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Potential biotechnological applications of Phytases from thermophilic moulds

Harshul Arora[1], Bijender Singh[2] and T. Satyanarayana[1*]
[1]*Department of Microbiology, University of Delhi, South campus, New Delhi-110021*
[2]*Department of Microbiology, M.D. University, Rohtak-124001 (Haryana)*
**Corresponding author Email: tsnarayana@gmail.com*
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ABSTRACT

Phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyzes the hydrolysis of phytic acid to myo-inositol and inorganic phosphate in a step-wise manner. Varied amount of phytic acid is present in food and feeds and acts as an antinutrient. Its reduction by enzymatic methods is, therefore, preferred because the physical and chemical methods of removal negatively affect nutritional value of foods. Phytase find applications in food and feed industries for decreasing the phytic acid content, liberating inorganic phosphate, improving digestibility by mitigating antinutritional factor and as therapeutics. Feed pelleting involves treatment at 80-85 ºC for a few seconds, and therefore, a thermostable phytase is in demand. Although a large number of microorganisms have been reported to produce phytase, thermophilic fungi produce thermostable phytases with resistance to denaturants. This review focuses on the production, characterization and biotechnological applications of native and recombinant phytases of thermophilic moulds.

Keywords: Thrmostable phytase, phytic acid, thermophilic moulds, anti-nutritional factor, phosphorus pollution, plant growth promotion

INTRODUCTION

Phytic acid (*myo*-inositol-hexakis-dihydrogen phosphate) is an organic form of phosphorus that accounts for 1 to 5 % by weight of edible cereals, legumes, pollen, oil seeds and nuts, constituting a large part of animal diet (Vohra and Satyanarayana, 2003). Phytates (salts of phytic acid) occur in the form of a polyanion at pH 1–6 with three to six negative charges, they accumulate in the crop, gizzard and proventriculus of poultry, including the stomach of humans and swine (Bebot-Brigaud *et al*.,1999; Rao *et al.*, 2009). Phytate acts as an anti-nutritional factor, as it causes mineral deficiency by chelating metal ions such as Mg^{2+} Ca^{2+}, Fe^{2+} and Zn^{2+}, it forms complexes with proteins, affecting their digestion and also inhibits enzymes like amylase, trypsin, tyrosinase and acid phosphatase. (Harland and Morris, 1995; Singh and Satyanarayana, 2011) [**Fig. 1**]. Thus it causes nutritional deficiencies in places where plant derived staple foods are used (Manary *et al.*, 2002). Monogastric animals (poultry, fishes, pigs and humans) cannot digest phytic acid efficiently due to lack of an adequate phytase, and it is excreted in faeces. When excreted in feaces, it is degraded by soil micro-organisms, releasing phosphorus in the soil that reaches aquatic bodies giving rise to eutrophication (Vats *et al.*, 2005). Although phytic acid can be removed by some physical methods (steeping, cooking and autoclaving) and chemical methods (acid hydrolysis ion and exchange), these methods decrease the nutritional qualities of foods. Thus reduction of phytic acid content in foods and feeds by enzymatic hydrolysis with phytase is desirable since it improves their nutritional value.

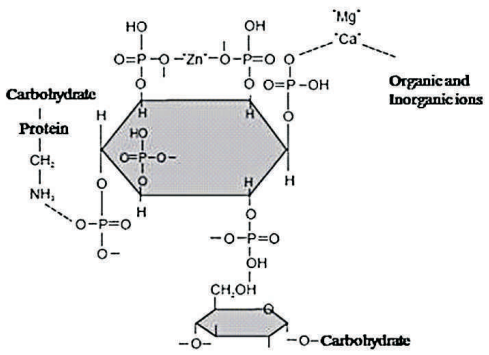

Fig 1. The structure of phytic acid showing chelation of cations and complexation with carbohydrates and proteins.

Phytases have always attracted attention of scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology. The supplementation of the diets for swine and poultry with inorganic phosphate can be removed by including adequate amounts of phytase along with an appropriate manipulation of other dietary factors (Han *et al.*, 1999). Therefore, the phosphate excretion of these animals may be reduced by about 50% (Lei *et al.*, 1993a;b; and Vohra and Satyanarayana, 2003). The feeding trials have shown the effectiveness of phytase supplementation for improving utilization of phytate-P and the phytate-bound minerals by poultry, swine and fishes (Lei and Stahl, 2001; Singh *et al.*, 2006; Cao *et al.*, 2007; Selle and Ravindran, 2007; 2008; Rao *et al.*, 2009).

Phytase thus has potential applications in feed and food industries. Phytase is present in many plants and animal tissues, and it is also produced by many moulds, yeasts and bacteria (Shieh and Ware, 1968; Howson and Davis, 1983; Vohra and Satyanarayana, 2003; Singh *et al.*, 2011; Singh and Satyanarayana, 2011.) In case of fungi, various species of *Aspergillus* (Shieh and Ware, 1968; Howson and Davis, 1983) and thermophilic moulds such as

Myceliophthora thermophila (Mitchell *et al.*, 1997), *Thermomyces lanuginosus* (Berka *et al.*, 1998) *Thermoascus aurantiacus* (Nampoothiri *et al.*, 2004) and *Sporotrichum thermophile (*Singh and Satyanarayana 2006a;b; 2008a;b;c*)* are known to secrete phytases. Thermophilic fungal phytases are relatively thermostable than their mesophilic counterparts (Pasamontes *et al*, 1997a), and filamentous fungi are well known to produce phytases in SSF (Pandey *et al.*, 2001; Bogar *et al.*, 2003; Chadha *et al.*, 2004; Singh and Satyanarayana, 2006a). Albeit a number of articles have been published on phytase production, characteristic features and applications (Vohra and Satyanarayana, 2003; Greiner and Konietzny, 2006; Vats and Banerjee, 2004; Kaur *et al.*, 2007; Rao *et al.*, 2009; Singh *et al.*, 2011), the thermophilic fungi have not been explored adequately like their mesophilic counterparts (Chadha *et al.*, 2004). The fungal SSF product contains not only phytase, but also other accessory enzymes, fungal protein and organic acids which increase feed digestibility and access to phytin present in plant cells (Pandey *et al.*, 2001; Bogar *et al.*, 2003; Singh and Satyanarayana, 2006a). In this review, we have discussed thermophilic mould phytases, their properties and multifarious applications.

CLASSIFICATION OF PHYTASES

Phytases have been classified on the bases of site of bond cleavage by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) into two categories viz. 3-phytase (EC3.1.3.8) which hydrolyses the phosphoester bond at third position of *myo*-inositol hexakisphosphate first, and is observed in microorganisms; and the 6-phytase (EC 3.1.3.26) that hydrolyses phosphoester bond at the sixth position of *myo*-inositol hexakisphosphate first, and is mostly discovered in plants and also in a few basidiomycetous fungi (Lassen *et al.*, 2001). Another phytase alkaline 5-phytase has been observed from lily pollen which starts phytate hydrolysis at D-5 position.

On the basis of pH for activity, phytases have been classified into two major classes: acid phytases and alkaline phytases. Acidic phytases have wider applications in animal foods and feeds as well and show broader substrate specificity. Likewise, based on the catalytic properties, phytases have also been grouped as HAP (histidine acid phosphatase), BPP (β-propeller phytase), CP (cysteine phosphatase) and PAP (purple acid phosphatase) (Mullaney and Ullah, 2003).

IDEAL PHYTASE

Phytase with desirable characteristics for applications in animal and feed industry would be called the ideal phytase. However, the following characteristics are kept in mind for their use in animal feed:

a. Active in stomach
b. Stability during processing and storage of feed
c. Resistant to proteases
d. Low cost of production
e. High yield and purity

Thus a single phytase may not serve as an ideal phytase for all species and in all cases. For example stomach pH of finishing pigs is more acidic than weaniling pigs (Radcliffe *et al.*, 1998), and thus, phytase with pH < 3 will work better in finishing pigs. For poultry, an enzyme active near neutral pH would be adequate i.e. pH 6.5 (Riley and Austic, 1984) and also at the acidic pH of stomach. Phytases used for aquaculture, due to their lower body temperature, require a lower temperature optimum than that of swine or poultry (Ramseyer *et al.*, 1999). Thus choice of organism for phytase production depends upon the target application.

SOURCES OF PHYTASE PRODUCTION

Sources of phytases include plants, animals and microorganisms (bacteria, yeasts and fungi). Microbial sources have, however, been proven to be better for the production of phytases on a commercial scale (Vohra and Satyanarayana 2003, Vats and Banerjee, 2004). Unlike bacterial and yeast phytases, which are intracellular and cell-bound (Vohra and Satyanarayana 2003), thermophilic fungal phytases are extracellular in nature (Mitchell *et al.*, 1997, Berka *et al.*, 1998). The report of first phytase was from *Aspergillus fumigatus* (Pasamontes *et al.*, 1997b). A phytase from *Thermomyces lanuginosus* with optimum activity at 65 °C and a pH of 6.0, (Berka *et al.*, 1998) also has been reported. Other thermophilic fungi which produce phytases are *Rhizomucor miehi (*ATCC22064), *Chaetomium thermophilum* (ATCC58420), *Thermomucor indicae-seudaticae* (ATCC28404) and *Myceliophthora thermophila (*ATCC48102) [Mitchell *et al.*, 1997], *Aspergillus fumigatus* (Pasamontes *et al.*, 1997b) *Rhizomucor pusillus* (Chadha *et al.*, 2004), *Thermoascus aurantiacus* (Nampoothiri *et al.*, 2004), *Thermomyces lanuginosus* (Berka *et al.*, 1998; Gulati *et al.*, 2007) and *Sporotrichum thermophile* (Singh and Satyanarayana 2006 a;b; 2008 a;b;c).

Thermophilic fungal phytases have been produced both by solid-state fermentation (SSF) and submerged fermentation (SmF) (Chadha *et al.*, 2004, Singh and Satyanarayana 2006 a; b; 2008 a; b;c) [**Table 1**]. Although commercial phytases have been reported using submerged fermentation (SmF), nowadays SSF is used because it has certain advantages over SmF (Pandey *et al.*, 1999; Pandey *et al.*, 2001). Chadha *et al.* (2004) isolated thermophilic fungi from composts and soils and screened *Rhizomucor pusillus, Scytalidium thermophilum, Melanocarpus albomyces, Chaetomium thermophile and Thermomyces lanuginosus* for phytase production. Among all the thermophilic fungal isolates, *Rhizomucor pusillus* produced highest level of phytase in the basal medium containing asparagine and corn steep liquor as nitrogen sources along with some micronutrients supplemented with wheat bran as an inducer after 48 h of incubation at pH 6.0 and 50 °C. Further optimization of phytase

Table 1. Sources and culture conditions for phytase production by thermophilic moulds.

Thermophilic mould	Temp.	pH	Fermentation	Reference
Myceliophthora thermophila	45	5.5	SmF	Mitchell *et al.* (1997)
Thermomyces lanuginosus	37	6.0	SmF	Berka *et al.* (1998)
Aspergillus fumigatus SRRC 322	37	5.0	SmF	Mullaney *et al.* (2010)
Thermoascus aurantiacus	45	5.5	SmF	Nampoothiri *et al.* (2004)
Rhizomucor pusillus	50	8.0	SSF	Chadha *et al.* (2004)
Myceliphthora thermophila Lomy 713	45	6.0	SSF	Hassouni *et al.* (2006)
Sporotrichum thermophile	45	5.0	SSF	Singh and Satyanarayana, (2006a)
Thermomyces lanuginosus TL-7	45	5.5	SSF	Gulati *et al.* (2007)
Sporotrichum thermophile	45	5.0	SmF	Singh and Satyanarayana (2008a)

production using the Box-Behnken factorial design resulted in improved phytase production. *Thermoascus aurantiacus* TUB F43 also produced phytase in semi-synthetic medium containing glucose and starch as carbon sources and peptone as a nitrogen source at 45 °C temperature, pH of 5.5 after 72 h of fermentation (Nampoothiri *et al.*, 2004). Mitchell *et al.* (1997) screened various thermophilic fungi for phytase production, and cloned and over-expressed the phytase of the thermophilic mould *Myceliophthora thermophila* in *Aspergillus niger*. *A. fumigatus* secreted a heat-stable phytase that was able to withstand temperatures up to 100 °C over a period of 20 min (Pasamontes *et al.*, 1997a). The phyA gene for an extracellular phytase from the thermophilic mould *Thermomyces lanuginosus* was cloned and heterologously over-expressed in *Fusarium venenatum* (Berka *et al.*, 1998).

Pasamontes *et al.* (1997b) reported cloning of a phytase gene from *Talaromyces thermophilus* having 61% sequence homology with that of *Aspergillus niger*. Hassouni *et al.* (2006) studied phytase production by *Myceliphthora thermophila* in solid-state fermentation using sugarcane bagasse, and maximum phytase production was recorded at 45°C and pH 6.0 after 36 h of incubation at a moisture level of 70 %. Phytase production by the thermophilic mould *Thermomyces lanuginosus* TL-7 was optimized using wheat bran as a substrate in SSF using a Box-Behenken factorial design of response surface methodology, and it resulted in maximum phytase production (Gulati *et al.*, 2007). Four thermophilic fungal strains including two strains each of *Sporotrichum thermophile* and *Humicola lanuginosa* were selected and screened for phytase production on sesame oil cake and wheat bran in solid state fermentation (Singh and Satyanarayana, 2006a). Phytase secretion by *Sporotrichum thermophile* was the highest in the sesame oil cake followed by that in the wheat bran and the mustard oil cake. The mould secreted maximum phytase levels at 45 °C, a substrate to moisture ratio of 1:2.5 and an a_w of 0.95 after 120 h. The supplementation of solid medium with glucose and ammonium sulfate further enhanced phytase secretion. *Sporotrichum thermophile* also secreted phytase in submerged fermentation in the synthetic medium containing starch, glucose, peptone and source at 45 °C and at pH 5.0 after five days of fermentation. Optimization of phytase production using statistical designs also resulted in a two-fold improvement in phytase production (Singh and Satyanarayana, 2006b). phytic acid along with micronutrients (Singh and Satyanarayana, 2008b) and in a cost-effective cane molasses medium supplemented with ammonium sulfate as a nitrogen source.

PURIFICATION AND CHARACTERIZATION OF THE THERMOPHILIC FUNGAL PHYTASES

Thermophilic fungal phytases have been purified using established methods such as salt/solvent precipitation followed by chromatographic separation (Wyss *et al.*, 1999; Wang *et al.*, 2007; Singh and Satyanarayana, 2009) . The phytases of the thermophilic moulds reported till now belong to the class of histidine acid phosphatases (HAP) (Singh and Satyanarayana, 2009). Phytases from thermophilic moulds are proteins with high molecular-weight proteins from 50 to 456 kDa (**Table 2**). The molecular sizes of thermophilic fungal phytases are smaller when compared to those of bacteria, yeast and mesophilic moulds except that of *Sporotrichum thermophile* which is a 456-kDa, glycosylated protein (Singh and Satyanarayana, 2009). Usually the phytases of thermophilic fungi are stable in acidic pH except for that of *Thermomyces lanuginosus*, which shows optimal activity at pH 3.0 and 7.5 (Berka *et al.*, 1998) [**Table 2**]. The phytase of *Aspergillus fumigatus* hydrolyzed

Table 2. Biochemical characteristics of phytases of thermophilic moulds

Source	MW (kDa)	pH_{opt}	T_{opt}	Reference
Myceliopthora thermophila	-	6.0	37	Mitchell *et al.* (1997)
Thermomyces lanuginosus	60	7.0	65	Berka *et al.* (1998)
Rhizomucor pusillus	-	5.4	70	Chadha *et al.* (2004)
Thermoascus aurantiacus	-	5.0	55	Nampoothiri *et al.* (2004)
Myceliophthora thermophila	-	5.5	70	Hassouni *et al.* (2006)
Thermomyces lanuginosus TL-7	54	5.0	70	Gulati *et al.* (2007)
Aspergillus fumigatus	88	5.5	55	Wang *et al.* (2007)
Sporotrichum thermophile	456	5.5	60	Singh and Satyanarayana, 2009

phytic acid optimally at pH 5.5 – 6.5, whereas p-nitrophenyl phosphate was optimally hydrolyzed at pH 5.0. Phytases from thermophilic moulds show optimal activity in the temperature range of 50 – 80 °C (**Table 2**). Thermophilic fungal phytases are highly thermostable than their mesophilic counterparts. An extremely thermostable phytase from *Aspergillus fumigatus* has been reported by Pasamontes *et al.* (1997a).The phytase of *Sporotrichum thermophile* retained 100 % its activity at 60 °C for 5 h with a $T_{1/2}$ of 16 h at 60 °C and 90 min at 80 °C. Phytases with broad substrate specificity are highly suited for food and feed applications (Wyss *et al.*, 1998). In comparison to bacterial phytases, which show specificity only towards phytate, the phytases of thermophilic moulds show broad substrate specificity with the highest affinity for phytate (Berka *et al.*, 1998; Wyss *et al.*, 1999; Chadha *et al.*, 2004; Gulati *et al.*, 2007; Wang *et al.*, 2007; Singh and Satyanarayna, 2009). The K_m value of the thermophilic fungal phytases reported till date ranges between 0.010 and 0.650 mM albeit low phytate K_m values have been reported for the phytases from *Thermomyces lanuginosus* (0.005 mM) (Chadha *et al.*, 2004). Metal ions generally do not show any major effect on the phytases from thermophilic moulds. The activity of the *Sporotrichum thermophile* (Singh and Satyanarayana, 2009), *Aspergillus fumigatus* (Wang *et al.*, 2007) and *Thermomyces lanuginosus* TL-7 (Gulati *et al.*, 2007) was inhibited moderately in the presence of metal ions. EDTA affects *Aspergillus fumigatus* phytase only, but no effect on other thermophilic fungal phytases (Gulati *et al.*, 2007). The phytases of *Sporotrichum thermophile* and *Thermomyces lanuginosus* are resistant to inactivation by detergents, organic solvents, chaotropic and oxidizing agents (Gulati *et al.*, 2007; Singh and Satyanarayana, 2009). The activity of *Sporotrichum thermophile* phytase was not affected by the tested organic solvents (Singh and Satyanarayana, 2009). Among all the surfactants, the tweens stabilize phytase of *Sporotrichum thermophile* (Singh and Satyanarayana, 2009) and *Thermomyces lanuginosus* (Gulati *et al.*, 2007), whereas SDS has shown inhibitory action on the phytase (Singh and Satyanarayana, 2009). The phytases for applications in the food and feed industries should be resistant to the action of the proteases present in the gastrointestinal tract. Thermophilic fungal phytases are relatively resistant to proteases when compared to the phytases of mesophilic microbes (Wang *et al.*, 2007; Singh and Satyanrayana, 2009). The phytase from the thermophilic mould *Aspergillus fumigatus* WY-2 was found to be insensitive to pepsin, and it retained about 90 % activity after protease treatment (Wang *et al.*, 2007). An extracellular HAP phytase of *Sporotrichum thermophile* is also resistant to pepsin, trypsin and sodium taurocholate (Singh and Satyanrayana, 2006b).

CLONING AND OVER-EXPRESSION OF THE THERMOPHILIC FUNGAL PHYTASES

Phytases are biotechnologically important, and hence, significant progress has been made on the biochemistry and molecular biological aspects of phytases in recent years. Attempts have been made to enhance the production of phytase economically that led to the use of recombinant DNA technology. Pasamontes *et al.* (1997b) cloned the phytase gene of the thermophilic moulds *Talaromyces thermophilus* and *Emericella nidulans.* The enzymes encoded by the *E. nidulans* and *Talaromyces thermophilus* sequences consisted of 463 and 466 amino acids, respectively, both of the predicted amino acid sequences displayed high identity to already reported phytases. By modeling all available fungal phytases, 21 conserved amino acids in fungal phyA phytases have been identified to form the substrate pocket. Potential glycosylation sites have been identified and compared with that of *Aspergillus niger.*

The phyA gene encoding an extracellular phytase from the thermophilic fungus *Thermomyces lanuginosus* has been cloned and expressed in *Fusarium venenatum* The product encoded was a primary translation product of 475 amino acids, which including a putative signal peptide of 23 amino acids and propeptide of 10 amino acids. The phytase showed a limited sequence identity with that of *Aspergillus niger* (Berka *et al.*, 1998). A phytase gene of 1.4 kb encoding the *A. fumigatus* phytase was expressed in *Pichia pastoris* as an active extracellular phytase The properties exhibited by the recombinant enzyme showed similarities to the same enzyme expressed in *Aspergillus niger.* The enzyme showed resistance to pepsin digestion but was sensitive to high levels of trypsin (Rodriguez *et al.*, 2000). The phytase gene (phyA) encoding a heat-stable phytase was cloned from *A. fumigatus* and was over-expressed in *A. niger.* The enzyme displayed high activity with phytic acid as a substrate in the pH range between 2.5 and 7.5, and on 4-nitrophenyl phosphate in the pH range between 3 and 5. This phytase could withstand temperatures up to 100°C over a period of 20 min retaining 90 % of its activity (Pasamontes *et al.*, 1997a).

A gene encoding a novel phytase from *Myceliophthora thermophila* was over-expressed in *Aspergillus niger.* The encoded phyA phytase protein showed 48 % identity to phyA of *A. niger* and had 21–29% identity with other histidine acid phosphatases (Mitchell *et al.*, 1997). A novel thermostable phytase gene from *A. fumigatus* WY-2 was expressed in *Pichia pastoris* GS115. The 1459 bp gene encoded a polypeptide of 465 amino acids. The recombinant phytase was purified to homogeneity and biochemically characterized. The purified enzyme had a molecular mass of 88 kDa with a specific activity of 51 Umg^{-1} (Wang *et al.*, 2007).

BIOPHYSICAL STUDIES OF THERMOPHILIC FUNGAL PHYTASE

Dendogram of the amino acid sequences of thermophilic fungal phytases is shown in **Fig. 2**. The protein sequence alignments from different thermophilic moulds and mesophilic fungi, bacteria and plants have shown the presence of a conserved heptapeptide (RHGXRXP) characteristic of histidine acid phosphatases in the sequence of all thermophilic and mesophilic moulds (Singh and Satyanarayana, 2011). On the contrary, this sequence was absent in bacterial (*Bacillus subtillis*) and plant (*Zea mays*) phytases. Four crystal structures of *Aspergillus fumigatus* phytase were obtained at a resolution higher than 1.7 A° using X-ray crystallography (Liu *et al.*, 2004). The evaluation of these structures revealed that a water molecule attacks the phosphamide bond during the process of hydrolysis. Crystal structure of the *Aspergillus fumigatus* phytase was also determined at a 1.5 Å resolution to resemble the other reported structures (Xiang *et al.*, 2004). Six N-glycosylation sites have been identified from the structure. The residue His59 was found to be partly phosphorylated, which confirmed the two-step catalytic mechanism involved in the acid histidine phosphatase family.

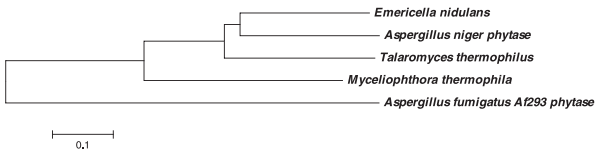

Fig.2. Dendogram showing phylogenetic relationships among amino acid sequence of phytases of thermophilic fungi

THE ROLE OF DISULFIDE BRIDGES IN PHYTASES

Disulfide bridges (DBs) are often found in extracellular proteins. Their role is to contribute to the proper folding of the molecule and preserve its 3-D structure (Abkevich and Shakhnovich, 2000). The site-directed mutagenesis has been utilized to investigate the contributions of individual DBs in several enzymes (Zhu *et al.*, 1995; Liu *et al.*, 2004; Hagihara *et al.*, 2002). The importance of DBs to phytases activity in fungal HAPhys has been already reported by (Ullah and Mullaney, 1996; Wang *et al.*, 2007). Berkmen *et al.* (2005) has also shown the role of a protein disulfide isomerase (DsbC) in the formation of nonconsecutive DB bonds by generating cysteine to serine mutations in *Escherichia coli* phytase. Mullaney *et al.* (2010) reported five DBs in *Aspergillus niger* phytase and mutated all the five DBs using site directed mutagenesis. One of the mutants, C71S became inactive while for other mutants, temperature pH and catalytic activity was significantly altered. The DB mutants slowed the catalytic rates, increased K_m and decreased K_{cat} indicating that their affinity for phytate had decreased noticeably. The properties of these misfolded phytases might result from the interaction between amino acids not normally in close proximity. This predicts that the mutation has changed the structure of catalytic center including the substrate binding site. X-ray crystallography studies of these mutants would further help in comprehending the relationship between the structural distortions and kinetic properties (Mullaney *et al.*, 2010).

THE APPLICATIONS OF THERMOPHILIC FUNGAL PHYTASES

The two important areas in which the applications of phytase have increased enormously include: a) elimination of phytate in the food and feed industries, and b) the preparation of myo-inositol phosphates for biochemical signaling investigations. Phytases also produce isomers of myo-inositol phosphates for physiological and kinetic studies. Some isomers of myo-inositol phosphates have shown metabolic effects like prevention of renal stone formation (Ohkawa *et al.*, 1984), and emendation of heart disease by controlling atherosclerosis and hypercholesterolemia (Potter, 1995). In addition D-myo-inositol-(1,2,6)-triphosphate has been shown to prevent the complications from diabetes and also in the treatment of persistent inflammation and cardiovascular disease (Siren *et al.*, 1991).

Phytases can be applied to food industry to produce foods with improved nutritional value and health benefits without change in properties (Greiner and Konietzny, 2006). The use of phytase can be further extended to the soil improvement, aquaculture, plant growth promotion and in the synthesis of peroxidase (due to a structural similarity of the active site of peroxidase and fungal phytase). The biotechnological applications of phytases from the thermophilic moulds are discussed below.

a) Applications in dephytinization of food ingredients

Phytic acid is the main storage form of phosphorus present in oil seeds, nuts, cereals, and legumes, which are commonly used as sources of food by monogastric animals such as poultry, fishes, pigs and humans. Thus it becomes necessary to breakdown this phytic acid because they lack adequate levels of phytase in their gastrointestinal tract. The supplementation of animal feed with *A. fumigatus* phytase resulted in the liberation of inorganic phosphate from the feed sample (Wyss *et al.*, 1999). Also the phytase of *Sporotrichum thermophile* can dephytinize wheat flour, soymilk and sesame oil cake well, by reducing phytic acid and liberating inorganic phosphate with optimal activity at 60 °C (Singh and Satyanarayana, 2006 a;b; 2008a;b).

b) Application in bread-making

The supplementation of phytase to the dough during fermentation could aid the reduction of phytic acid with improved levels of proteins and reducing sugars. When the *Sporotrichum thermophile* phytase was added to the dough, it liberated a higher amount of inorganic phosphate, sugars and soluble protein as compared to

the control bread made with commercial enzymes. The phytic acid content of wheat flour also decreased with supplementation of phytase (Singh and Satyanarayana, 2008b).

c) Hydrolysis of insoluble phytates in plants

Plants can not directly utilize organic phosphorus compounds, and therefore, organic phosphorus compounds in the soil must first be dephosphorylated by phosphatases or phytases prior to the incorporation (Tang *et al.*, 2006; Singh and Satyanarayana, 2010). Phytate in the soil is either adsorbed to soil components, or is complexed with cations. The phytase of *Sporotrichum thermophile* can hydrolyze a variety of insoluble phytates (Singh and Satyanarayana, 2010). Inorganic phosphate from the Ca^{2+}, Mg^{2+} and Co^{2+} phytates are released more efficeinetly than the Al^{3+}, Fe^{2+}, Fe^{3+} and Zn^{2+} salts of phytic acid. The rate of hydrolysis of these salts was higher at 60 °C than at 26 °C (Singh and Satyanarayana, 2009). Tang *et al.* (2006) had also observed a similar trend for the hydrolysis of insoluble phytate salts by three types of phytases (fungal, bacterial and wheat phytases). The main mechanism for removing free phytate could be the formation of metal complexes by these acids, because in comparison to malate and oxalate, carrying two carboxyl groups each, citrate carries three carboxyl groups, and thus, it has a higher capacity to form complexes with cations (Tang *et al.*, 2006).

d) Promotion of plant growth

The deficiency of phosphorus in soil is an international crisis for agricultural production. On the other hand, most soils do contain significant amounts of total soil phosphorus in inorganic and organic forms (Tang *et al.*, 2006; Singh and Satyanarayana, 2010). Phytic acid is the leading form of soil phosphorus in the form of organic phosphorus. Thus phytate is the principle stored form of phosphorus in plant seeds and is a proven anti-nutritional factor and a chief phosphorus pollution source in animal manure. There is an adequate literature on the role of phytase in improving the plant growth and phosphate reduction. The growth and also the inorganic phosphate content of plants were found to be better than control plants supplied with inorganic phosphate (Singh and Satyanarayana, 2010). The effect of different concentrations of sodium phytate was evaluated in liquid cultures. Sodium phytate (5 mg per plant) was found to be appropriate for liberating enough phosphorus for the growth of a seedling. Plant growth, root/shoot length and the inorganic phosphate content of the test plants were higher than that of the control plants. An enzyme dose of 20 U per plant was appropriate to release enough inorganic phosphate to sustain plant growth. Plant growth, root/shoot length and the inorganic phosphate content of test plants were higher than control (Singh and Satyanarayana, 2010). The compost prepared by the combined activity of the native microflora of wheat straw along with *Sporotrichum thermophile* promoted the growth of plants (Singh and Satyanarayana, 2010). The inorganic phosphate content of wheat plants was also found to be higher in contrast to those cultivated on compost, prepared with either only native microflora or *Sporotrichum thermophile.* The difference in plant growth-promoting effect could be seen after 10 days, and it further became prominent after 30 days (Singh and Satyanrayana, 2006; 2010).

e) Role of phytase in combating environmental pollution and aquaculture

Phosphorus is an essential component in animal and plant production. Ruminants support microflora which releases enzymatically the inorganic phosphorus from phytic acid. However, monogastrics such as pigs, chickens and humans, do not produce adequate levels of phytase.Therefore, the phytic acid phosphorus is biologically unavailable, and the phytic acid is thus, excreted in the faeces (Mullaney *et al.*, 2010). Finally this phytic acid is enzymatically cleaved by soil and water-borne microorganisms. The released phosphorus is transported into water bodies and causes eutrophication which results in oxygen depletion due to excessive algal growth. Pretreatment of animal feed with phytases in this manner will increase the availability of inorganic phosphorus, thereby improving the nutritional value of food and will also help in eradication of phosphorus pollution (Vats *et al.*, 2005). Phytases are well known to reduce pollution caused by the excess of phosphorus accumulation in the soil and water (Nahm, 2002). The phosphorus excretion can be decreased by 30% by replacing feed phosphate with phytase. The addition of microbial phytase in the diet of fish can help in surmounting this problem. It makes the chelated phosphorus accesible to fish, and decreases the fecal excretion, which in turn reduces the environmental pollution. The use of phytase in feed lowers the necessity of mineral supplementation, decreasing the cost of feed.

CONCLUSIONS AND FUTURE PROSPECTS

Phytases are well known for their essential role in reducing the phosphorus levels in manure and diminishing the need for the addition of phosphorus in the nutritional regime of monogastrics. The use of phytases to supplement animal feed is rising very fast, because of their environment friendly nature and their potential applications in human nutrition. None of the microbial phytases reported till date have all the desired features for biotechnological applications. The phytases from different sources possess different biochemical properties. Therefore, there is a need to find novel phytases from thermophilic microbes especially from thermophilic moulds. The thermophilic moulds are well known for secreting a variety of hydrolytic enzymes including phytase. The phytases of thermophilic moulds show a broad range of substrate specificity, which is important for the food and feed industries for ameliorating the nutritional value of foods and feeds.

Thermophilic mould phytases are thermostable, acid-stable and protease-insensitive, and they could be useful as food and animal feed supplements and in soil amendment for plant growth promotion. Phytase genes from thermophilic moulds can be cloned, over-expressed and scaled up to bring down cost of production and for making it economical. Transgenic plants expressing thermophilic mould phytases would help in producing grains with reduced levels of phytic acid. All the strategies could be used for generating unique phytases for improving the nutritional value of foods and feeds, and combating environmental phosphorus pollution. Thermophilic fungal phytases present a multitude of opportunities for producing and utilizing them. Extensive efforts are, however, needed from scientists worldwide in this endeavour.

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