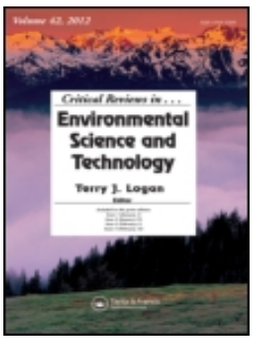

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Microbial production of phytases for combating environmental phosphate pollution and other diverse applications

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ABSTRACT

Concerns of phosphorus pollution and its impact on environments have driven the biotechnological development of phytases. Phosphoric acid, inositol phosphate, or inositols are produced after hydrolysis of phosphate from phytate, initiated by phytase. Research over the last two decades on microbial phytases has deepened our understanding of their production, optimization, and characterization. Despite the wide availability of phytase producing microorganisms, only a few have been commercially exploited. The current high cost of phytases, inability to withstand high temperatures (>85°C), a limited pH range, and poor storage stability are a major bottleneck in the commercialization of phytases. The development of novel phytases with optimal properties for various applications is a major research challenge. In this paper, recent advances in microbial phytase production, application of tools to optimize higher enzyme production, and characterization of phytases along with potential biotechnological applications are reviewed. Additionally the development of phytase assay methods and functions of phytate and phytate degradation products are discussed.

KEYWORDS

Phytic acid; monogastric animals; antinutrients; fermentation; protein engineering

1. Introduction

Supplementation of inorganic phosphorus to nonruminant animal feed and excretion of phosphorus from cereal phytate into effluents creates a global ecological problem leading to eutrophication of water bodies (Azeem et al., 2015; Vats et al., 2005). To safeguard the environment, and improve the phosphorus nutrition in farm animals, a sustainable approach is required (Lessl et al., 2013; Prasad et al., 2015). Phosphorus is stored in all plant seeds in the form of phytic acid, accounting

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for 60–90% of the total phosphorus of many cereals. Phytate as a polyanionic molecule (inositol hexa- and pentaphosphates) that chelates to positively charged nutrients (metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}). The molecule can act as an antinutritional factor by forming complexes with proteins affecting digestion and also inhibiting certain digestive enzymes such as α-amylase, trypsin, acid phosphatase and tyrosinase (Harland and Morris, 1995). Some animals (swine, poultry, and preruminant calves) do not have phytate-phosphorus hydrolyzing enzymes in their gastrointestinal tracts (Rao et al., 2009; Singh et al., 2011; Ravindran, 2012). Therefore, the unutilized feed phosphorus ends up in manure, and through surface runoff reaches water bodies causing water pollution. To provide proper nutrient requirements for these animals, inorganic phosphorus is supplemented in the diets. This supplementation represents the third largest expense of animal diet and a significant impact on both agriculture and the environment could be made by improving the animal's utilization of plant phosphorus (Wasaki et al., 2009; Richardson et al., 2009a; Richardson et al., 2009b; Kumar et al., 2015b).

Approximately 60% of organic phosphorus is found in the soil that exists in the form of phytates. In the plant rhizosphere organic phosphorus is hydrolyzed by various microbial enzymes including phosphatases and phytase (Yadav and Tarafdar, 2003, 2007). Phosphatases can cleave the monophosphoester bonds in numerous organophosphate compounds except those in phytic acid. Both enzymatic and nonenzymatic methods may be used for effective degradation of the phytic acid (Greiner and Konietzny, 2006) but only the enzymatic method is addressed here. Phytic acid is hydrolyzed by phytases—*myo*-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases, EC 3.1.3.8 and EC 3.1.3.26—to a series of myo-inositol phosphate derivatives and inorganic phosphate (Yadav and Tarafdar, 2003, 2007).

For phytate degrading activity has been found in a number of microorganisms, plants, and animal tissues leading to the isolation, purification and characterization of the enzymes followed by large-scale production (Haros et al., 2007; Hill et al., 2007). Phytases from filamentous fungi, particularly from *Aspergillus niger* are well characterized and are used as the major sources of microbial phytase in feed application. The first commercial phytase was introduced into the market in 1991. In 2005 the value of the market had increased to €150 million (Haefner et al., 2005; Greiner and Konietzny, 2006) with the current global market estimated at $350 million/annum and account for more than 60% of the total feed enzyme market (Cowieson and Cooper, 2010). The current inclusion rate of phytase in all diets for swine and poultry is approximately 70% (Lei et al., 2013). Kemme et al. (1997) demonstrated that 0.1 g of inorganic phosphorus addition can be replaced by 500–1000 units of phytase and reduce total phosphorus excretion by 30–50%. Phytases also have great potential in processing and manufacturing of food for human consumption but have only been utilized as an animal feed additives in the diets of monogastric animals (Jorquera et al., 2008). Furthermore, alteration of the molecular and catalytic properties of microbial phytases has enhanced uptake of

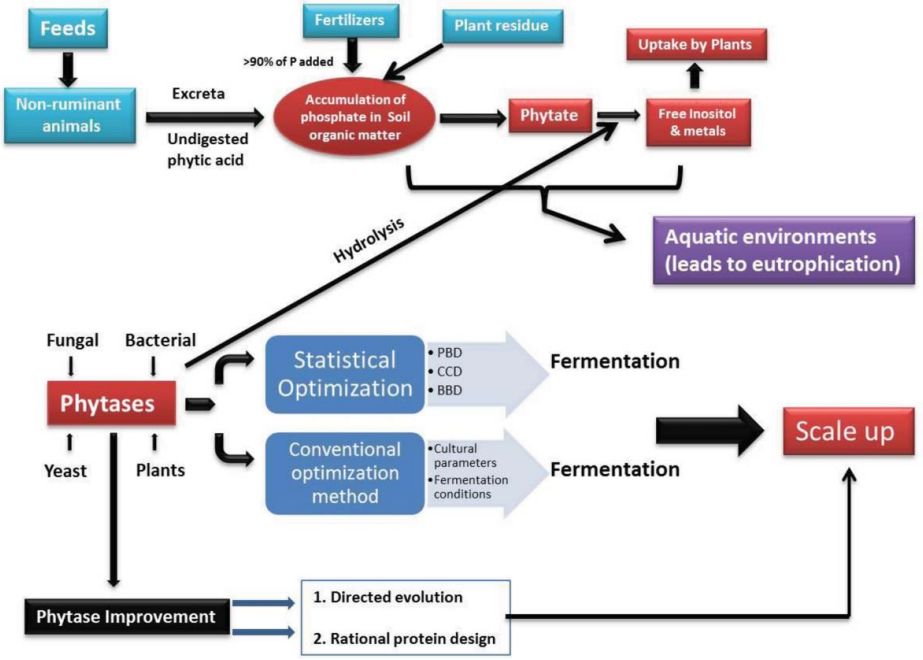

Figure 1. Strategies to maximize phytase production and its role in phytate degradation.

phytate-bound minerals and phytate-phosphorus by pigs, poultry, and fish (Selle and Ravindran, 2008; Rao et al., 2009; Zeng et al., 2014). Their application also enhances amino acids, vital minerals, and dietary carotenoids accessibility in the feed (Farhat-Khemakhem et al., 2012). Low specific phytase activities and poor thermal tolerance are the main constraints limiting current commercial use, and preclude the extensive use of these enzymes in animal feeds. Strategies are outlined in Figure 1, which can be used to improve the phytase production. Superior thermostability and catalytic efficiency of a phytase obtained from the thermophilic fungus *Thermomyces lanuginosus* compared to that of *A. niger* phytase (Berka et al., 1998; Gulati et al., 2007) points to a possible solution to these constraints.

Many thorough and informative reviews on different aspects of phytases (characteristics of phytate-degrading enzymes or phytases and their identification and production) and their application in food and feed industries are published previously (Greiner and Konietzny, 2006; Selle and Ravindran, 2007; Fu et al., 2008; Jorquera et al., 2008; Afinah et al., 2010; Singh and Satyanarayana, 2010; Caipang et al., 2011; Singh et al., 2011; Singh and Satyanarayana, 2011; Yao et al., 2012; Joshi and Satyanarayana, 2015). However, the development of phytase assay methods and use of tools to optimize phytase production are rarely discussed. In this review we focus on phytase assay methods and statistical optimization to enhance phytase production. The latest developments in phytase research and their application to various industries are discussed.

2. Classification and distribution of phytases

Different and improved phytases have been mainly isolated from microbes and plants and classified on the basis of their catalytic mechanisms (β-propeller phytase [BPP], histidine acid phosphatases [HAP], purple acid phosphatases [PAP], or cysteine phosphatases [CP]), stereospecificity of phytate hydrolysis (3- or 6-phytases), and pH optima (alkaline or acid phytases). A number of systems have been used to classify phytases. In one system the enzyme is classified as either a 3-phytase (EC3.1.3.8) or 6-phytase (EC3.1.3.26) based on hydrolytic activity; 3-phytases initially hydrolyze the C3-position of phytic acid, yielding inorganic phosphate (Pi) and 1,2,4,5,6-pentakisphosphate whereas 6-phytases initiate hydrolysis at the C6-position yielding Pi and 1,2,3,4,5-pentakisphosphate. And 3-phytases are produced mainly by microorganisms and filamentous fungi while 6-phytases are mostly found in plants (Reddy et al., 1982). In another system, the phytases are classified into four groups: BPP, HAP, CP, and PAP, which is based on amino acid sequences, reaction mechanisms, three-dimensional conformation, and biochemical properties. HAP phytases have been found in microbes and plants whereas BPP phytases are found in *Bacillus* and some other bacteria. PAPs were found in animals, plants, and fungi while CPs were found in *Selenomonas ruminantium* (Cheng and Lim, 2006; Lim et al., 2007). Each of these phytase families has distinct catalytic and unique structural features that use phytate as a substrate in various environments.

3. Microbial production of phytases

Production of microbial phytases can be achieved by two main processes (Vats and Banerjee, 2004), namely submerged fermentation (SmF; Nampoothiri et al., 2004) and solid-state fermentation (SSF; Roopesh et al., 2006). SSF system has many advantages (minimum wastewater output, involve less energy, easier aeration, simple fermentation media, and reduced bacterial contamination) and has gained much interest recently as a method of choice (Vats and Banerjee, 2004). Different microbes producing a substantial level of acid phosphatases/phytases that include strains from the genera *Enterobacter, Serratia, Citrobacter braakii* (Cao et al., 2007), *Rhizobium* (Abd-Alla, 1994), *Pseudomonas* sp. (Hosseinkhani et al., 2009), *Bacillus* sp. (Joseph and Raj, 2007), *Proteus, Klebsiella ruijuan* (Mittal et al., 2011), *Pseudomonas fragi* (In et al., 2004), *Pseudomonas aeruginosa* p6 (Sasirekha et al., 2012), and *Discosia* sp. (Rahi et al., 2009), *Sporotrichum thermophile* (Singh and Satyanarayana, 2010), *Emericella rugulosa* (Yadav and Tarafdar, 2003, 2007), and some other fungi. Studies revealed that the bacterial phytases are more thermostable than fungal phytases. Some other desirable properties include resistance to proteolysis and greater substrate specificity, and catalytic efficiency (Jorquera et al., 2008). Table 1 shows the production of phytases by different microbes in SmF and SSF. Studies have been conducted where gene has been transferred from strain to other strain to enhance the enzyme production

and some of its characteristics. A novel phytase gene was transferred by Shi et al. (2009) from *A. niger* N-3 and expressed in *Pichia pastoris*. At least 45% activity was retained at 90°C for 5 min by the purified enzyme. Dual optimum pH values were obtained at 2.0 and 5.5, with activity at pH 2 was about 30% higher than that at pH 5.5, which is more similar to conditions in the stomachs of mongastric animals. Promdonkoy et al. (2009) identified two novel thermostable phytases in *Aspergillus japonicus* BCC18313 (TR86) and BBC18081 (TR170), respectively. The high thermostability demonstrated for both enzymes is partly dependent on glycosylation and more than 50% activity is retained after heating at 100°C for 10 min. Hong et al. (2011) isolated and characterized a thermostable phytase from *B. subtilis* CF92 found in cow feces. The purified phytase displayed optimal activity at 60°C and pH 7. Mittal et al. (2011) isolated an extracellular phytase producing *Klebsiella* sp. bacterium from poultry farm soil. The maximum phytase activity of 395 IU/ml at was at 55°C and pH ranging from 3.5 to 5.5. Recently, Coban and Demirci (2014) screened a number of microbes and have optimized culture conditions for the phytase production in SmF and found that the productivity is higher as compared to SSF. They found that *Aspergillus ficuum* (NRRL 3135) was the most productive strain. Phytase thermostability is one of the major desirable factors to consider as high temperatures are used during the preparation of pelleting of animal feed.

4. Biochemical characterization and purification of phytases

Biochemical characterization of phytases showed that their estimated molecular weight ranged between 35 and 700 kDa depending upon the microbial source. Phytases are usually most active within temperature ranges of 45–60°C and pH ranges of 2.2–8.0. Based on optimal pH for catalytic activity, a majority of phytases belong to either the acid or alkaline class of phytases. Generally, fungal phytases display activity between pH 4.5 and 5.6 while those of bacterial origin are optimally activity between pH 6.5 and 7.5 (Caipang et al., 2011). Phytases produced by *Bacillus* generally have optimum pH levels at 6.5–7.5. However, *B. subtilis* CF92 phytase displayed optimal activity at pH 7 with the purified enzyme relatively stable between pH 4 and 8 (Hong et al., 2011) while phytase from *Bacillus* sp *KHU-10* was fairly stable from pH 6.5 to 10.0 (Choi et al., 2001). Variation in the molecular structure of the protein from the various sources could be the reason for wide differences in pH optima.

Temperature studies revealed that phytases have high enzyme activity in the range of 50–70°C whereas the optimum temperature is 45–60°C (Vats and Banerjee, 2004). Phytase from the thermophilic fungus *Rhizomucor pusillus* has been shown to be optimally active at 70°C (Chadha et al., 2004), while phytase produced from *R. oryzae* has an optimal temperature of 45°C (Rani and Ghosh, 2011). Phytase from *Bacillus* spp has an optimal temperature of 80°C (Dechavez et al., 2011) while the enzymes from *Yersinia intermedia* (Huang et al., 2006),

Table 1. Biochemical properties (optimum pH and temperature, MW, and enzyme activity) of microbial phytases (2007–2015).

Microbial strain	Fermentation	Optimum temperature (°C)	Optimum pH	M.W. (kDa)	Enzyme Activity	Reference
Aspergillus flavus	SSF	45	7	30	34 U g^{-1} to 112.25 U g^{-1} fermented substrate	Gaind and Singh (2015)
Bacillus licheniformis PFBL-03		55	6	36	14.7 U/ml	Fasimoye et al. (2014)
Alcaligenes sp.	SmF	60	7–8	41	6.202 U/ml	Vijayaraghavan et al. (2013)
Klebsiella pneumoniae 9-3B	–	50	4	45	—	Escobin-Mopera et al. (2012)
Enterobacter sakazakii ASUIA279	–	45–55	4.5	43	—	Farouk et al. (2012)
Volvariella volvacea (Straw mushroom)	–	45	5	14	—	Xu et al. (2012)
Bacillus subtilis US417	SmF and SSF	—	—	—	112 U/g	Kammoun et al. (2012)
Rhizopus oryzae	SSF	45	1.5 and 5.5	34	148.77 U/gds	Rani and Ghosh (2011)
Klebsiella sp. DB3	SmF	50	5.5	—	3.15 U/ml	Mittal et al. (2011)
Bacillus subtilis CF92	—	60	7	46	—	Hong et al. (2011)
Rhizopus oligosporus (DSMZ 1964)	—	60 for RO1 and 55 for RO2	3.0 and 4.5 two intracellular phytases (RO1 and RO2)	45.0 ± 5.0	—	Azeke et al. (2011)
Aspergillus niger NCIM 563	SSF	55	6	—	154 U/g	Bhavsar et al. (2011)
Pichia pastoris	SmF	—	—	—	161.64 U/ml	Liu et al. (2011)
Aspergillus niger FS3	SSF	60	5.0–5.5	—	170.4 U/ml	Spier et al. (2011)
Lichtheimia blakesleeana URM 5604	SSF	34	6	—	264.68 U/g	Neves et al. (2011)
Mitsuokella jalaludinii	–	55–60	4.0–5.0	—	—	Lan et al.(2011)
Saccharomyces cerevisiae sp.	SmF	35	—	—	0.62 U/ml	Ries and Macedo (2011)
Pichia anomala	—	60	4	64	—	Vohra et al. (2011)
A. ficuum	—	67	1.3	65.5	—	Zhang et al. (2010)
Aspergillus niger St-6	SmF	30	6	—	850 U/ml	Tahir et al. (2010)
Shigella sp. CD2	SmF	60	5.5	—		
Bacillus sp.	—	67–73	6–7	—	—	Tran et al. (2010)
Aspergillus niger	SmF	60	6.5	—	46.33 U/ml	Thyagarajan and Namasivayam (2010)

(*Continued*)

Table 1. (*Continued*)

Microbial strain	Fermentation	Optimum temperature (°C)	Optimum pH	M.W. (kDa)	Enzyme Activity	Reference
Aspergillus niger	SmF	40	5.5	—	1.075 U/ml	Raza et al. (2010)
Fusarium verticillioides	SmF	50	5	—	0.78 U/ml	Marlida et al. (2010)
Aspergillus niger CFR 335	—	—	—	—	1.26 Umg^{-1}	Gunashree et al. (2010)
Pseudomonas sp.	SmF	28	7–3.5		749.2 U/ml	Hosseinkhani et al. (2010)
Aspergillus niger 11T53A9	SSF	55	5	—	133 U/mg^{-1}	Greiner et al. (2009)
Malbranchea sulfurea	SSF	70	5.6	—	2.84 U/g	El Gindy et al. (2009)
Aspergillus niveus	SSF	70	5.6	—	3.4 U/g	El Gindy et al. (2009)
Erwinia carotovora var. carotovota	—	40	5.5	45.3	—	Huang et al. (2009a)
Pedobacter nyackensis	—	45	7	38	24.4 U mg^{-1}	Huang et al. (2009b)
Dickeya paradisiaca		55	4.5, 5.5	—	769 U mg^{-1}	Gu et al. (2009)
Saccharomyces cerevisiae CY	SmF	37	4	—	135.09 mU/mg DCW	In et al. (2009)
Kodamea ohmeri BG3	SmF	28	5	—	557.9 mU/ml	Li et al. (2008)
Flammulina velutipes	—	45	3–9	14.8	—	Zhu et al. (2007)

Obesumbacterium proteus (Zinin et al., 2004), *Enterobacter* sp.4 (Yoon et al., 1996), *K. pneumoniae* 9-3B (Escobin-Mopera et al., 2012), *Pseudomonas* sp. JPK1 (Park and Cho, 2011) are optimally active in the range of 45–60°C.

Metal ions are essential trace elements that affect the fermentation process. During fermentation, it is difficult to ascertain whether phytase activity is inhibited by binding of metal ions to phytic acid or to the enzyme itself. Phytase from *Enterobacter sakazakii* was strongly inhibited by Zn^{2+}, Fe^{3+}, Cu^{2+}, fluoride, molybdate, vanadate, and phosphate (1 mM; Farouk et al., 2012). Cations Fe^{3+}, Cu^{2+}, and Zn^{2+} at 0.5 mmol/l also decreased phytase activity in sonicated cells of *Enterococcus hirae* by 99.4%, 90.7%, and 96.5%, respectively while Mg^{2+} increased activity by 11.0% (Marounek et al., 2009). Similarly the activity of phytase produced by *K. pneumoniae* 9-3B was inhibited by Zn^{2+} and Fe^{2+} (Escobin-Mopera et al., 2012) and slightly stimulated by Ca^{2+} and the chelating agent ethylene diaminetetraacetic acid (EDTA). Whereas phytase activity from *Bacillus* is metal ion dependent as the enzyme needed calcium for activity and stability (Fu et al., 2008). Tran et al. (2011) reported that both enzyme stability and activity of a recombinant alkaline phytase obtained from *Bacillus* sp. MD2 is affected by divalent metal ions (Ca^{2+}). Enzyme activity inhibition by excess Ca^{2+} was overcome by adding EDTA. However, excess EDTA reduced the activity by promoting chelation of the metal essential for the activity. This observation was supported by Hong et al. (2011) and Kerovuo et al. (2000) in separate studies. Results showed that *B. subtilis* phytase was activated in the presence of EDTA and significantly inhibited by metal ions (Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}). Zhang et al. (2010) reported that phytase activity from *A. ficuum* NTG-23 was slightly stimulated in the presence of EDTA but not significantly affected by metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. Partially purified *Aspergillus niveus* phytase was reported to be inhibited by Mg^{2+}, Mn^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+}, and Fe^{2+} but the activity was enhanced by low concentrations of Fe^{3+} and high concentrations of Na^{+}, Li^{+}, Ca^{2+} (El-Gindy et al., 2009). The metal ions Zn^{2+}, Fe^{2+} and Fe^{3+} enhanced the phytase activity of *Malbranchea sulfurea* while Na^{+}, Li^{+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, and Cd^{2+} inhibited its activity. High concentration of Ca^{2+} slightly enhanced the enzyme activity while low concentration decreased it (El-Gindy et al., 2009).

Substrate specificity and affinity are two chief characteristics of phytases and it is mostly related to the substrate physiological nature. Phytase enzymes shows wide substrate specificity and capable of hydrolyzing the following substrates; guanosine monophosphate, guanosine triphosphate, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, ρ-nitrophenyl phosphate, phenyl phosphate, 1-naphthyl phosphate, nicotinamide adenine dinucleotide phosphate, 2-naphthylphosphate, galactose 1-phosphate, a-glycerophosphate, b-glycerophosphate, pyridoxalphosphate, glucose 6-phosphate, glucose 1-phosphate, fructose 1,6-diphosphate, fructose 6-phosphate, o-phospho-l-serine, and pyrophosphate (Oh et al., 2004). Phytase enzymes easily hydrolyze phytate to myo-inositol monophosphate without accumulating intermediates. Low specific activity is generally

coupled with broad substrate specificity (Konietzny and Greiner, 2002). Phytases with a narrow range of substrate specificity conversely result in accumulation of myo-inositol tris- and bisphosphate during hydrolysis of phytate coupled with a slow release of phosphate (Konietzny and Greiner, 2004).

The purification of phytases is accomplished using frequent biochemical techniques such as acetone precipitation, ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography (Konietzny and Greiner, 2002). The purification process is generally performed with the aim of characterizing the enzyme for its potential use in industry. Only fungal phytases and those produced by bacteria belonging to the genera *Enterobacter, Pseudomonas*, and *Bacillus* are extracellular in nature and have been easily extracted from the culture filtrate (Liu et al., 1998). Intracellular phytase must be extracted before purification, leading to additional cost added to the production process. A large number of phytases have been purified and characterized from different microorganisms. A 32.6 kDa phytase from *Cladosporium* sp. FP-1 has been purified to electrophoretic homogeneity by gel filtration and by using ion-exchange chromatography (Quan et al., 2004). On the basis of size exclusion chromatography and SDS-PAGE analyses a purified phytase from *K. pneumonia* 9-3B was characterized as a monomeric protein with an estimated molecular weight of 45 kDa. The purification strategy involved ion exchange chromatography followed by gel filtration and resulted in respectively 240- and 2,077-fold purification of the enzyme (Escobin-Mopera et al., 2012). A phytase of 46 kDa from *B. subtilis* CF92 was purified via ethanol precipitation, anion-exchange chromatography, and gel filtration chromatography (Hong et al., 2011). Partial purification of a phytase from *P. aeruginosa* P6 was achieved by ammonium sulfate precipitation followed by dialysis. The specific activity of the crude enzyme was 31.86 U. mg^{-1} and this was increased to 70.77 U. mg^{-1} following partial purification (Sasirekha et al., 2012). Phytase has also been purified from other sources such as *Lactobacillus sanfranciscensis* CB1 (De Angelis et al., 2003), *A. niger* (Sariyska et al., 2005), *P. pastoris* FPHY34 (Guo et al., 2007), and *Mucor indicus* MTCC 6333 (Gulati et al., 2007).

5. Analytical methods for phytase determination

The determination of phytase enzyme activity is relies on the analysis of total released phosphate but phytase activity in its purest form represents released product per time from inositol hexaphosphate (IP6) only. Microbial preparations for the analysis contain mixtures of phosphatases and organic phosphate compounds; hence some released phosphate in enzymatic assays may originate from nonphytase phosphatases degrading nonphytate molecules. Moreover, even purified enzyme extracts assessed via Pi release may result in errors, as commercial IP6 commonly contains contamination of other inositol phosphates, and furthermore the products of phytase IP6 hydrolysis by phytase can serve as substrates for additional hydrolysis by the phytase (Qvirist et al., 2015). Phytase activity

determinations may seem straightforward, but can be deceptive and involve a number of uncertainties. Assessing phytase activity in a comparable way is therefore important for screening, selecting and improving enzymes as well as in producing organisms. In most of the phytase assays, the measurement of orthophosphate liberated from phytate by enzymatic action is used to determine the degree of phytate-degradation and phytase activity. Several analytical methods are available in the literature for quantitative and qualitative determination of phytase activity, but no single method is enough. These assay methods are further complicated by the fact that neither the degradation products, nor phytate substrates can be measured with precision (IPS Program Committee, 2010).

Rapid screening of phytase producing microorganisms necessitates suitable enzyme assays. Use of a differential plating medium to detect the phytase activity may be used as quick initial indicator. Nonspecific chromogenic phosphatase substrates (phosphate based) can be utilized for screening, but this method lacks the desired specificity. Subsequently a more specific method for determining the phytase activity based on the disappearance of sodium phytate complex or precipitated calcium as an indication of enzyme activity was established (Bae et al., 1999). However, this method is not satisfactory for screening of phytase activity in anaerobic bacteria because the chance of a false positive reaction due to the acid produced by the bacteria. To overcome the problem of acid induced false results, Bae et al. (1999) developed a two-step counter staining technique where the agar medium is first flooded with cobalt chloride solution followed by ammonium molybdate/ammonium vanadate solution, which reprecipitates acid solubilized phytate. Quantification methods that began with Heubner and Stadler (1914) have previously been given great attention. Several methods have been developed with variation in this method (Rather, 1917; Earley and DeTurk, 1944; Samotus and Schwimmer, 1962; Oberleas, 1964), but the method of Fiske and Subbarow (1925) has been the commonly used method in many laboratories. However, in the Fiske and Subbarow (1925) method of analysis, there is no separation of other inositol phosphate esters and the method is nonspecific and labour intensive. Several of the colorimetric phosphate analysis methods used today (Sanikommu et al., 2014; Caputo et al., 2015) are modified versions of a method presented by Fiske and Subbarow (1925). In the original method, acid ammonium molybdate solution was added to a phosphate-containing sample to form the yellow phosphomolybdic acid, subsequently reduced by 1-amino-2-naphtol-4-sulfonic acid (ANSA) to molybdenum blue, which can be determined spectrophotometrically. Since ANSA is difficult to prepare in solution (Fiske and Subbarow, 1925), and may be toxic, modified methods have been developed to exclude ANSA. One alternative is spectrophotometric detection of the formed yellow phosphomolybdic acid (Heinonen and Lahti, 1981; Greiner and Alminger, 1999) without reduction to molybdenum blue. Another alternative is to exchange ANSA for a different reducing agent, such as ferrous sulfate (Bae et al., 1999), stannous chloride (Dickman and Bray, 1940) or ascorbic acid (Henriksen, 1965) to produce molybdenum blue.

HPLC allowed more specific methods to be developed to determine phytate and degradation products. The earliest methods used a reverse phase column that was rapid but lacked sensitivity and accuracy and provided limited product separation (inositol phosphate; Tangendjaja et al., 1980). The development of strong anion exchange columns, replacing the reverse phase columns, further improved the separation of the possible fractions and thus improved the sensitivity and specificity. An HPLC approach combining an ion-exchange procedure (Harland and Oberleas, 1977) with a uBondapak CIS column (Tangendjaja et al., 1980) for the determination of phytic acid was reported (Graf and Dintzis, 1982). Subsequently a new method of phytase determination was developed by Latta and Eskin (1980) and was further modified by Rounds and Neilsen (1993) and Oberleas and Harland (2007).

In a collaborative study to validate a colorimetric assay for determination of microbial phytase activity in feed, a trial has been conducted by many laboratories. The performance of the phytase enzyme, when added as a feed supplement for phytate hydrolysis *in vivo* is considerably different from *in vitro* assay conditions. Development of reliable *in vitro* assay by the spectroscopic method for phytase activity is further complicated by detection of degradation products (Clements, 2011). Weaver et al. (2009) determined the effects of assay conditions on activity evaluations of two phytases (*A. niger* PhyA and *E. coli* AppA2) and compare the biochemical characteristics at a pH of 3.5, which is found similar to the stomach. In this study, three major phytase assay methods (Engelen et al., 2001; Gizzi et al., 2008) were used: the molybdenum blue method (method 1; Chen et al., 1956; Han et al., 1999; Augspurger et al., 2003), the molybdo-vanadate method (Engelen et al., 1994; Leeson et al., 2000; Huang et al., 2006) and the acetone phosphomolybdate method (method 3; Heinonen and Lahti, 1981). The determination of activities with these three assay methods of AppA2 compared to PhyA activities showed much greater variation pointing to caution in interpreting the feeding efficiency based on assay conditions.

In most of the commonly used assay methods, determinations of phytase activity in *in vitro* studies have been performed with IP6 as the substrate with either the amount of Pi released or the degradation of IP6 being followed. Though in the *in vivo* method, phytic acid does not exist in the free sodium salt form, which is not measured accurately (Tran et al., 2011). Furthermore, determination of the quantity of IP6 degradation is time-consuming and tedious. This method also necessitates the use of chromatography with multiple devices because none of inositol monophosphate-inositol pentaphosphate (IP1–5), IP6, and Pi can be sensed directly by fluorescence or absorbance, which limited the development of this technique for high-throughput screening (Chen and Li, 2003; Ishiguro et al., 2003).

The most commonly used method is based on the measurement of Pi released from IP6 by the action of phytase. In this assay, color development is directly proportional to the amount of Pi released and depends on various factors that need to be carefully taken into consideration during the analysis. In another method,

phytase activity is measured kinetically using ρ-nitrophenyl pyrophosphate or ρ-nitrophenyl phosphate as a substrate, where yellow color production is measured spectrophotometrically. However, in this assay, it is hard to differentiate whether the yellow color is coming from substrate hydrolysis by the action of phosphatases or by phytase. Furthermore, all phytases do not show good activity toward these two artificial substrates (Choi et al., 2001).

Tran et al. (2011) reported a nontoxic, simple, and fast kinetic method adapted for high throughput for assaying phytase using phytic acid protein complex (IP_6 – lysozyme) as a substrate. The principle of this method is to monitor the decrease in turbidity at 600 nm due to hydrolysis of the phytic acid-protein complex by phytase. This kinetic method is useful for a variety of phytases over a wide pH range. Determination of phytase activity by using kinetics and influence of unreduced phytic acid on phosphate estimation by three commonly used methods such as the AOAC (Engelen et al., 2001), Cooper and Gowing (1983), and Fiske and Subbarow (1925) methods were compared and reported recently by Sanikommu et al. (2014). Their results show that the AOAC method is most suitable to estimate the phytase activity in the presence of phytate in the medium. Readings taken during the second hour of incubation is apparently influenced by the presence of phytic acid. The method of Cooper and Gowing (1983) is labor intensive and is prone to give error values at higher concentrations.

6. Optimizing phytase production

The fermentation process depends on various physical and chemical parameters. High production of phytases depends on optimizing parameters of the culture medium such as pH, temperature, source of carbon, source of nitrogen, and agitation. Traditional optimization involves standardizing parameters using one factor at a time approach and by keeping the other parameters constant; these methods are time-consuming and labor intensive. Experimental designs based on statistical principles are useful tools to optimize, model and control of phytase production during fermentation (Singh and Satyanarayana, 2008). Application and use of response surface methodology (RSM) have received increased popularity recently to optimize growth parameters during different stages of fermentation and biotechnological processes (Vohra and Satyanarayana, 2002; Téllez et al., 2003; Tsen et al., 2009; Chakraborty et al., 2011; Jain et al., 2011; Modha and Pal, 2011). These statistical methods have been found to enhance phytase production at a reduced cost (Ries and Alves Macedo, 2011). Three main steps are involved in these statistical optimization protocols: (a) the experiments are designed statistically, (b) the coefficients in a mathematical model are estimated, and (c) the response is predicted and the adequacy of the model is checked. In this model, additional information such as observed vs predicted response is generated. The levels of the variables under analysis, giving maximum/optimized response are then calculated using the mathematical model, which is further tested experimentally (Maddox

and Richert, 1977). Statistical tools required for this include Plackett and Burman's design (PBD), central composite design (CCD), and evolutionary operation factorial design. RSM may be used for optimization of experiments after being designed using CCD (Baş and Boyacı, 2007). CCDs are formed from two level factorials (second-order models) by the addition of just enough points to estimate curvature and interaction effects. Numerous experiments have shown the use of this statistical approach with considerable success for optimization of phytase production (Ries and Alves Macedo, 2011; Sasirekha et al., 2012). Rani and Ghosh (2011) in their experiment used CCD to optimize mutual interactions between selected variables under experimentation (ammonium sulfate, mannitol, and K_2HPO_4/Na_2HPO_4) and their corresponding optimum concentrations and got an overall 8.41-fold increase in phytase production. Kammoun et al. (2012) used PBD to optimize phytase production by *B. subtilis* US417 in both SSF and SmF. A fivefold improvement in SmF and a fourfold improvement in SSF were achieved when such variables as inoculum size, methanol, and yeast extract (as a nitrogen source) were identified to control phytase production. Some successful examples of statistical optimization are presented in Table 2.

7. Biotechnological improvements of phytase production

Production of phytases and their acceptance in the industry largely depend on three factors, ability to release phytate-P in the digestive tract, thermostability during feed processing and storage, and cost effectiveness. Developments of effective phytases depend on identifying new microbial or plant sources of phytase production and genetic modification of the enzyme. Large numbers of phytases have been characterized to date and some of them available commercially (Cao et al., 2007). Commercial phytases have been developed and supplemented in the diets of swine, poultry, and fish in a number of countries, including Europe, North America, and Asia in order to reduce phosphorus pollution of animal waste. These phytases have three major constraints: (a) high susceptibility to proteolysis in the stomach, (b) suboptimal catalytic efficiency, and (c) inadequate stability to resist heat inactivation during feed pelleting. Phytex, a newly established company, has developed thermostable phytase with a potential to take up a significant portion of the world market. An effective and heat-stable phytase may also help to fight against nutritional deficiency of iron and zinc, a health problem that affects approximately 30–50% of the world population. The staple foods in developing countries are primarily of plant origin. The high abundance of phytate in these foods significantly reduces absorption of dietary iron and zinc by chelating with these elements and rendering them unavailable for digestion. Using young anemic pigs as an *in vivo* model, it has been demonstrated that effective release of phytate-bound iron in corn and soya for hemoglobin synthesis by supplementing phytase to feed (Stahl et al., 2004). Other investigators have shown a similar effect of phytase in humans (Rao et al., 2009, Kumar et al., 2010b; Luo et al., 2010). Production of phytase in a

Table 2. Phytase production using different statistical optimizing tools.

Microorganisms	Optimization tools	Achievements	Reference
Thermomyces lanuginosus	Central composite design (CCD) of response surface methodology (RSM).	Optimization of four variables (i.e., temperature, age of seeding culture, initial pH, and aeration area). An overall 10.83-fold improvement in phytase activity (0.30–3.248 U) was recorded.	Berikten and Kivanc (2014)
Aspergillus niger var. *phoenicis* URM 4924	2^2Central composite design and response surface methodology.	Enhancement of phytase activity up to 7.8-fold (from 1.04 to 8.09 U/ml)	Nascimento et al. (2013)
Bacillus subtilis 168	Plackett-Burman (PB) and Box-Behnken (BB) design.	The maximum phytase activity of 47 U ml^{-1} (73-fold higher over native strain US417 before optimization) was achieved in the presence of 12.5 g l^{-1} of yeast extract (YE) and 15 g l^{-1} of ammonium sulfate with shaking speed of 300 rpm.	Farhat-Khemakhem et al. (2012)
Bacillus subtilis US417	PB design were optimized by RSM	High enzyme production of 112 U/g (5 fold improvement) of wheat bran.	Kammoun et al. (2012)
Klebsiella sp. DB3	CCD	Optimum levels of variables (orange peel bran 2.0%, sucrose 2.0%, and ammonium dihydrogen phosphate [$NH_4H_2PO_4$] 0.1%, and pH 5.5) that supported maximum enzyme activity. 5.25-fold enhancement of enzyme production was recorded.	Mittal et al. (2011)
Aspergillus niger FS3	PB design and central composite rotational design (CCRD)	Optimum level of variable to maximize phytase production were; temperature (30°C), initial moisture content (65%), Na-citrate buffer concentration (0.3M), initial pH (5.0), and urea concentration (1.5%). 4.3-fold increases in phytase production were achieved successfully.	Spier et al. (2011)
Saccharomyces cerevisiae MTCC 5421	Central composite experimental design	Design based on five levels of three factors namely incubation temperature, incubation period and pH level presented that *S. cerevisiae* could produce phytase (200 U/ml) at following set parameters; 36 hr, 30°C, and pH 3.5.	Roopashri and Varadraj (2014)
Rhizopus oryzae	Plackett-Burman design and CCD	Optimized conditions to maximize phytase production (w/w, mannitol, 2.05%; ammonium sulfate,	Rani and Ghosh (2011)

(*Continued*)

Table 2. (*Continued*)

Microorganisms	Optimization tools	Achievements	Reference
		2.84% and phosphate, 0.38%). 8.41-fold improvement in phytase production was achieved.	
Recombinant *Pichia pastoris*	Plackett-Burman design and CCD	The optimal concentrations of the three components, leading to a maximal extracellular phytase activity of 161.64 U/ml, were K_2SO_4 13.25 g/l, $CaSO_4$_$2H_2O$ 1.03 g/l, and $MgSO_4$_$7H_2O$ 17.94 g/l.	Liu et al. (2011)
Saccharomyces cerevisiae	22 full-factorial CCD and RSM	Tenfold improvement in the production of phytase.	Ries and Macedo (2011)
Aspergillus niger NCIM 563	PB design and BB design	Improved phytase production (3.08-fold) from 50 IU/g dry moldy bran (DMB) to 154 IU/g DMB after optimization was attained.	Bhavsar et al. (2011)
Paecilomyces variotii	CCD methodology	Maximum activity of phytase was 350 U/gds after 72 hr at 66% (v/w) and 5.8% (w/w) of moisture and tannic acid conc., respectively.	Madeira et al. (2011)
Sporotrichum thermophile	PB design and 24 full factorial CCD of RSM	Starch (0.4%), Tween-80 (1.0%), peptone (0.3%), and sodium phytate (0.3%) supported maximum enzyme activity (overall 3.73-fold improvement).	Singh and Satyanarayana (2008)
Thermomyces lanuginosus mutant (TL-7)	BB factor factorial design	Wild type strain CM was found to produce maximum amount of phytase (4.33 units/g DW substrate) and mutant TL-7 produces phytase using BB design 32.19 units/g of substrate.	Gulati et al. (2007)

highly purified form using a relatively low-cost system would increase commercial application. However, any single phytase may never be able to meet all conditions of application. Some of the newly characterized and expressed microbial phytases are summarized in Table 3. Main challenges are to develop commercially attractive phytase that should be thermostable and can withstand temperature during the pelleting process of 60–90°C as pig and poultry feed are pelleted. Most of the phytases available today generally unfold at temperature between 56 and 64°C so increasing the thermostability of the enzyme using different approaches are actively investigated (Vats et al., 2005). A considerable number of phytases from various molds have been identified, cloned and overexpressed, that is the first step for the development of a thermostable phytase (Pasamontes et al., 1997). Wyss et al. (1998) evaluated the thermostability properties of three HAPs, *A. niger* phytase, *A. fumigatus* phytase, and *A. niger* optimum pH 2.5 acid phosphatase. After heat denaturation at 85°C, the recovery of enzymatic activity was considerably higher for *A. fumigatus* phytase than for *A. niger* phytase or pH 2.5 acid

Table 3. Newly characterized and expressed microbial phytases.

Origin	Expression host	Mr (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Phytase from *A. niger* NII 08121	*Kluyveromyces lactis* GG799 cells	≥140	—	100 for 1 hr	Ushasree et al. (2014)
Janthinobacterium sp. TN115 (symbiotic bacterial strain from the gut contents of *Batocera horsfieldi* larvae [Coleoptera: Cerambycidae], a phytase-encoding gene (phyA115) was cloned and expressed in *E. coli*.	*Escherichia coli*	64	8.5	45	Zhang et al. (2011b)
Two types of phytases (PhyH49 and PhyB49) in *Serratia* sp. TN49 that was harbored in the gut of *Batocera horsfieldi* (Coleoptera) larvae was cloned and expressed in *E. coli*.	*Escherichia coli*	–	pH 5.0 (PhyH49) and pH 7.5–8.0 (PhyB49)	60 (PhyH49) and 45 (PhyB49)	Zhang et al. (2011a)
Escherichia coli app Aphytase gene	Chloroplast genome of the model microalga, *Chlamydomonas reinhardtii*, and isolated homoplasmic plastid transformants.	—	4.5	60	Yoon et al. (2011)
Neosartory aspinosa BCC 41923	*Pichia pastoris*	52	5.5	50	Pandee et al. (2011)
Aspergillus ficuum	*Lactobacillus casei*	39.2	5.0	40–80	Zuo et al. (2010)
Pichia pastoris GS115-phyA	*Yarrowia lipolytica* po1h	130	5.5	55	Chen et al. (2010)
Bacillus phytase C	*Pichia pastoris*	39	—	—	Guerrero-Olazaran et al. (2010)
Dickeya paradisiaca	*Escherichia coli*	—	4.5 and 5.5	55	Gu et al. (2009)
Kodamaea ohmeri BG3	*Escherichia coli* BL21 (DE3)	51	—	—	Li et al. (2008)
Hansenula fabianii J640 (Hfphytase)	*Pichia pastoris*	49	4.5	50	Watanabe et al. (2009)
Aspergillus niger N-3	*Pichia pastoris*	60–70	2.0 and 5.5	55	Shi et al. (2009)
Pectobacterium wasabiae	*Escherichia coli* BL21 (DE3)	45	5	50	Shao et al. (2008)

phosphatase. They confirmed the refolding ability of *A. fumigatus* phytase after heat denaturation might positively affect its pelleting stability.

Development of transgenic plants with an expression of phytase enzyme may offer opportunity to deal with the problem of phosphorus pollution and can save

the extra cost associated with phosphate amendment. Acceptable amounts of phytase production in transgenic plants could meet daily requirements in food and feed (Richardson, 2001; Richardson et al., 2001; Mudge et al., 2003; Rao et al., 2009). Chen et al. (2008) transferred a gene from *Aspergillus niger* phyA2 using a construct driven by the maize embryo-specific globulin-1 promoter in maize seeds. These phytase expression lines may be used for the development of new maize hybrids to alleviate the impact of animal farming on the ecology and environment and improve phosphorus availability. Transgenic seeds of phytase producing canola have been directly used in animal feed (Chen et al., 2008).

8. Recent progress in phytase research

Several types of phytases have been produced from different sources and characterized in the last decade. Puhl et al. (2009) have identified a new protein tyrosine phosphatase–like inositol polyphosphatases (IPPase) from *Megasphaera elsdenii* similar to known protein tyrosine phosphatase–like IPPases based on its stereospecific reaction of cleaving myo-inositol hexakisphosphate at specific positions. Subsequently Kumar et al. (2011) isolated and identified thirty-three isolates capable of producing phytase. The best isolate gave a titer of 31.76 U/ml at pH 6.0 in SSF using rice bran and 1% maize flour as a substrate a titer of 36.21 U/g was achieved at pH 6. The effect of a temperature increase from 20 to 40°C among the isolates resulted in an enhancement of phytase production of approximately 15% while there was a decrease in production of about 29–68% at 60°C. Optimal pH of the production medium was observed at pH 4.0 and 6.0. Production of an extracellular phytase by *Enterobacter sakazakii* ASUIA279 was optimized using RSM with full factorial faced centered CCD. Elhadi et al. (2011) compared the phytase activity from different bacteria in which *Klebsiella, E. coli*, and *Aspergillus* phytases displayed optimal activity in an acidic pH range while *Bacillus* phytase at neutral pH. Comparative temperature profile showed *Bacillus* phytase more resistant to heat treatment. *E. coli* phytase showed the highest K_m and V_{max} values compared to the other phytases. Dechavez et al. (2011) reported the potential application of bacillus species as a feed supplement to improve the bioavailability of phosphorus in aquaculture. This study investigated the ability of four species of *Bacillus* (*B. pumilus, B. coagulans, B. megaterium*, and *B. licheniformis*) to hydrolyze phytate, and the biochemical properties of the phytases from these strains. Among all, *B. megaterium* displayed the highest enzyme activity. The phytases obtained in crude form from different *Bacillus* strains were optimally active at pH from 5.5 to 7.0, relatively stable up to pH 10 and activity was retained up to 80°C. These properties are favorable for an animal feed application. Escobin-Mopera et al. (2012) purified a bacterial phytase (monomeric protein, ~45 kDa, optimum pH 4, and temperature 50°C) from *Klebsiella pneumoniae* 9-3B using size exclusion chromatography and SDS-PAGE. In another study, Sasirekha et al. (2012) optimized culture conditions for maximizing phytase production by *Pseudomonas spp*. They found that the

enzyme was stable between pH 4 and 10 and temperatures of 30°C to 50°C. Optimal pH and temperatures were 6 and 37°C, respectively, with a maximum phytase activity of 98.76 U/ml after 24 hrs of incubation. Maximum phytase production was observed in a medium supplemented with 13.6% rice bran at a temperature of 39.7°C, pH of 7.1, 320 rpm of agitation, and 0 vvm of aeration (Hussin et al., 2012). Gaind and Singh (2015) isolated a thermotolerant *Aspergillus flavus* strain from the rhizosphere of *Pisum sativum* and found that the purified phytase enzyme had a molecular weight of 30 kDa and a pH optimum in neutral range. This phytase enzyme might have application in supplementation of poultry/marine food. Borgi et al. (2015) reviewed the current status and future prospects of phytases from *bacillus* strains. The phytase from *B. licheniformis* possesses attractive biochemical properties appropriate to improve the profitability of several biotechnological processes.

Wild-type microbes generally have low phytase activity, which is not favorable if the enzyme is to be commercialized. To overcome this problem, phytases have been cloned into different expression hosts. A thermostable phytase gene, phyA, was isolated from *Aspergillus aculeatus* (RCEF 4894) and expressed in *Pichia pastoris*. The recombinant thermostable phytase exhibited high activity when Na phytate was used as substrate at a pH range of 2.5–6.5 and was able to resist the temperature of up to 90°C for 10 min (Ma et al., 2011). The high thermostability of phytases coupled with broad pH optima suggested it as a promising candidate for feed-pelleting applications. PBD and Box-Behnken designs were used for fermentation (Farhat-Khemakhem et al., 2012), 47 U/ml of phytase activity was achieved by combining heterologous expression using a cloning vector carrying the pAMb1 replication origin.

Statistical optimization technique was used to optimize the production of phytase and xylanase on citrus pulp by using different culture variables such as the effects of pH, temperature, and moisture. Results showed that the major factor contributing to enzyme production was pH. An optimal phytase activity of 264.68 U/g at pH 6 was observed after 48-hr incubation with an initial moisture level of 50% (Neves et al., 2011). Ushasree et al. (2014) reported the functional expression of phytase from *A. niger* NII 08121in *Kluyveromyces lactis*. This enzyme was thermostable and retained 69% and 37% activity at 90 and 100°C for 10 min respectively and stable at the same temp for 1 hr. Coban et al. (2015) enhanced the phytase production in SmF by using a novel fermentation technique by testing the effect of the various microparticles on *A. ficuum*. Borgi et al. (2014), characterized the recombinant phytase from *B. licheniformis* ATCC 14580 (PhyL). This enzyme (42 kDa) was optimally active at 70–75°C and pH 6.5–7.0. A novel phytase-producing rumen bacterium *Mitsuokella jalaludinii* was isolated, purified, and characterized by Lan et al. (2011). The cell-bound phytase had an optimal pH between 4 and 5 and an optimal temperature between 55 and 60°C and was highly specific toward the substrate sodium phytate. Analysis of the effect of metal ions showed

that the enzyme was strongly inhibited by Cu^{2+}, Fe^{3+}, Zn^{2+}, and Fe^{2+}, slightly stimulated by Mn^{2+} and Ca^{2+} and significantly stimulated by Ba^{2+}.

Review articles published by Singh et al. (2011) and Yao et al. (2012) discussed the latest developments in phytase research with special reference to enzyme mechanism, three-dimensional structure, and improving bioavailability to support the broader application of phytases in crop and animal production. Details about phytase structure are necessary in order to design ideal phytases that suit the desired biotechnological applications. The crystal structure of phytase from *Klebsiella* sp. ASR1 has been determined and bears similarity to other histidine-acid phosphatases with an active site a unique binding mode for its substrate phytate located in a positively charged cleft between the polypeptide domains (Böhm et al., 2010). The structural details are necessary and provide information regarding substrate binding and catalytic mechanism.

The use of directed evolution approaches to improve the enzyme properties for wide application in the industry has increased considerably (Kumar and Singh, 2013). Phytases possessing qualities such as thermostability, wide pH range, and storage stability are very difficult to find in screened isolates from nature. To achieve phytases with improved and desired properties, two different strategies are suggested: directed (molecular) evolution and rational protein design (Böttcher and Bornscheuer, 2010). In a study by Garrett et al. (2004) site saturation mutagenesis technology, which is a part of directed evolution approach, was used to detect mutations that augmented enzymatic performance and creating an optimized phytase gene. This technology was based on the generation of an array of all possible combinations of single-site mutations in an enzyme. The main goal of this approach was to increase thermostability and maintenance of high substrate turnover. After combining, these single site mutations led to the development of an improved thermostable phytase with an enhanced gastric stability appropriate for the commercial market as an animal feed supplement. Shivange et al. (2012) reported the cloning, characterization, and directed evolution of the *Yersinia mollaretii* phytase (Ymphytase). Ymphytase has a tetrameric structure with positive cooperativity (Hill coefficient was 2.3) and a specific activity of 1,073 U/mg, which is ~10 times higher than widely used fungal phytases. The thermostability of phytase can be improved by multiple amino acid exchanges, each of which help in slight increases in the unfolding temperature of the protein. Studies based on the semi-rational protein engineering based on three-dimensional structure and sequence alignment have attempted to improve characteristics of phytases of *A. fumigatus* and *A. niger* (Bei et al., 2009). In this work, phytase was divided into seven different fragments and equivalent regions were swapped to construct an array of chimeras. These functional chimeras expressed in the *P. pastoris* that resulted in the production of novel phytases with high thermostability.

9. Phytase applications

Hydrolysis of phytic acid is necessary for better utilization of bounded phosphorus, minerals, and trace elements in phytic acid complexes. Recently diverse applications other than in feed supplements have been found for phytases. Microbial phytases have been applied to human and animal foodstuffs to improve mineral bioavailability and for food processing but their use in environmental protection, fish nutrition, and plant nutrition is poorly explored. The following section focuses on phytase application in various fields.

9.1 *Environmental protection*

To protect the environment, legislation has been passed in response to increased public awareness of the impact of animal agriculture on environments. Supplementation with inorganic phosphorus and excretion of high phosphorus by monogastric animals creates a problem of phosphorus pollution into water bodies. Golovan et al. (2001) developed transgenic mice expressing *E. coli* appA phytase gene in the salivary gland resulting in the secretion of a biologically active 55-kD glycosylated protein with a low pH optimum and with protease resistance. Secretion of the phytase in saliva resulted in a substantial reduction of fecal phosphorus and the result pointed to a way to deal with the phosphorus pollution generated from animal agriculture.

Subsequently, Golovan et al. (2001) developed transgenic pigs that expressed phytase in the salivary gland, which completely digested dietary phytate phosphorus and reduced fecal phosphorus output thereby showing possible environmental pollution reduction by the pork industry.

9.2 *Preparation of myo-inositol phosphates*

Various pharmaceutical applications such as inositol phosphate, phospholipids, in the mobilization of calcium from intracellular reserves and in trans-membrane signaling have potential development (Billington, 1993). Siren (1995, 1998) proposed the use of specific inositol triphosphates as pain killers. These specific inositol triphosphates have also been of use to treat cardiovascular diseases (Siren et al., 1992).

The chemical synthesis of myo-inositol phosphates is performed at extreme temperatures and pressures (Billington, 1993). The enzymatic production of *myo*-inositol phosphate derivatives is a potential alternative to chemical synthesis as phytases hydrolyze *myo*-inositol hexaphosphate sequentially. Siren (1986), reported that phytase from *S. cerevisiae* by enzymatic hydrolysis resulted in the production of D-*myo*-inositol 1,2,5-triphosphate, *myo*-inositol 1,2,3-triphosphate, D-*myo*-inositol 1,2,6-triphosphate, and L-*myo*-inositol 1,3,4-triphosphate. Various *myo*-inositol phosphates have been efficiently produced using immobilized

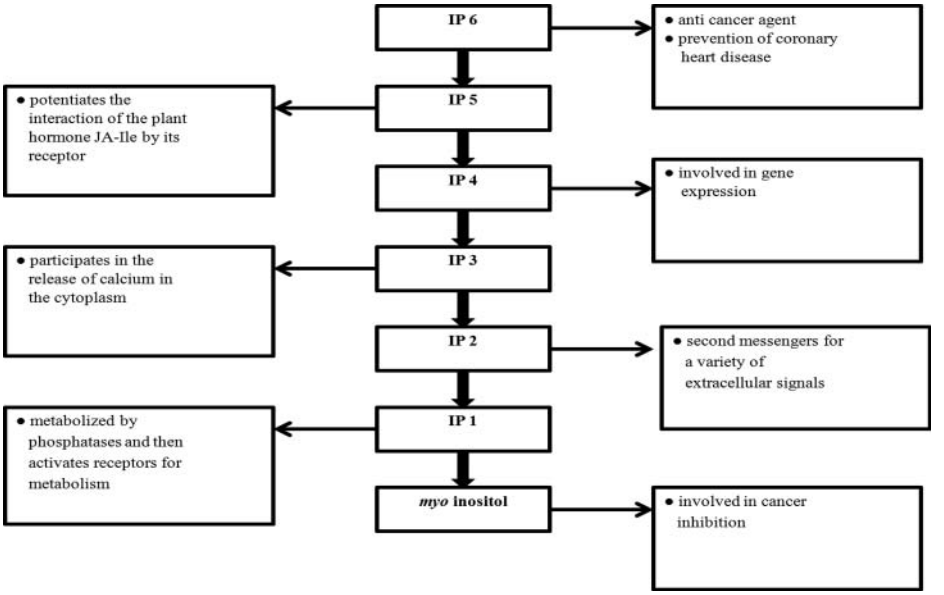

Figure 2. Functions of phytate and phytate degradation products. Vucenik and Shamsuddin (2003) and Husna et al. (2010): IP 1 and IP 6; Bacic et al. (2010): IP 6; Watson et al. (2012) and Steger et al. (2003): IP4; Sheard et al. (2010) and Mosblech et al. (2011): IP5; Patergnani et al. (2011) and Somlyo and Somlyo (1994): IP3; Carey et al. (2004) and Abel et al. (2001): IP2.

phytases (Ullah and Phillippy, 1988; Greiner and Konietzny, 1996). Functions of phytate and phytate degradation products are presented in Figure 2.

9.3 *In human nutrition and disease prevention*

Plant-based food products are composed of various nutrient and antinutrient factors. Among the antinutrient component, phytic acid is of prime concern for health management and human nutrition. Phytate is not utilized by human beings because of the absence of a phytase enzyme in the intestine and is a significant antinutrient component of food. The presence of phytate in food affects the bioavailability of many divalent and trivalent mineral ions Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, $Fe^{2+/3+}$, and Cu^{2} (Fredlund et al., 2006; Kumar et al., 2010b). Furthermore, phytate also binds with amino acids, positively charged proteins and multivalent cations or minerals present in the foods to form complexes. The resulting complexes are difficult for humans to hydrolyze during the digestion process and nutritionally less accessible for absorption. These complexes also affect many functions by altering the protein structure, and by reducing enzymatic activity, protein solubility, and proteolytic digestibility. Addition of phytase during food processing and consumption could alleviate some of the negative aspects of phytate in plant-based foods (Kumar et al., 2010b). Dephosphorylation of phytate is important to improve nutritive value of food and feed by increasing the bioavailability of essential dietary minerals through the release of phosphate groups from the inositol ring. This has

resulted in decreasing the mineral binding strength of phytate (Sandberg et al., 1999). However, adding phytase during food processing and preparation to reduce the amount of phytate in food has increased the cost of food and feed and limits commercial application on a large scale. Development of whole wheat bread with less phytic acid content and more mineral bioavailability achieved greater nutritional value of the product. Addition of commercial fungal phytase obtained from *A. niger* in the dough ingredients leads to an improvement of the bread quality by affecting its shape, volume, and also confers softness to the crumb. In this process bread quality is improved by the action of phytase indirectly on α-amylase activity (Greiner and Konietzny, 2006). For increased application of phytase in human nutrition, yeasts with high phytase activity have been identified as an ideal candidate for use in the manufacture of whole meal bread with high bioavailable mineral availability. Yeasts (*Saccharomyces cerevisiae, Pichia kudriavzevii, Pichia occidentalis, Candida humilis*, and *Kazachstani aexigua*) isolated from sourdough breads with phytase activity were found to optimally leaven bread dough at pH 5.5 and 30°C. *S. cerevisiae* L1.12 produced the highest amount of phytase, with a specific extracellular activity of 10.6 U/10^{10} CFU. (Nuobariene et al., 2012). Application of phytase in the food industry seems to have great potential benefits nutritionally as well as economically. However, efficacy of phytase must be tested before applying commercially available phytase in food application.

9.4 *Animal feed supplement*

Numerous research experiments have shown that supplementation of microbial phytases to feed enhance utilization of phytate-phosphorus and the phytate-bound minerals by monogastric animals (Lei and Stahl, 2000). Addition of adequate amounts of phytase along with the appropriate amount of inorganic phosphorus supplementation can reduce phosphorus excretion of these animals up to 50% (Lei et al., 1993a; Lei et al., 1993b).

9.5 *For crop plants*

According to an estimate, world assets of inexpensive rock phosphate may be exhausted by 2050, limiting phosphorus available for crop production (Vance et al., 2003). Improving the phosphorus acquisition will have some major impacts on both the environment and agriculture (George et al., 2009; Wasaki et al., 2009; Richardson et al., 2009a; Richardson et al., 2009b). Generally, plant phosphorus uptake is associated with plant roots, performed by arbuscular mycorrhizal fungi. These evolved symbiotic associations between fungi and plants considered as a useful plant strategy for plant growth under a range of abiotic and biotic stress conditions (Kumar et al., 2010a; Kumar et al., 2015a).

Phytic acid, which acts as a source of phosphorus is not easily accessible to plants because it either adsorbs to various soil components or forms a complex with cations. Phosphate-solubilizing microorganisms are commonly present in the

rhizosphere and play a vital role in supplying phosphorus and other nutrients to plants. Various microbes are able to hydrolyze this organic form (phytic acid) of phosphorus by secreting phytases. Potential phosphorus source, phytate, and its derivatives have received attention from several research groups aiming to improve the phosphorus use efficiency in plants by genetic transformation and further release the phosphorus from organic phosphorus containing composite. The production of transgenic plants using genetic engineering raises safety concerns (Ahmad et al., 2012). Yip et al. (2003) showed that the transformation of a tobacco line by a neutral *Bacillus* phytase exhibited phenotypic changes in seed development, flowering and to phosphate deficiency. Developed transgenic lines revealed an increase in fruit and flowers and lower inositol phosphate ratio (IP6/IP5) in seed and promoted growth under phosphate-deprived conditions as compared to the wild type. Mudge et al. (2003) showed that transgenic *Arabidopsis* plants, when grown on a low phosphate medium secreted phytase from the roots only. Expression of extracellular phytase from *Medicago truncatula* in transgenic *Arabidopsis* had a significant improvement in organic phosphorus utilization and plant growth (Xiao et al., 2005). In addition to the expression of phytase in whole transgenic plants, another approach would be to express the phytase gene specifically in root hairs which are directly involved in mineral nutrients uptake. These approaches may be a positive solution to deal with the environmental phosphorus problem and improve sustainable agriculture.

9.6 *Phytase for bioethanol production*

Demand for the production of fuel-grade ethanol using agricultural raw materials, continues to grow on a global basis. Recently, the use of thermostable phytase, which breaks down phytic acid in corn and indirectly related to the production of bioethanol. This removal of phytic acid solves a waste disposal problem, simultaneously creates a higher value added ethanol coproduct, and improves the overall efficiency of ethanol production (Shetty et al., 2008). Phytase incubation in E-Mill dry grind corn processing may assist in increasing co-product values as well as lead to increased ethanol concentrations (Khullar et al., 2011). A method for using a thermostable phytase for eliminating or reducing phytic acid or salts of phytic acid in an alcohol production process has recently been reported (Solbak et al., 2015).

10. Concluding remarks and future direction

Evidence of a decline of global supplies of rock phosphate and inorganic phosphates is emerging suggesting these reserves may be depleted in 50–100 years. The use of phytase in monogastric diets is expected to increase in the future and will be driven by ongoing changes in the world of animal agriculture. It is likely that new microbial phytases will be developed in the foreseeable future, with greater capacity to degrade the majority of phytate in broiler diets. To develop an ideal phytases

that suit industrial needs (catalytically efficient, proteolysis resistant, thermostable, and cost effective) will be a significant challenge. Moreover, a search for phytases in extremophiles with desirable properties could help meet the challenge. In this direction, the use of directed evolution and rational protein design approaches will be required to develop a given phytase for a given process. Expression of phytase in transgenic plants, and animals and development of low-phytate crop could be a solution. Evidence suggests that preparations comprises with multiple enzyme cocktails may provide a competitive strategy for increased nutrient utilization in poultry diets. Such enzyme mixtures with high thermostability and a broad pH range could represent the next generation of feed enzymes rather than using pure single enzymes. However, the quest for more effective phytases will continue with emphasis on a broad pH activity profile, thermal tolerance and enhanced stability under the pH conditions of the stomach. Expanded research on discovering new phytases from environmental samples, engineering ideal phytases based on three-dimensional structures and development of less-expensive expression systems will be required in near future. Therefore, there is an exceptional prospect for new research and development coupled with omics platform aimed at developing, enhancing the sustainability and lessening the environmental impact of agricultural production.

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