Konietzny, 2006; Rao et al., 2009). The major food supplements in animal food are derived from plant sources such as cereals, legumes, soybean, etc. The presence of phytate in plant foodstuffs causes mineral deficiency due to the chelation of metal ions (De Boland et al., 1975). The presence of phytic acid in rapeseed causes Zn, Mg and

Ca deficiency in chickens (Nwokolo and Bragg, 1977).

Canola meal contains 4 to 6% phytic acid, which reduces the nutrition value of the meal. The phytic acid has been shown to bind with multivalent cations, and hence, reduce their bioavailability. The addition of phytase to high phytate containing diets improves the absorption and utilization of phosphorus (Hughes and Soares, 1998). Dietary phytase also improves the nutritive value of canola protein concentrate and decreases phosphorus output in case of rainbow trout (Forster et al., 1999). Similar reports have been documented for different species like rainbow trout (Rodehutscord and Pfeiffer, 1995), channel catfish (Li and Robinson, 1997), African catfish (Van Weerd et al., 1999), common carp (Schafer et al., 1995) and *Pangasius pangasius* (Debnath et al., 2005). Robinson et al. (2002) reported that 250 units of phytase per kilogram of diet could effectively replace dicalcium phosphate supplement in the diet of channel catfish without affecting growth, feed efficiency or bone phosphorus deposition.

Phytic acid is well known to make complexes with various cations as well as with proteins (Wise, 1983). Phytase added to diets improves the bioavailability of copper and zinc in pigs (Adeola et al., 1995) and poultry (Yi et al., 1996). Microbial phytase also improves the apparent absorption of magnesium, zinc, copper and iron in pigs (Selle and Ravindran, 2007). Similar results have also been reported for fishes (Cao et al., 2007). Phytase addition increases the concentration of minerals like magnesium, phosphorus, calcium, manganese and zinc in plasma, bone and the whole body (Vielma et al., 2004). Yan and Reigh (2002) demonstrated that the phytase supplementation improved the retention of calcium, phosphorus and manganese by catfish fed with an all-plant protein diet. The phytase supplementation in the diets significantly improved the digestibility of minerals, total-P, phytate-P and gross energy (Cheng and Hardy, 2002). The experimental studies in animals and humans have shown that phytic acid rich diets can cause zinc deficiency. Phytic acid does not inhibit copper absorption, but has a modest inhibitory effect on manganese absorption (Lonnerdal, 2000).

The treatment of fish feed with phytase was found to improve protein digestibility and retention in fishes (Cheryan, 1980; Storebakken et al., 1998; Papatryphon et al., 1999; Boling et al., 2001; Cheng and Hardy, 2002; Usmani and Jafri, 2002; Vielma et al., 2004; Sajjadi and Carter, 2004; Debnath et al., 2005; Baruah et al., 2005; Ai et al., 2007; Altaff et al., 2008; Hassan et al., 2009). The inclusion of phytase to broilers diets increased the coefficient of phosphorus retention and reduced the presence of this element in poultry birds, thus, indicating a favorable environmental effect (Ahmad et al., 2000; Brenes et al., 2003; Juanpere et al., 2004; Murugesan et al., 2005; Vohra et al., 2006; Ahmadi et al., 2008; Pillai et al., 2009). Microbial phytases positively affected the pigs' performance and their daily gain, and further, the feed conversion ratios were ameliorated by organic acids (Jongbloed et al., 2000; Walz and Pallauf, 2002; Revy et al., 2005; Kim et al., 2005; Pomar et al., 2008; Akinmusire and Adeola, 2009; Hill et al., 2009).

The role of phytases in dephytinization and bread making

The presence of phytates in plant food stuffs (De Boland et al., 1975) is well known. Moulds commonly used in oriental food fermentation have been examined for their ability to produce phytase. Tempeh is a popular oriental fermented food made from soyabeans inoculated by moulds (*Rhizopus oligosporus*) in the koji process. The digestibility, vitamin contents and flavour of soyabean were improved by the mould fermentation (Fardiaz and Markakis, 1981). Dietary phytase is inactivated during cooking so the phytate digestion is very poor, thereby affecting mineral absorption. The addition of *A. niger* phytase to the flour containing wheat bran increased iron absorption in humans (Sandberg et al., 1996). The use of phytase was suggested for producing low phytin bread. Also, phytic acid has positive effects. It exerts an antineoplastic effect in animal models of both colon and breast carcinomas. The presence of undigested phytate in the colon may protect it against the development of colonic carcinoma (Iqbal et al. 1994).

By adding mould phytases during bread making, dough phytate could be almost completely eliminated. Caransa et al. (1988) reported that phytase supplementation could accelerate the process of steeping required in the wet milling of corn, thereby improving the properties of corn steep liquor. Supplementation of phytase from a thermophilic mould, *S. thermophile*, improved the bread quality with concomitant reduction in phytate (Singh and Satyanarayana, 2008c). Phytase released inorganic phosphate from calcium, magnesium and cobalt phytates (Singh and Satyanarayana, 2010).

The effect of the supplementation of exogenous phytase to four different bread formulations on the bread quality was assessed by Haros et al. (2001a, b). The supplementation of bread with phytase shortened the fermentation period. There was a considerable increase in the specific bread volume, which is an improvement in the crumb texture and the width/height ratio of the bread slice (Knorr et al., 1981). The chapathi dough with reduced phytic acid levels was developed using a mutated strain of the yeast *Candida versatilis* and it resulted in 10 to 45% reduction in phytate levels (Bindu and Varadaraj, 2005). Wheat flour, sesame oil cake and soymilk were efficiently dephytinized by *S. thermophile* phytase with concomitant reduction in phytic acid content and liberating inorganic phosphate (Singh and Satyanarayana, 2006a; 2008a, 2008b). Similarly, the cell-bound phytase of *P. anomala* resulted in dephytinization of soymilk (Kaur and Satyanarayana, 2010).

Semisynthesis of peroxidase

Peroxidases are ubiquitous enzymes that catalyse a wide variety of selective oxidations with hydrogen peroxide as the primary oxidant (van de Velde et al., 2000). The active site of vanadium chloroperoxidase from *Curvularia inaequalis* closely resembled that of the acid phosphatases and the apoenzyme of vanadium chloroperoxidase exhibits phosphatase-like activity (Hemrika et al., 1997). The combination of phytase with vanadate produced an effective semi-synthetic peroxidase. The effect of pH on the vanadate phytase-catalysed oxidation of thioanisole revealed that the pH optimum coincided with that of phytase. Optimisation led to a maximum enatiomeric excess (ee) of 68% obtained in formate buffer at 4.0°C. The vanadium-incorporated phytase was stable for over three days with only a slight decrease in activity.

A cross-linked enzyme aggregate of 3-phytase was transformed into peroxidase by incorporation of vanadate (Correia et al., 2008). The cross-linked aggregate phytase showed similar efficiency and asymmetric induction as the free enzyme. Moreover, the cross-linked aggregate phytase can be reused at least three times without significant loss of activity. Some other acid, phosphatases and hydrolases were tested for peroxidase activity, when incorporated with vanadate ion. Phytases from *Aspergillus ficuum*, *A. fumigatus* and *A. nidulans*; sulfatase from *Helix pomatia*; and phospholipase D from cabbage, catalyzed the enantioselective oxygen transfer reactions when incorporated with vanadium. However, phytase from *A. ficuum* was unique in catalyzing the enantioselective sulfoxidation as compared to others.

Plant growth promotion

Phosphorus deficiency in soil is a major constraint for agricultural production worldwide. Large proportion of soil P exists in the organic form, of which phytic acid is the pre-dominant form. There are a large number of reports explaining the role of phytase in improving the growth of the plants and reducing the phosphorus pollution. A β-propeller phytase from *Bacillus subtilis* was constitutively expressed in tobacco and *Arabidopsis*, and it was shown to be secreted from their roots (Lung et al., 2005). In transgenic tobacco, phytase activities in leaf and root extracts were 7 to 9-fold higher than those in wild-type. A 4 to 6-fold higher extracellular phytase activity had been recorded in transgenic plants. In sterile liquid culture, using 1 mM sodium phytate as the sole P source, the transgenic tobacco lines accumulated 1.7 to 2.2 times more shoot biomass than the wild-type plants after 30 days of growth with concomitant increase (27 to 36%) in shoot P concentration. Similar observations have been recorded in the transgenic *Arabidopsis*, explaining the mobilization of soil phytate into inorganic phosphate for plant uptake (Lung et al., 2005). Yip et al. (2003) showed that the tobacco line transformed with a neutral *Bacillus* phytase exhibited phenotypic changes in flowering, seed development, and response to phosphate deficiency. The transgenic line showed an increase in number of flower and fruit, lesser seed IP6/IP5 ratio, and enhanced growth under phosphate-starvation conditions as compared to the wild type.

The transgenic *Arabidopsis* plants secreted phytase only from roots when grown on a medium under low phosphate conditions (Mudge et al., 2003). The growth rates and shoot P concentrations of plants were similar when grown on the medium containing phytate or phosphate as the P source. Phytase and phosphatases producing fungi were used as seed inoculants, to help attain higher P nutrition of plants in the soils containing high phytate phosphorus (Yadav and Tarafdar, 2003). The efficiency of different organic P compounds' hydrolysis by different fungi indicated that the fungi have enough potential to exploit native organic phosphorus to benefit plant nutrition. Transgenic *Arabidopsis* plant expressing an extracellular phytase from *Medicago truncatula* led to significant improvement in organic phosphorus utilization and plant growth (Xiao et al., 2005). Using phytate as the sole source of phosphorus, dry weight of the transgenic *Arabidopsis* lines were 3.1 to 4.0-fold higher than the control plants and total phosphorus contents were 4.1- to 5.5-fold higher than the control, suggesting the great potential of heterologous expression of phytase gene for improving plant phosphorus acquisition and for phytoremediation. The growth and phosphorus nutrition of *Arabidopsis thaliana* plants supplied with phytate was improved significantly after the introduction of phytase gene from *Aspergillus niger* (Richardson et al., 2001). Li et al. (2007) showed that both wild type *Bacillus mucilaginosus* and transgenic (containing phytase gene) strains promoted the tobacco plant growth under greenhouse study and field experiments.

The plant growth promotory effect of an extracellular phytase of a thermophilic mould, *Sporotrichum thermophile,* has been reported recently (Singh and Satyanarayana, 2010). Both phytase, as well as the mould, promoted the growth of wheat seedlings. The growth and inorganic phosphate content of the plants were better than the control. Sodium phytate (5 mg plant^{-1}) was adequate for liberating enough phosphorus for the growth of the seedlings. The plant growth, root/shoot length and inorganic phosphate content of test plants were better than the control plants. An enzyme dose of 20.0 U plant^{-1} was found to adequately liberate enough amount of inorganic phosphate required for supporting plant growth. The plant growth, root/shoot length and inorganic phosphate content of test plants were higher than the control (Singh and Satyanarayana, 2010). The compost prepared by the combined action of native microflora of wheat straw along with *S. thermophile*

promoted the growth of plants. The inorganic phosphate content of the wheat plants was also high as compared to those cultivated on the compost prepared either with only native microflora or *S. thermophile*. These approaches can be applied as a strategy for boosting the productivity in agriculture and horticulture.

Miscelaneous applications

Preparation of myo-inositol phosphates

There is a continuous demand of inositol phosphates and phospholipids, which play an important role in cell signalling pathways (Billington, 1993). Enzymic hydrolysis of phytic acid using *S. cerevisiae* resulted in the production of 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, 1986). Greiner and Konietzny (1996) prepared inositol 1,2,3,4,5-pentakisphosphate, inositol 2,3,4,5-tetrakisphosphate, inositol 2,4,5-triphosphate and inositol 2,5-biphosphate using immobilized phytase from *E. coli.* Inositol phosphate derivatives can be used as enzyme stabilizers (Siren, 1986), enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potential drugs, and as chiral building blocks.

Pulp and paper industry

It has been observed that the removal of plant phytic acid could be important in the pulp and paper industry (Liu et al., 1998). A phytase with activity at elevated temperatures could have the potential as a biological agent to hydrolyse phytic acid during pulp and paper processing. This process will not produce any carcinogenic and toxic byproducts. Therefore, the use of phytases in pulp and paper processing could be ecofriendly and would help in the development of cleaner technologies (Liu et al., 1998).

Combating environmental phosphorus pollution

Phosphorus is an essential ingredient in animal and plant production; however, too much or too little P can be a problem both for animal production and the environment. Researchers all over the world are finding ways for poultry to better utilize P, thus increasing productive efficiency and protecting the environment. The ruminants sustain the microflora that enzymatically releases inorganic phosphorus from phytic acid, though, monogastrics such as humans, chickens and pigs produce little or no phytase in the intestine. Hence, the phytic acid phosphorus is unavailable and the phytic acid is excreted in their feaces (Mullaney et al., 2000). Phytic acid present in the manure of these animals is enzymatically cleaved by soil and water-borne microorganisms. The phosphorus thus released is transported into the water bodies causing eutrophication. This results in oxygen depletion due to excessive algal growth. Pretreatment of animal feed with phytases will increase the availability of inorganic phosphorus, thereby improving the nutritional status of food and also help in combating phosphorus pollution. Phytases are very well known to reduce pollution caused by excess of phosphorus accumulation in soil and water (Nahm, 2002). The excretion of phosphorus can be reduced by 30%, via replacing feed phosphate with phytase and by equally calculated digestible P content. The addition of phytase to the feed of piglets gives positive results in some experiments such as a significant increase in growth rate and feed intake and a significantly better feed conversion ratio in comparison with the conventional feed. The supplementation of phytase in corn and soybean meal diets was additive, significantly improving P digestibility and dramatically decreasing P excretion to reduce the potential impacts of P from pig manure on the environment (Hill et al., 2009).

Microbial phytase supplementation in the diet of fish can overcome this problem. It makes the chelated phosphorus available to fish, and hence, there is less faecal excretion, thereby reducing environmental pollution. The environmental benefits of using this enzyme in fish feed are thus listed:

1. Reduced requirement of the mineral supplements, thereby reducing chances of excess inorganic phosphorus getting into the aquatic system.
2. Reduced organic phosphorus, that is, phytic acid outputs.

Use of phytase in feeds reduces or sometimes eliminates the necessity of mineral supplementation, which also decreases the cost of feeds. Although phytase was first used for environmental reasons, it is now realized that there are a range of other nutritional and health benefits from using these enzymes.

CONCLUSIONS AND FUTURE PERSPECTIVES

Besides effectively tackling phosphorus pollution in the areas of intensive livestock rearing, phytases have considerable potential in commercial applications. The applications of phytases in improving human health and in synthesis of lower inositol phosphates have increasingly attracted attention. A significant progress has been made in phytase research during the last few decades. The phytases, which exhibit desirable activity profile over a broad pH range, excellent thermal stability, and broad substrate specificity, are more promising for commercial exploitation. Modern day technologies

(molecular biology and genetics) could be utilized for the development of staple foods with higher and improved bioavailability of the minerals and proteins. Genetic engineering techniques could be employed for the generation of consensus phytases with improved and desirable properties for applications in food and feed industries (Lehman et al., 2000a, b, c). Adding phytase to the animal diets not only improves the bioavailability of proteins and minerals, but also aids in combating environmental phosphorus pollution in the areas of intensive live stock management.

Transgenic plants of corn, rice, barley and soybean with low phytic acid have been generated; and this could be a novel approach for reducing micronutrient malnutrition and animal waste phosphorus. Further research efforts are needed to understand the molecular biology and genetics of phytic acid accumulation during seed development and feasibility and effectiveness of employing this approach at the community level (Mendoza, 2002). The transgenic plants harboring the microbial phytase genes could also be used to improve soil fertilization and nutrient availability to plants. With the collaborative efforts of phytase scientists from different fields, it would be possible to design and develop an ideal phytase for animal nutrition, human health and environmental protection.

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