Heterologous expression of yeast and fungal phytases: Developments and future perspectives

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The phytases, which hydrolyse inositol hexaphosphate (IP6), are used as animal feed supplements for ameliorating phosphorus availability and assimilation, and mitigating anti-nutrient effects of phytates. Phytases have been reported from several yeasts and fungi. The phytase titres attainable from the wild yeast and fungal strains are very low and, therefore, attempts have been made to clone and overexpress them in heterologous hosts. The enzymes have also been improved by site directed mutagenesis and directed evolution to suit the applications. This review focuses on the progress made so far in cloning and expression of yeast and fungal phytases in heterologous hosts and the research needs.

Keywords: Acid stability, heterologous expression, inositol hexaphosphate, phytase, recombinant phytase, thermostability

Introduction

Phytate [inositol hexaphosphoric acid (IP6)] is an organic form of phosphorus found in plant products, such as, cereal grains, legumes, oilseeds and others, in which it represents more than two-thirds of the total $P_1$. Phytates are not digested by monogastric animals and human beings because of inadequate levels of phytase in their gastrointestinal tract and thus pass through faeces. Soil and aquatic microbes act on phytates and release inorganic phosphate that, in areas of intensive animal rearing, leads to eutrophication of aquatic bodies. Due to the presence of six phosphate groups, phytic acid is a strong metal ion chelator (Fig. 1). Because of strong affinity to metal ions, phytates chelate various nutritionally important metal ions, such as zinc, iron, calcium, magnesium and others, and thus lower their bioavailability (Fig. 1). Phytate acts as an antinutrient that causes mineral deficiencies in populations where staple food is phytate-rich legumes and cereals. In the past few decades, attempts have been made to mitigate the problems caused by phytates in foods and feeds by supplementing them with microbial phytases.

In order to fulfil their P requirement, animal feeds are supplemented with other forms of phosphorus like calcium salts of phosphate. Such supplementation increases the overall feed cost and can culminate into a severe cause of water pollution. An effective solution to overcome poor phytate phosphorous utilization in monogastrics is supplementation of feeds and foods with exogenous phytase.

The recent reviews on phytases have dealt with the production, characteristics and applications of native phytases. This review focuses on the heterologous expression of fungal and yeast phytases and the characteristics of recombinant phytases, attempts made in ameliorating them by molecular approaches and their utility in dephytinization.

Phytate: A Major Form of Organic Phosphorous

Phosphorus is an essential nutrient for all living organisms and is the third most abundant mineral in
the animal body. This versatile element is found in all cells and participates in every metabolic pathway. Phosphorus plays diverse functions in living systems being part of cell membrane structure in the form of phospholipids, in energy transfer as ATP, an important element in nucleic acids and as an important element of bone structure\textsuperscript{13}. Life cannot be imagined without phosphorus\textsuperscript{14}.

In soil, phosphorus exists both in inorganic and organic forms. Phosphorus is abundant in soils but it is not present in a form readily available for uptake by plants. According to Lopez-Bucio and coworkers\textsuperscript{15}, 70\% of the cultivable land in the world is either acid or alkaline, in which phosphorus tends to form compounds, which are not readily available to plants\textsuperscript{16}. One of the most abundant forms of organic phosphorus in soil is inositol phosphates, which constitute up to 50\% of the organic phosphorus\textsuperscript{17}.

In mature seeds of the most traditional crops, about 75\% of the total phosphorus is found in the form of phytic acid (phytate P)\textsuperscript{18}, which is hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol. Phytic acid (IP6) is a major storage form of phosphorus in the plant tissues. Inositol pentaphosphate (IP5), tetra- (IP4) and triphosphate (IP3) are also called phytates. The molecular formula is C\textsubscript{6}H\textsubscript{26}O\textsubscript{32}P\textsubscript{6} with molecular mass of 660.04 g mol\textsuperscript{-1}. It represents a sum equivalent to about 50\% of all phosphorus applied as fertilizer worldwide\textsuperscript{19}, an amount that would satisfy a major fraction of animals’ dietary requirement for phosphorus.

The total phosphorus found as phytic acid phosphorus in grains fed to non-ruminants is simply wasted. 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 the increasing public concern regarding environmental protection and biotechnology, attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology. The addition of exogenous phytase to the diets can improve the assimilation of dietary phosphate that leads to amelioration in performance parameters different from those associated with an increase in the phosphate utilization\textsuperscript{27,29}. The interaction between protein and IP6 is dependent on pH. The binary complexes of protein-IP6 are formed at lower pH, while ternary complexes of protein-IP6- mineral form at pH near neutrality\textsuperscript{25,27,32}. The complex formation of minerals due to chelating property of phytate can reduce their involvement as cofactors of enzymes, particularly under suboptimal dietary levels.

According to \textit{in vitro} studies performed by Knuckles \textit{et al}\textsuperscript{23}, the esters of inositol phosphates inhibit digestibility of casein by proteolytic enzyme pepsin and the inhibition is dependent on the degree of phosphorylation of the inositol ring. Positive effects of phytate include protection from various types of cancers\textsuperscript{34}, which is mediated through inhibition of the cellular signal transduction\textsuperscript{35},

**Effects of Phytic Acid**

The presence of phytates exerts both negative and positive effects on nutritional and environmental health. Phytate-P is largely unavailable for assimilation by monogastrics due to lack of endogenous phytase, the enzyme that catalyzes the hydrolysis of phytate. Phytase secreted by microorganisms in the alimentary tract is effective in hydrolysing phytate in ruminant animals\textsuperscript{20}. Since microflora in monogastric animals and human beings is predominantly located in the large intestine, it can be understood that most of the vegetable phosphate liberated from phytate remains unabsorbed and excreted. Due to increase in the livestock population in various regions, manure applied to the soil is exceeding that leads to the accumulation of phosphate in soil\textsuperscript{21}. Excess soil phosphorous could lead to eutrophication of waters bodies and leaching of phosphate into ground water could also occur\textsuperscript{22}. Inositol hexaphosphate is reasonably reactive due to the presence of 12 dissociable protons with pK\textsubscript{a} values ranging from 1.5 to 10\textsuperscript{23}.

The reactivity of inositol phosphate isomers depends on the conformation and configuration of the molecule and the pH of its environment\textsuperscript{23}. The phosphorylated myoinositol (IP6) is a potent chelator of many mineral ions\textsuperscript{24,25}. As a metal chelator, it forms insoluble salts at intestinal pH that results in reduction in the availability of minerals for absorption in the body fluids\textsuperscript{26-28}. Furthermore, the availability of nutritionally important amino acids is also compromised in the presence of inositol hexaphosphate in the diet\textsuperscript{27,29}. Some \textit{in vitro} studies have shown that phytate-protein complexes are degraded very slowly by proteolytic enzymes\textsuperscript{30}, and even some enzymes, such as, pepsin, amylópsin, and amylase, are inhibited by phytates. Phytates may also interfere with the digestibility of lipid and starch\textsuperscript{31}.

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increase in activity of natural killer cells\textsuperscript{36}, stimulation of cell differentiation genes\textsuperscript{37,38} and antioxidant properties\textsuperscript{39}. Apart from this, phytate consumption also helps in the treatment of diabetes mellitus\textsuperscript{39,40}, coronary heart disease\textsuperscript{41} and dental caries\textsuperscript{42}. Phytates are known to reduce the chances of renal stone formation\textsuperscript{43}, HIV-1 infection\textsuperscript{44} and heavy metal toxicity.

**Methods of Reducing Phytic Acid Content in Foods and Feeds**

Dephosphorylation of phytate improves nutritional quality of diets as the removal of anionic phosphate groups from the IP6 reduces mineral chelating strength of phytate that results in the improvement of assimilation of essential minerals\textsuperscript{45}. Phytic acid content of food can be reduced by some physical (autoclaving, cooking & steeping), chemical (ion exchange & acid hydrolysis) and food processing (soaking, germination & fermentation) 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 this does not affect their nutritional value and, thus, phytases find a very important application in nutrition. The dephytinizing capacities of phytases depend upon the origin due to differences in their inherent phytate-degrading activities\textsuperscript{46}. The commercial phytase products have been mainly used as animal feed additives in diets, largely for swine and poultry, and to some extent for fish. In spite of its immense potential in processing and manufacturing of food for human consumption, there is no phytase product that has found its way to the market for human food application. Many researchers have reported a convincing improvement in food products by adding microbial phytase in food processing, for example, bread-making\textsuperscript{47,48}, plant protein isolates\textsuperscript{49}, corn wet milling\textsuperscript{50} and the fractionation of cereal bran\textsuperscript{51}.

**Phytase**

Global phosphate mineral deposits are non-renewable. Phosphorous captured in the form of phytate is an optional phosphorous source, which can be efficiently converted to available phosphorous by phytases. Phytases (myo-inositolhexaphosphate phosphohydrolase) hydrolyze phosphonooester bond of phytic acid and liberate myo-inositol and inorganic phosphates through various myo-inositol phosphate intermediates that mitigates its anti-nutritional properties. Phytases are synthesized in various microbes, plants and animals\textsuperscript{11,52–54}. Phytase is produced by a large number of microorganisms including bacteria, filamentous fungi and yeasts. Various yeast species have been reported to produce phytase either intracellularly (Cryptococcus laurentii strain AL\textsuperscript{55}), cell bound (Candida krusel\textsuperscript{56} & Pichia anomala\textsuperscript{57}) or extracellularly (Arxula adeninivorans\textsuperscript{58}, Kluyveromyces lactis\textsuperscript{59}, P. anomala\textsuperscript{59}, Rhodotorula gracilis\textsuperscript{60}, Saccharomyces cerevisiae\textsuperscript{61}, Schwanniomyces occidentalis\textsuperscript{62} & Torulaspora delbrueckii\textsuperscript{63}). Phylogenetic relationship among phytases of various yeast species is shown in Fig. 2. Fungi, such as Aspergillus niger\textsuperscript{63,64}, A. terreus\textsuperscript{64}, A. fumigatus\textsuperscript{65,66}, A. oryzae\textsuperscript{67}, A. caesipitosus\textsuperscript{68}, Emericella nidulans\textsuperscript{69}, Myceliophthora thermophila\textsuperscript{64} and Thermomyces lanuginosus\textsuperscript{60}, have been reported to produce phytases. The phylogenetic relationship among various fungal phytases is shown in Fig. 3.

**Classification of Phytases**

In consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (ICBN), Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) has classified phytases into three categories. 3-Phytases (EC 3.1.3.8) constitute the first category, which first hydrolyse the ester bond at the 3 position of myo-inositol hexakisphosphate, and are mainly of microbial origin. Second category comprises 6-phytases (EC 3.1.3.26), which first hydrolyse the ester bond at the position 6 of phytic acid content in foods and feeds by enzymatic hydrolysis using phytase is desirable, since this does not affect their nutritional value and, thus, phytases find a very important application in nutrition. The dephytinizing capacities of phytases depend upon the origin due to differences in their inherent phytate-degrading activities. The commercial phytase products have been mainly used as animal feed additives in diets, largely for swine and poultry, and to some extent for fish. In spite of its immense potential in processing and manufacturing of food for human consumption, there is no phytase product that has found its way to the market for human food application. Many researchers have reported a convincing improvement in food products by adding microbial phytase in food processing, for example, bread-making, plant protein isolates, corn wet milling and the fractionation of cereal bran.

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myo-inositol hexakisphosphate. These are typical for plants, although this has recently been reported in some Basidiomycete fungi. Third category contains 5-phytases, which start phytate hydrolysis at the D-5 position and, hence, are classified as 5-phytases. So far, only one enzyme has been reported from this class, which is an alkaline phytate-degrading enzyme from Lily pollen.

Based on the pH for enzymatic activity, phytases have been broadly classified into two major classes: acid phytases and alkaline phytases. Acidic phytases have been explored more compared to alkaline phytases because of their applicability in foods and animal feeds, their broader substrate specificity and acid stability. Besides two systems of classification, depending on their structure and catalytic mechanism, phytases are also classified as HAP (histidine acid phosphatase), BPP (β-propeller phytase), CP (cysteine phosphatase) and PAP (purple acid phosphatase).

HAPs (EC 3.1.3.8) are the most commonly studied phytases, which share a common active site motif (RHGXRXP), a catalytically active dipeptide (HD) and employ a two-step mechanism consisting of a nucleophilic attack on the phosphorous atom by the histidine of the active site motif, followed by hydrolysis of the resulting phospho-histidine intermediate. The positive charge of the guanido group of the arginine residue in the conserved tripeptide RHG interacts directly with the phosphate group in the substrate, thus making it more susceptible to nucleophilic attack; while the histidine residue serves as a nucleophile in the formation of covalent phosphohistidine intermediate. The aspartic acid residue (from the C-terminal HD-motif) protonates the group, leaving the substrate. The substrate specificity of all known HAPs for phytic acid varies from one to another.

BPPs (EC 3.1.3.8), first identified in Bacillus species, have six-bladed β-propeller structures. BPPs show a novel mechanism for hydrolyzing its substrate. Crucial amino acid moieties, which are involved in the conformation of two phosphate binding sites and six calcium binding sites, are conserved in all BPPs. This class of enzymes is mainly useful in aquaculture due to activity at neutral pH and at the lower body temperature of fish. Most freshwater monogastric and agastric fishes have a digestive tract with a neutral environment; this includes cyprinid fishes, a group of commercially important freshwater species. Thus, a phytase useful in aquaculture should have high activity at neutral gut pH and at the lower body temperature of fish.

A novel class of phytases, cysteine phosphatases, is predominantly produced by anaerobic bacteria of the rumen. In contrast to monogastrics, ruminants can utilize phytate from a plant-based diet more efficiently because of the presence of the bacteria in their rumen, which can hydrolyse phytate very efficiently. Yanke et al. have identified an anaerobic rumen bacterium Selenomonas ruminantium capable of producing phytase. The structure and proposed catalytic mechanism of this phytase suggested it to be a member of the cysteine phosphatase (CP) superfamily. Its deduced amino acid sequence contains the active site motif HCXXGXXR (T/S) and other substantial similarities with protein tyrosine phosphatase (PTP), a member of the CP group. Cysteine phytases are active at acidic pH, mostly at pH 4.5. These are, therefore, classified as acid phytases (pH optima of 4.0-5.0).

Purple acid phosphatases (PAP) are binuclear metallohydrodrolases that are found in plants, animals...
and fungi. PAPs harbour a binuclear metal centre of Fe(III)–M(II), where M can be Fe, Zn or Mn. A tyrosine to Fe(III) charge transfer transition imparts PAPs their characteristic purple colour. The type of bivalent metal ion is dependent on the type species and is Fe(II) in animals and Zn(II) or Mn(II) in plants. PAPs have been expressed in various eukaryotes and prokaryotes, and fungi. A bacterial PAP has not yet been characterized. Hegeman and Grabau isolated PAPs from the cotyledons of germinating soybeans (GmPhy). GmPhy has the active site motif of a PAP. The mol wt of GmPhy (70-72 kDa) is in the range of mol wt of other plant PAPs. The GmPhy is the only known PAP reported to have significant phytase activity. A. niger NRRL 3135 PAP has been reported to display only a minimum ability to utilize phytate as a substrate.

**Heterologous Expression of Microbial Phytases**

Heterologous expression systems offer several advantages in protein expression for industrial applications. The choice of a heterologous host depends on the objective of the study. Among various systems, bacteria (Escherichia coli, Streptomyces sp. & Lactobacillus lactis), yeast (S. cerevisiae & P. pastoris), fungi (Aspergillus spp.), animal cells (insect cell lines, e.g., baculovirus in Autographa californica (moth); mammalian cell lines, e.g., Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cell line) and plant systems can be used for heterologous protein expression. Microbes are known to produce phytases with broad substrate specificity, but most of them exhibit low specific activities. In the enzyme industry, low yield and high cost of enzyme production have been constraints in the direct use of theses enzymes in animal diets. With the advancements in heterologous microbial expression systems, large-scale phytase production is now possible at an affordable cost.

Presently in India, dicalcium phosphate (DCP) is used in animal feeds to fulfill phosphorous requirement. The substitution of DCP with phytase could replace 50-60% DCP. It has been estimated that 1 kg DCP can be replaced with 25 g of phytase and, thus, the demand for phytase in animal feed industry could be around 4000 tonnes per year. In order to reduce the phytase production cost and substituting phytase produced by microbial fermentation with that of plant-produced phytase, a number of attempts have been made to express bioactive phytases in plants. Phytases from different sources have been expressed in various transgenic plants, such as tobacco leaf, tobacco seed, alfalfa leaf, Arabidopsis roots, rice, and wheat grains, and many others (Table 1). Fungal and yeast phytases with the desirable properties have also been cloned and expressed in various microbial expression systems (Table 2).

**Conserved Amino Acid Sequences in Phytases**

Primer design is greatly assisted if conserved sequences and sequences exhibiting similarity to that of known phytase can be identified. Histidine acid phytases have conserved heptapeptide sequence (RHGXRP) and a dipeptide (HD) in the primary structure of phytase protein. Figs 4 and 5 show the positions of these conserved heptapeptide and dipeptide sequences in fungal and yeast phytases. Acid phosphatases from both prokaryotes and eukaryotes have been shown to share two regions of primary sequence similarity, each centred around a conserved histidine residue. According to SWISS-PROT protein domain data base, there are two regions: [LIVM]-X-[LIVMA]-X(2)-[LIVM]-X-R-H-[GN]-X-R-X-[PAS] and [LIVMF]-X-[LIVMFFAG]-X(2)-[STAG]-H-D-[STANQ]-X-[LIVM]-X(2)-[LIVMFY]-X(2)-[STA]. Disulphide bridges increase both stability and heat tolerance in proteins. Mullaney and Ullah have reported that fungal histidine acid phosphatases exhibiting phytase activity contained a conserved eight cysteine motif, which appears to participate in disulphide bond formation. Fig. 6 compares the position of eight cysteine residues in A. niger HAP with HAP of other yeast and fungal phytases.

**Approaches Used For Amplification of Phytase Genes**

The cloning of eukaryotic genes is difficult as compared to that of prokaryotic genes as most of the former are interrupted by introns, which are removed during RNA processing. The cloning of a eukaryotic gene with an intron will alter the reading frame of the gene and will result in the expression of a non-functional protein. To get rid of introns, the researchers have used different strategies for cloning and expression of eukaryotic genes. Lassen and coworkers constructed and screened cDNA libraries of four basidiomycete fungi (Agrocybe pediades, Ceriporia sp., Peniophora lycii & Trametes pubescens) for the selection of phytase encoding cDNA. The libraries were constructed in E. coli DH10B and then S. cerevisiae was transformed by the recombinant plasmids, and the clones were subsequently screened for phytase activity. Finally all
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<td>Expression was under control of CaMV35S promoter but all the constructs were modified for extracellular secretion using an extracellular targeting sequence from the carrot extension (ex) gene</td>
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</tr>
<tr>
<td>A. niger, P. lycii</td>
<td>Nicotiana tabacum</td>
<td>Roots</td>
<td></td>
<td></td>
<td>108</td>
</tr>
</tbody>
</table>

Phytases were expressed in *A. oryzae*. Berka *et al*° constructed genomic DNA library using bacteriophage cloning vector λZipLox and *E. coli* Y1090ZL cells. The screening of the library of recombinant plaques was done by plaque hybridization method using the radio-labelled phytase probe. The identified phytase gene was cloned in pDM181 expression vector for phytase expression in *Fusarium venenatum* under *F. oxysporum* trypsin gene promoter. Phytase genes of *E. nidulans* and *Talaromyces thermophilus* were also identified by genomic DNA library construction and southern hybridization.° Genomic DNA library was constructed using pBluescriptII KS (-) and *E. coli* TG-1 cells and confirmed the presence of an intron in phytase genes by sequence analysis. Xiong *et al*°
Table 2—Heterologous expression of yeast and fungal phytases in microbial hosts

<table>
<thead>
<tr>
<th>Source of the gene</th>
<th>Gene</th>
<th>Host used</th>
<th>Vector used</th>
<th>Temp. Opt. (°C)</th>
<th>pH Opt</th>
<th>Specific activity (U mg⁻¹)</th>
<th>References</th>
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<tbody>
<tr>
<td>Phytases from yeasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Debaryomyces castellii</em> CBS 2923</td>
<td>PhytDc</td>
<td><em>P. pastoris</em> X33</td>
<td>pPICZα B &amp; pGAPZα B</td>
<td>60</td>
<td>4–4.5</td>
<td>182</td>
<td>109</td>
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<td><em>Kodamaea ohmeri</em> BG3</td>
<td>Phy1</td>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>pET24a (+)</td>
<td>65</td>
<td>5</td>
<td>16.5</td>
<td>110</td>
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<td><em>P. anomala</em></td>
<td>Pphy</td>
<td><em>S. cerevisiae, A. adeninivorans &amp; Hansenula polymorpha</em></td>
<td>pYES2.1-V5-His-TOPO, Xplor1-URA3-SwARS-FMD, pBS-TEF-PHO5</td>
<td>60</td>
<td>4</td>
<td>1.56</td>
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<td><em>P. anomala</em></td>
<td>PPHY</td>
<td><em>P. pastoris</em></td>
<td>pPICZαA</td>
<td>60</td>
<td>4</td>
<td>-</td>
<td>112</td>
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<tr>
<td>Phytases from fungi</td>
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<tr>
<td><em>A. ficuum</em></td>
<td>-</td>
<td><em>E. coli</em></td>
<td>pIAβ 8 &amp; pGAPZαA</td>
<td>50</td>
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<td><em>A. ficuum</em> (BCRC 32870)</td>
<td>phyA</td>
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<td><em>P. pastoris</em> GS115</td>
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<td><em>S. cerevisiae</em></td>
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<td><em>S. cerevisiae</em></td>
<td>pYES2</td>
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<td>phyI</td>
<td><em>P. pastoris</em></td>
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<td><em>P. pastoris</em> X33</td>
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<td>-</td>
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<td><em>A. niger sp.</em></td>
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<td><em>P. pastoris</em></td>
<td>pPICZα A</td>
<td>55</td>
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<td><em>E. nidulans, Talaromyces thermophilus</em></td>
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<td>pBluescript II KS (-)</td>
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<tr>
<td><em>Neosartorya spinosa</em> BCC 41923</td>
<td>-</td>
<td><em>P. pastoris</em> KM71</td>
<td>pPIC9K</td>
<td>50</td>
<td>5.5</td>
<td>38.62</td>
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<td><em>Obesumbacterium proteus</em></td>
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<td><em>E. coli</em></td>
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<td>40–45</td>
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<td><em>P. lycii</em> 6-phytase</td>
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<td><em>P. pastoris</em></td>
<td>pPIC9K</td>
<td>50</td>
<td>4.5</td>
<td>-</td>
<td>125</td>
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<tr>
<td><em>P. lycii, Agrocybe pediades, Ceriporia sp. &amp; Trametes pubescens</em></td>
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<td><em>A. oryzae</em></td>
<td>pHD414</td>
<td>-</td>
<td>-</td>
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</table>

synthesized a gene sequence based on nucleotide sequence of the native phytase of *P. lycii*; 20 oligonucleotide sequences were used to synthesize complete gene in a single PCR reaction. This synthetic gene was subsequently amplified by using end primers and used for expression in *P. pastoris*. In an attempt to express *PhyA* gene of *A. fumigatus* and *A. awamori* in *A. awamori*, Martin et al. employed pGEXA expression vector. The genomic DNA was used for the amplification of *PhyA* genes from both the fungi. The mRNA was captured using a poly (dT) column and prepared cDNA by RT-PCR for amplifying the phytase gene of *A. niger* 113. While Li et al. used Rapid Amplification of C-DNA Ends- (RACE-) PCR for amplifying the full-length phytase gene from *Kodamaea ohmeri* BG3. For amplification of *P. anomala* phytase gene (PPHY), specific primers (which were designed based on consensus sequence of phytases) were used to amplify partial gene sequence, which was followed by restriction ligation of genomic DNA to use reverse primers for full length gene amplification. Heterologous Expression of Fungal and Yeast Phytases

The addition of microbial phytases to food and feed is a usual practice to mitigate environmental and nutritional problems caused by phytates. This practice requires production of high titres of phytase by the microbial sources. Heterologous expression offers solution to this problem by generating recombinant
**Fig. 4**—Multiple sequence alignment (MSA) of amino acid sequences of fungal phytases [phytase of *Thielavia heterothallica* (ID PHYA_THIHE, AC O00107), *T. thermophilus* (IDvO00096_TALTH, AC O00096), *A. niger* (ID PHYA_ASPNG, AC P34752), *A. fumigatus* (ID PHYA_ASPFU, AC O00092)] have been retrieved from Uniprot (Universal Protein Resource database, website: http://www.uniprot.org). The conserved heptapeptide and dipeptide are shown in black boxes.

Microbes capable of producing very high enzyme titres as compared to their wild type counterparts. Watanabe et al.\(^\text{131}\) reported that a recombinant *Pichia* strain could produce 23 g L\(^{-1}\) of recombinant phytase. The yield of *A. fumigatus* phytase expressed in *Pichia* was 729 mg L\(^{-1}\) of purified protein, exhibiting specific activity of 43 U mg\(^{-1}\)\(^\text{132}\). *P. pastoris* offers several advantages over other microbial expression hosts, such as, genomic integration of the transgenes, availability of expression vectors where cheap methanol can be used both as inducer and carbon source, no requirement of antibiotic once the stable integrant has been constructed and very high culture densities can be achieved for high protein expression\(^\text{133}\). This budding yeast offers utilization of various alternative promoters, such as, pAOX1, pFLD1, pEPEX8, pYPT1, pDHAS, pICL1, pTEF and pGAP, which are regulated by the carbon sources incorporated in the production medium\(^\text{134-136}\) and a few, such as, pFLD1, are regulated by nitrogen source\(^\text{137}\).

Apart from microbial production of phytases, the recombinant plants harbouring phytase gene offer an added advantage as these genetically modified plants can be used as feed material without any need to add phytase from external source. Maize seeds are a major ingredient of commercial feed used in pork and poultry\(^\text{138}\). Recombinant maize has been produced by
Fig. 5—Multiple sequence alignment of amino acid sequences of different yeast phytases [phytases of *S. cerevisiae* Fosters O (GenBank Acc. no.: EGA63258.1), *S. capriotii* (GenBank Acc. no.: ABN04184.1), *D. castellii* (PDB: 2GFI_A), *Cyberlindnera fabianii* (GenBank: BAH58739.1), *Wickerhamomyces anomalus* (GenBank: FN641803.1) *Yarrowia lipolytica* CLIB122 (GenBank: CR382129.1)]. Conserved heptapeptide and dipeptide sequences are shown in black boxes.

A group of Chinese researchers that produces phytase 2200 U kg\(^{-1}\) of maize seeds\(^{138}\). This titre was 50-fold higher than the native maize plant and was stable across at least four generations\(^{138}\). Similarly the construction of a recombinant microalga *Chlamydomonas reinhardtii* has been reported producing phytase of *E. coli* (AppaA) in its chloroplasts\(^ {139}\). Microalgal cell hydrolysate can directly be used as feed additive. It has been observed that it contains 10 U g\(^{-1}\) dw. Upon feeding recombinant microalga based diets to young broiler chicks, the excretion of faecal phytate was reduced with increase in inorganic phosphate\(^ {139}\).
Biochemical and Molecular Attributes of Recombinant Phytases

pH and Temperature Optima

The phytase of *A. niger* 113 expressed in *P. pastoris* exhibited two pH optima, at pH 2.0 and pH 5.0. Similarly, in another study, PhyA of *A. niger* had two optimal peaks at pH 2.5 and 5.5, and it was optimally active at 60°C. However, the optimum pH and temperature for *A. fumigatus* phytase were 5.5 and 60°C, respectively. Further, the phytase of *Debaryomyces castelli* CBS 2923 expressed in *P. pastoris* exhibited pH optima of 4.0, similar to the native phytase. This phytase was optimally active at 60°C. The recombinant phytase (PHY1) of *K. ohmeri* BG3 was optimally active at pH 5.0 and 65°C; while the recombinant phytase of *P. anomala* was optimally active at pH 4.0 and 60°C like that of the native phytase. On the other hand, the phytase coexpressed with a xylanase in *P. pastoris* was optimally active at 55°C and pH 5.0.

Kinetic Characteristics of Recombinant Phytases

The recombinant phytase of *P. anomala* expressed in *Hansenula polymorpha* has $K_m$ and $k_{cat}$ of 0.25 mM (phytate) and 18133 s$^{-1}$, respectively. While the phytase of *A. fumigatus* expressed in *P. pastoris* displayed $K_m$ of 1.38 mM for pNPP and 2.84 mM for sodium phytate. Similarly high values of turnover rate ($k_{cat}$) and substrate specificity constant ($k_{cat}/K_m$) for pNPP have been reported for *A. fumigatus* recombinant enzyme. Recently Joshi and Satyanarayana reported heterologous expression of *P. anomala* phytase in *P. pastoris*. The recombinant phytase exhibited high substrate specificity for calcium phytate as evident by its low $K_m$ (0.2 mM) with $V_{max}$ of 78 n moles mg$^{-1}$ s$^{-1}$.

Molecular Mass and Glycosylation of Recombinant Phytases

Molecular mass of the recombinant *A. niger* 113 phytase was 66-80 kDa, which was larger than the actual molecular mass of *A. niger* phytase. The molecular mass of the recombinant enzyme was more compared to the native due to glycosylation. Ragon et al. expressed *D. castelli* CBS 2923 phytase in *P. pastoris* and reported difference in glycosylated and deglycosylated recombinant phytases. Phytase phyA2 expressed in *P. lycii* ranged between ~70 kDa and ~110 kDa, and upon deglycosylation, its molecular mass on SDS-PAGE was 60 kDa. Deglycosylation reduced molecular mass of phyA of *A. niger* from 95 to 55 kDa. Li et al. found that phytase gene ORF of *K. ohmeri* BG3 consisted of 1389 bp. The calculated molecular mass of this phytase was 51.9 kDa, which had a putative signal peptide of 15 amino acid residues. When Li et al. expressed this phytase in *E. coli*, the molecular mass of the recombinant enzyme was 51.9 kDa, which had a putative signal peptide of 15 amino acid residues. When Li et al. expressed this phytase in *E. coli*, the molecular mass of the recombinant enzyme was 51.9 kDa, which had a putative signal peptide of 15 amino acid residues.
phytase was 51 kDa that differed from that of the native phytase. The molecular mass of native K. ohmeri BG3 phytase was 92.9 kDa, which can be attributed to many N-glycosylation sites present in the primary sequence of the protein as well as other processes during secretion. Monomeric recombinant phytase from P. anomala exhibited molecular mass of ~70 kDa, while deglycosylated form exhibited a molecular mass of ~53 kDa. Thus, the excess molecular mass is due to N-linked glycans.

Thermostability

A. niger 113 phytase expressed in P. pastoris retained 70% residual activity after 5 min heating at 90°C, while the recombinant enzyme exhibited 75.2, 74.1 and 68.8% residual activities after 15 min exposure to 80, 85 and 90°C, respectively. Synthetic 6-phytase gene of P. lycii, which was heterologously expressed in P. pastoris, retained only 25% of its initial activity after 10 min exposure to 80°C. Han et al. reported that the deglycosylated PHY1 of K. ohmeri BG3 retained 93% residual activity after the treatment at 60°C for 1 h, and it lost total activity upon treatment at 80°C for 1 h. A phytase (PhyA-2A) coexpressed with xylanase showed optimum activity at 55°C. PhyA-2A retained 70% residual activity after exposure to 90°C for 5 min. Thermostability of recombinant phytase of A. fumigatus was dependent on pH and buffer used as it exhibited higher thermostability at 65 and 90°C, when acetate buffer was used as compared to that in citrate buffer.

Resistance to Proteolytic Action

In in vivo conditions, the actual functional site of phytase is the stomach, where pH instantly increases to 5.5 just after food intake and, thereafter, it decreases to pH 2.0. Pepsinogen is secreted from stomach cell lining, which activates pepsin by the release of HCl in the stomach. An ideal phytase used as a food and feed additive needs to possess a strong release of HCl in the stomach. An ideal phytase used as a food and feed additive needs to possess a strong release of HCl in the stomach. A phytase (PhyA-2A) coexpressed with xylanase showed optimum activity at 55°C. PhyA-2A retained 70% residual activity after exposure to 90°C for 5 min. Thermostability of recombinant phytase of A. fumigatus was dependent on pH and buffer used as it exhibited higher thermostability at 65 and 90°C, when acetate buffer was used as compared to that in citrate buffer.

Crystal Structure of Phytases

Crystal structures of phytases from different sources have been solved. The crystal structure of a protein can reveal unexplored truths about functioning of the proteins and give answers for their special biophysical characters. The phytases from A. niger and A. fumigatus have 66% sequence identity, but they differ in their thermostability; this was attributed to the removal of repulsive ion pair interaction due to substitutions with polar residues along with the formation of hydrogen bonds. Apart from hydrogen bonding, a helical capping at C-terminal also contributed to the stability of A. fumigatus phytase. Crystal structure of A. fumigatus phytase was solved at 1.5 Å resolution. Electron density experiment revealed the presence of six predicted N-glycosylation sites in the phytase. Xiang et al. observed a partially phosphorylated His 59 residue in this phytase that indicated two step catalytic mechanism.

Based on the crystallographic studies, thermostable and thermolabile enzymes can be modified in vitro by rational design. Phytases from different microbial sources have been crystallized. As far as fungal and yeast phytases are concerned, only one yeast phytase and two fungal phytases have been crystallized (http://www.rcsb.org/pdb); the yeast phytase comes from D. castellii CBS 2932. Crystal structure of the phytase from D. castellii (PhytDc) was solved at 2.3 Å resolution that belongs to space group P6₂2₂. The three dimensional structural analysis revealed that this is a dimer containing five NAG molecules along with 628 water molecules. D. castellii CBS 2923 phytase (PhytDc) is a glycoprotein that exhibits thermostability up to 65.85°C, which cleaves six IP6-bound phosphate groups. The PhytDc belongs to histidine acid phosphatases since it contains the characteristic sequence motif RHGXRXXP. The structure of PhytDc can be divided into 2 parts: a small a-domain and a large a/b-domain with a
6-stranded $\beta$-sheets. The primary amino acid sequence of PhytDc exhibits 36% identity with acid phosphatase of *A. niger* (PAAn), 25% with the phytase A of *A. niger* (PhytAn) and 23% with phytase of *A. fumigatus* phytase A (PhytAf). The N-terminal regions of PhytDC and PAAn are extended (Fig. 7A), which are involved in dimer and tetramer formation. A consensus phytase designed based on 13 fungal phytases have also been crystallized (Fig. 7B). This consensus phytase exhibits normal catalytic activity but unexpectedly shows 15-22°C increase in unfolding temperature; its structure was resolved at 2.9 Å. The substrate specificity of this novel consensus phytase is similar to that of *A. fumigatus* and *E. nidulans*. Lehman and coworkers observed stabilization of a surface loop in the consensus phytase, which is one of the highly variable phytases but exhibits consensus sequence as T249, S250, D251, A252, T253. Liu et al. studied the crystal structure of *A. fumigatus* phytase both at pH 5.5 and 7.5. The *A. fumigatus* phytase structure was resolved at higher than 1.7 Å. The presence of a larger catalytic pocket explains the broader substrate specificity of *A. fumigatus* phytase than to that of *E. coli*. Liu and coworkers observed three water molecules at the substrate binding site, which were considered to be responsible for pH dependent activity of *A. fumigatus* phytase (Fig. 7C).

Molecular Approaches for Ameliorating Properties of Phytases

Although various phytases have been reported from different microbial sources, none of them possess all the properties of an ideal phytase. An ideal phytase suitable as food and feed additive should be thermostable to withstand the high temperature during feed pelleting process, and should be adequately acid-stable and protease resistant to withstand high acidic pH of the stomach and proteases, respectively. Biotechnologists have tried to tailor phytases for the acquisition of important properties. Kim et al. introduced mutations in the phytase of *A. niger* and could identify one mutein, which exhibited shift in the

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**Fig. 7(A-C)—3-D structures of yeast [A, adopted from ref. 152] and fungal phytases [B & C, adopted from ref. 153 and 154].** [A] Cartoon structure of *D. castelli* phytase (blue colour) overlapped with *A. niger* (grey colour) phytase sequence where their N-termini are coloured pink and cyan, respectively. [B] Consensus phytase sequence. [C] Reaction product phosphate groups attached to *A. fumigatus* active site.
pH optima towards acidic range. Similarly mutagenesis was used to increase the thermostability of phytase of *A. niger* by Lehman and colleagues. They increased thermostability by 7°C along with other favourable changes. A few mutant phytases have made their way to market. An *E. coli* phytase Phy9X, a product of genetic engineering, has exhibited improved properties and made its way to the market. This enzyme is being sold under the brand name of QuantumTM 605. In one of the mutagenesis studies of phytases, Mullaney *et al.* performed mutation in *A. niger* phytase for alteration of disulphide bridges. Five disulphide bridges (DB) present in the phytase were subjected to mutation forming cysteine residues, and heterologous expression of the mutated gene was carried out in *P. pastoris*. The effect of alteration of every DB was studied in terms of *T*<sub>opt</sub>, *pH*<sub>opt</sub> and substrate specificity (*K<sub>m</sub>). Alteration in DBs completely abolished the activity, while altering disulphide bond 1, 3/4 and 5 lowered the *T*<sub>opt</sub> to 53, 37 and 42°C, respectively. The pH profile of the mutated phytases was changed in such a way that a peak at 2.5 and 5 was abolished on removing DB at 1, 3 and 5. The *K*<sub>m</sub> of mutant phytases did not change significantly, but the turnover of the muteins was significantly lowered.

Utility of Recombinant Phytases in Dephytinization

Last few decades have witnessed increased utilization of microbial phytases in the field of animal nutrition, where plant based diets are used. Phytases have also found application in various other fields, such as, aquaculture, nutrition, where plant based diets are used. Phytases have also made their way for these applications. As mentioned earlier, Quantum TM 605 is one of the recombinant phytases available in the market. Several other unaltered microbial phytases are also sold in the market under different brand names, such as, Finase (source: *A. awamori*; production strain: *Trichoderma reesei*), SP (source and production strain: *A. oryzae*), Allzyme phytase (source and production strain: *A. niger*), Natuphos (source and production strain: *A. niger*), AMAFERM (source and production strain: *A. oryzae*), Bio-feed Phytase (source: *P. lycii*; production strain: *A. oryzae*), Phyzyme (source and production strain: *A. oryzae*), Avizyme (source: *A. awamori*; production strain: *T. reesei*), ROVABIO (source: *Penicillium simplicissimum*; production strain: *P. funiculosum*), Roxazyme (source and production strain: *A. oryzae*). Phytic acid complexes with carbohydrates, proteins and other dietary elements alter their properties. Joshi and Satyanarayana have demonstrated dephtyinization of phytic acid complexed with soy proteins by recombinant phytase of *P. anomala* (rPPHY). Two of the soy protein components, which are known to be highly allergenic in nature, exhibit differences in biophysical properties when they are dephtyinized. Utilizing this observation, they have successfully fractionated β-conglycinin from glycmin. By using rPPHY in whole bread preparation, reduction in phytic acid content up to 75% has recently been reported. A recombinant β-propeller phytase from *B. licheniformis* exhibited digestion efficiency for commercial feed and soybean meal. Sanz-Penella *et al.* have shown the utilization of microbial phytase in dephytinizing infant cereals for the first time. This observation suggests the potential use of phytases in human nutrition too.

Future Perspectives

The worldwide interest on phytases has led to the development of recombinant microbial phytase producers and plant bioreactors. Being a low value and high volume product, low cost of phytase production is required. Many of the native phytases lack either one or the other requisite property. The *in vitro* manipulations in microbial phytases and recombinant DNA technology have led to the generation of phytases with improved properties. A phytase with all the desirable characteristics, such as, thermostability, acid stability, protease insensitivity, adequate activity at animal body temperature and high specific activity in a single phytase is still a challenge. Phytases have been explored for their utility as a food and feed additive, but other applications, such as in soil amendment, environmental protection and enzymatic synthesis of lower inositol phosphate isomers are yet to be adequately explored.

Conclusion

The reduction of phytates in foods and feeds is of a major concern for monogastrics like humans, pigs, poultry birds and fishes because they mainly depend on the plant based diets and do not produce adequate levels of phytate-degrading enzymes in their alimentary tracts. As phytates act as anti-nutrients, the reduction in phytates is an essential step in the processing foods and feeds. Although a number of yeast and fungal phytases have been cloned and
expressed in different heterologous systems, the requirements for an ideal phytase have not yet been fulfilled. A few attempts have also been made to evolve phytases with the desirable characteristics by site-directed mutagenesis or directed evolution. Further developments in microbial phytases are needed in order to evolve an ideal phytase.

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