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Review Article

Microbial Phytases: Restoration of Phosphorus Balance in Ecosystem

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Received: January 28, 2017; **Published:** March 03, 2017

Abstract

Phosphorus being an element of nucleic acid and phospholipid is an essential macronutrient for all living organisms. It is required for various biological functions such as energy metabolism, activation of metabolic intermediates and signal transduction cascades through regulation of enzymes. Phytic acid, a phosphorylated derivative of myoinositol is a major storage form of organic phosphorus in plant seeds. The hydrolytic products of phytic acid play diverse roles in signal transduction, as osmoprotectants and constituent of cell wall. It also chelates other mineral nutrients and limits there bioavailability in agro-ecosystem. The accumulation of phosphorus as phytic acid and continues depletion of its inorganic sources from ecosystem could lead to eutrophication of minerals in water bodies and phosphorus imbalance in terrestrial ecosystem. Phytases a group of enzymes found primarily in microorganisms and plants, catalyze the hydrolysis of phytic acid and recycle phosphate in available form back into ecosystem. This review is focused highlighting phytases producing microbes in restoring ecological balance of phosphorus.

Keywords: Phytase; Microbes; Phytic acid

Introduction

Micronutrients are not only essential for plant growth and development but are also integral part of human and animal health [4]. Phosphorus (P) is a basic component of life like nitrogen. Adequate levels of phosphorus are critical to the growth and development of all organisms for a range of functions such as energy generation and metabolic regulation including a part of macromolecular structure. The low concentration and poor mobility of phosphorus in soil, due to strong interactions with soil constituents limit its availability to the plants [37,76]. The use of inorganic phosphorus fertilizers are also quite inefficient with only less than 20% of applied inorganic (Pi) being absorbed by plants. The remaining Pi either becomes immobile in the soil or leaches into and pollutes nearby surface waters [63]. The massive use of inorganic phosphorus (Pi) containing fertilizers and the suboptimal availability in agriculture soil is a major area of concern. Further accessible reserves of rock phosphate which is a major source of inorganic phosphorus are projected to be exhausted by the end of this century [95].

According to Environmental Protection Agency (EPA) report of Ireland on water pollution, nutrient loss from farms, fisheries and to less extent by sewage was considered one of the biggest reasons leading to eutrophication by excess P and nitrogen is considered to be a main factor [12]. The typical total P range in agriculture soils is approximately 0.20 - 2.0 g/kg [56]. Phosphorus in soils is either dissolved (< 0.1%) and mostly exists as ortho-phosphate, inorganic polyphosphates and organic forms whereas solid form which largely unavailable constitute the dominant form. The dissolved P is typically less than 0.1 percent of the total soil P [54] and organic P in solid form either exist a minerals bound inorganic P or organic P bound to living or dead organisms [11]. Phytic acid also designated as phytate, constitute major source of organic phosphorus in cereals legumes and oil seed crops [25]. During seed development and maturation, phytic acid is deposited in spherical inclusions known as globoids [32,64]. In wheat bran, the main components of the globoids is composed of phytic acid (40%), protein (46%), and several minerals [7,59]. The accumulation of organic phosphate such as phytic acid and salt of phytate limit the availability of phosphorus to plants in an ecosystem [36,37]. Phytic acid and its derivatives also act as an antinutrient factor in monogastric animals feed by forming insoluble complexes with protein and chelating divalent cations [100] such as calcium, copper, and zinc needed by the animal [3,48-50]. This undigested phytate is then excreted in the manure, thereby causing phosphate pollutions [95] and other phosphorus related pollution problems [17] particularly in aquatic ecosystem like deficiency or death of organisms like fish associated with blooms of toxin-producing microbes which already have attracted public and governmental concern.

Until the 1980, Europe was largest consumer of P fertilizers and in Western Europe approximately 79% of P supplied is used for fertilizer in agricultural sector followed 11% as feed additive for animals and 7% for detergents [11]. The loss of phosphorus in agriculture soil and its eutrophication in aquatic ecosystem is biggest source of pollution. The hydrolysis in process of recycling of phosphomonoesters in biological system is an important process and linked to regulation of different metabolic events and a wide variety of cellular signal transduction pathways [1]. According to recent studies, European P inputs via chemical fertilizers and animal feed can be reduced if P is recycled [72]. Microbes can play major role in this challenge and achieving the 5 R's formula on restoring the national P balance not only in Europe but other parts of the world [98] through phytase, siderophores organic anions secretion which can significantly increase the availability of available P in an ecosystem and handling water pollution.

Phytic acid

Phytic acid (Figure 1) a primary storage compound of phosphorus in seeds constitutes upto 80% of the total seed phosphorus and contributing as much as 1.5% of seed dry weight [65]. Maximal phytic acid levels are achieved at seed maturity immediately preceding desiccation [65]. The negatively charged phosphate in PA strongly binds to metallic cations, making them insoluble, thus limiting their availability as nutritional factors. Phytic acid and its derivatives are also implicated in various cellular events such as RNA export, DNA repair, signalling, endocytosis and cell vesicular trafficking [8,26]. In soybeans (*Glycine max*), phytic acid is deposited in protein bodies (protein storage vacuoles) as a complex of chelated minerals and protein known as phytin [64]. During seed germination, phytic acis is hydrolysed by the endogenous phytases for providing nutrients to rapidly growing seedling [35,52]. In some areas of the world, where the predominant diet pattern is vegetarian or animal meat is available in only small amounts and crops like legumes are major source diets, enriched with the organic phosphorus. Therefore, legumes can be considered as foods with health benefits, but their phytate contents can limit the availability of minerals. A comprehensive analysis of different roles of phytate in human has been done by Kumar and several other workers [46,61].

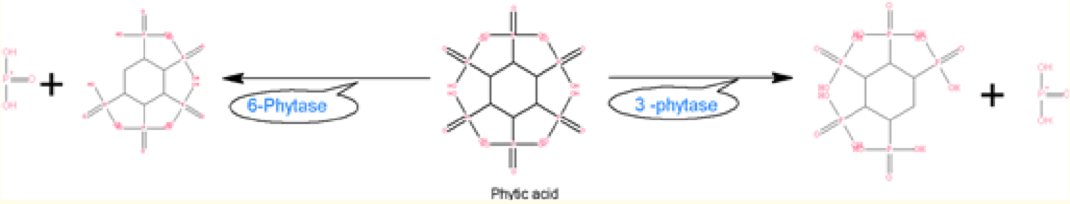

***Figure 1:** Structure and classification of phytases.*

Dynamics of phosphorus in ecosystem

Both organic and inorganic form of phosphorus differs in their behavior and fate in soils [33,87]. The inorganic form can constitute for 35 - 70% whereas organic form constitute upto 30- 65% of the total P in soils [34]. Further, the major stable form of organic form includes inositol phosphates and phosphonates whereas orthophosphate diesters, labile orthophosphate monoesters, and organic polyphosphates constitute its active form [18,86]. The organic form of phosphorus can be released through mineralization processes mediated by soil organisms and plant roots in association with phosphatase secretion and thus available for recycling in ecosystem. This process is extremely complex and in modern terrestrial ecosystem which is mostly dominated by agriculture and human activities, the concentration of available inorganic phosphorus in soil seldom exceeds 10 μM [5,60], much lower than that in plant tissues [94] where the concentration is approximately 5 to 20 mM [66]. To combat low concentration and poor mobility available phosphorus in soils, phosphorus fertilizers are applied or alternatively in the form of manure can be applied. In manure, inorganic form of phosphorus accounts for 50 - 90%. It also contained large amounts of organic phosphorus in the form of phospholipids and nucleic acids [86]. Further, organic acids available in manure can dissolve Ca^{2+} phosphate, and efficiently weaken the nanoparticle stability of HAP, by controlling the free Ca^{2+} availability and thereby the nucleation rate (Figure 2).

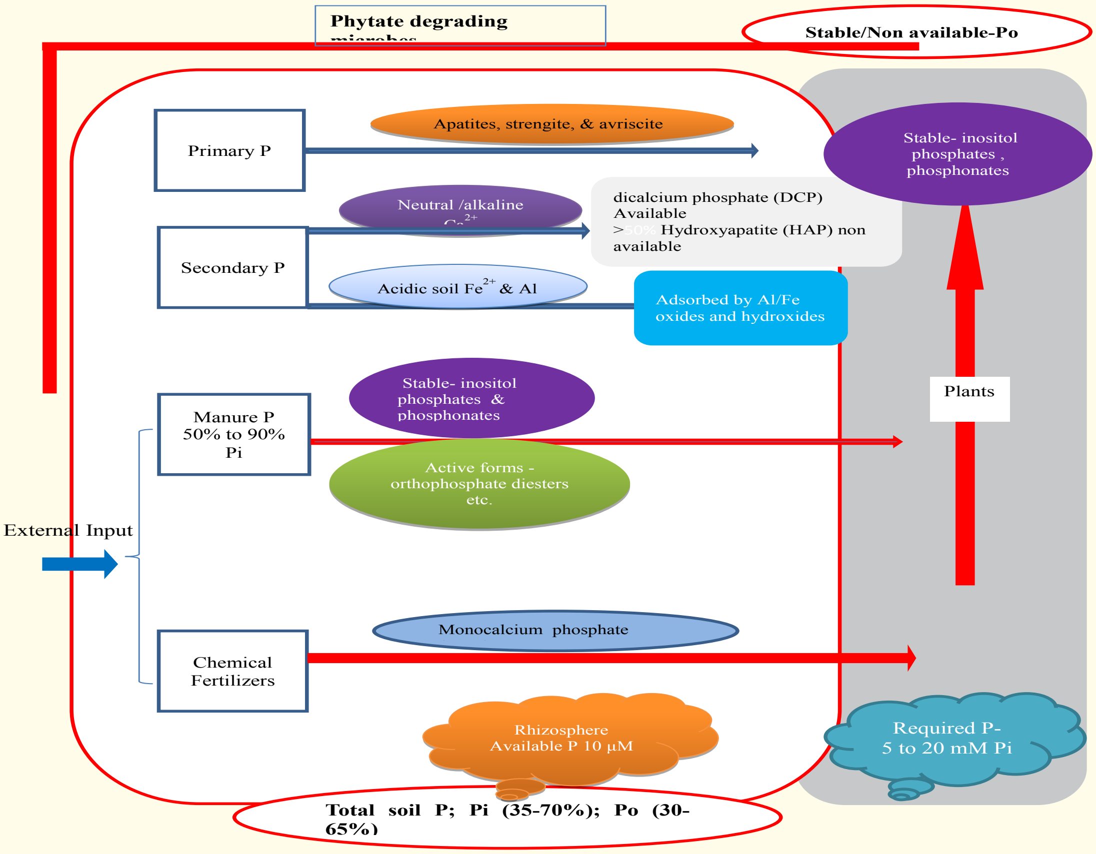

Figure 2: *Pictorial representation of phosphorus (P) cycle. In an ecosystem, available phosphorus (PO4) both from inorganic and organic sources is utilized by the plants for various metabolic processes. The available phosphorus is utilized by plants and converted into a stable form of phosphorus i.e. phytic acid and other biomolecules during plant growth and seed development. Different phytate degrading microbes like bacteria, fungi and yeasts play a pivotal role in recycling organic form of phosphorus back into ecosystem. These microbes can decrease the dependence on inorganic sources to meet the demand of phosphorus in soil.*

Microorganisms are in fact a key driver in regulating the mineralization of phytate in soil and their presence within the rhizosphere may compensate plants inability to otherwise acquire P directly from phytate. Bacteria with ability to mineralize phytate have been isolated from rhizosphere. Various microbes can facilitate absorption equilibrium alteration in P acquisition by plants from rhizosphere through different mechanisms [67] including increased form of orthophosphate P or organic P either directly or indirectly through microbial turnover in soil [73] by solubilizing and mineralizing P from sparingly available forms of soil inorganic and organic P in soil [55,66] through phosphatases, efflux of organic anions, and protons, siderophore production [67,68,75]. The sources of organic anions, siderophores and protons are effective in increasing the availability of adsorbed orthophosphate or organic P from precipitated forms including Ca phosphates by chelating ions associated with complexed P in soil and thus facilitating their release. Also soil microbes produce a range of phosphatases which are known to have capacity to utilize P from various forms of organic P and reviewed extensively in several studies [12,41]. The organic P form of may be hydrolyzed upto 60% by phosphatases [13] and the phosphatases of microbial origin are in particularly more effective than plants for P release [81].

Classification of phytases

According to IUPAC-IUBMB currently three classes 3- phytases (EC 3.1.3.8), 6-phytases (EC 3.1.3.26) and 5-phytases (EC 3.1.3.72), based on the position of specificity of the initial hydrolysis of phytic acid of phytases has been recognized [8] which are further divided into: acid and alkaline phytases [41]. On the basis of structure four classes; histidine acid phosphatase (HAP), cysteine phytase, purple acid phosphatase, and beta-propeller phytase (BPP) of phytases have been characterized [49]. Subsequently, a commonly accepted phytase nomenclature has been proposed based on three- dimensional structures and catalytic mechanisms: histidine acid phytase (HAPhy), β-propeller phytase (BPPhy), purple acid phytase (PAPhy), and protein tyrosine phytase (PTPhy) to delineate them respectively [50].

Microbial phytases and their distribution

The hydrolysis of phytic acid to myoinositol and phosphoric acid is an important metabolic process in any biological system. The dephosphorylation of free or bound inositol phosphate is mainly driven by phytases. Since, the first exploration in 1907 [80] a wide distribution of phytases have been reported in plants, animals and microorganisms [2,51]. Among them phytases of microbial origins are of particular importance due to their ease of genetic manipulation and large scale production [2]. The phytate-degrading bacteria are widely distributed and diverse in nature [39]. Bacterial phytases are most promising for biotechnological application [61,96] and alternative to fungal enzymes because of their higher substrate specificity, greater resistance to proteolysis and better catalytic efficiency [2,41,71]. A number of species and strains of *Bacillus* (Table 2) have been exploited for extracellular [23-25,27,31,44] and recombinant phytase production [25,31]. So far phytases of different bacterial isolates like *Bacillus* and *E. coli* etc. have been explored for their potential in feed additive. Simultaneously attempts have been made on improving the genetic stability and improving their catalytic efficiency through site directed mutagenesis (Table 1). Besides *Bacillus*, a number of other bacteria such as *Anabaena, Gloeobacter, Streptomyces, Flavobacterium, Prosthecochloris, Desulfuromonas* enterobacteria, anaerobic rumen bacteria, pseudomonads and *Azospirillum* (Table 1) from terrestrial and aquatic environments have been explored for their ability to degrade organic phosphorus using culture independent and dependent techniques, analysis of the phytase microbial diversity [39,41,57,96].

Fungal and yeast phytase

Over 200 fungal isolates belonging to *Aspergillus, Mucor, Penicillium, Rhizopus* and other genera have been tested for phytase production [28,38] (Table 1). *Aspergillus niger* has been identified as the most active producer and so far a number of commercially available phytases are largely sourced from its strains [38,80]. The phytases from these strains are relatively heat and acid-stable, a pre-requisite in context of feed heat treatment and stability in the upper digestive tract, respectively [14,15]. Phytases encoding genes have been amplified from *Peniophora lycii, Agrocybe pediades* and *Trametes pubescens*. Over expression of cDNA encoding these genes in *Aspergillus oryzae,* revealed temperature optima between 40 - 60°C and pH optima between 5.0 - 6.0, except for the *P. lycii* phytase, for which pH optimum 4.0 - 5.0 was reported [47]. Among the best-known commercial phytases added to animal feed 'Natuphos' (Gist Brocades, the Netherlands), a recombinant phytase produced by expressing the *phyA* gene from *Aspergillus niger* NRRL 3135 in *A. niger* CBS 513.88 [15]. The physicochemical characteristics of 124 kDa phytase purified from *Rhizopus oligosporus* ATCC 22959 likely to render it of potential industrial interest. Limited studies have been done on the phytases of yeast origin such as *Saccharomyces cerevisiae* and *Schwanniomyces castellii* [74]. The S. castellii had a higher phytase potential than other phytase-producing yeasts. The enhanced phytase activity by *S. cerevisiae* strain has been reported in mineral cultured medium and concentrations of carbon source act as inducer of phytase production [70].

Applications of phytases

The importance of microbial phytases in biotechnology industries has been recognized in various fields and reviewed extensively in several studies [75]. The research on phytases persisted for over a hundred years and it has grown exponentially during the past two to three decades [50]. Phytases have different roles in different organisms. In plants, phytases are induced during germination where as in microorganisms they are most frequently induced in response to phosphate starvation. Phytase are also involved in chelating K^+, Mg^{2+} Zn^{2+} and Ca^{2+} bound to phytic acid Degradation of PA and the release of phosphorous and minerals have been described as great interest to human and animal nutritionists as well as ecologists (Figure 3). The different roles of phytases are summarized in Table 1.

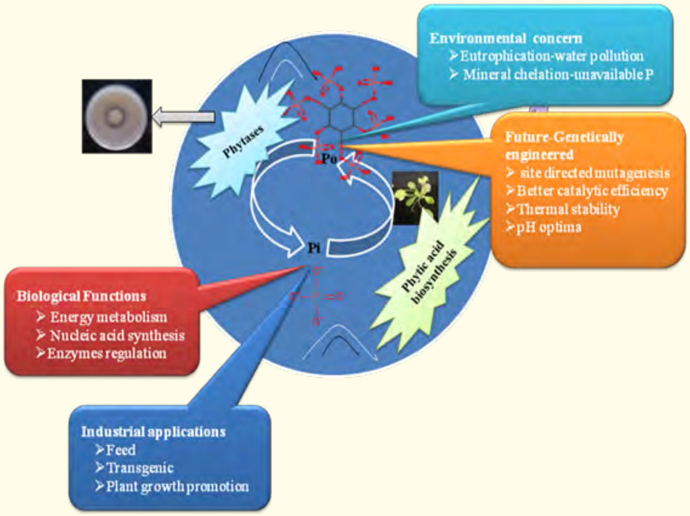

Figure 3: *An overview high lightening biological roles, industrial applications, environmental concerns of phosphorus and future scope of phytate degrading enzymes using genetic engineering. The eutrophication of phytic acid can leads to water pollution and P deficiency. Microbial phytases play a pivotal role in degrading organic phosphorus and recycle it back to ecosystem which to some extent could decrease the external application of fertilizers to meet its deficiency.*

S. No.	Microbes	Host strain	Description of study	References
1	*Citrobacte. freundii*	*P. pastoris*	The codon optimized and modified phytase gene (phyA-mod) showed a 50% increase in phytase activity	[30] Gordeeva., *et al*. 2010
2	*Penicillium* sp	-	The thermal stability, optimal pH, temperature and protease resistance has been modified for additive in industry.	[103] Zhao., *et al*. 2010
3	*Bacillus licheniformis*	-	beta-propeller phytase is stabilised using site directed mutagenesis	[85] Tung., *et al*. 2008
4	*Escherichia coli* AppA2	-	The catalytic efficiency was improved compared to wild type	[42] Kim., *et al*. 2008
5	Aspergillus niger (PhyA)	-	Through Site directed mutagenesis pH range was shifted to 5.5 to 4	[102] Zhang and Lei, 2008
6	*Aspergillus terreus*	-	Thermostability of phytases *A. terreus* was enchanced similar to *A. niger* was restored through ionic interactions and hydrogen bonds.	[40] Jermutus., *et al*. 2001

7	*Aspergillus niger* PhyB	-	Through Site directed mutagenesis pH range was shifted to 2.3 to 3-3.5	[97] Weaver., *et al*. 2007
8	*Aspergillus niger* PhyA	-	Using site directed mutagenesis pH optima was shifted to match the stomach condition which can be used in animal feed	[43] Kim., *et al*. 2006
9	*Aspergillus niger*	*Saccharomyces cerevisiae INVSc1*	The phytase activity for *A. niger* NRRL 3135 phytaseA has been increased using site directed mutagenesis	[58] Mullaney., *et al*. 2002
10	*Aspergillus fumigatus*	-	Site directed mutagenesis was used to improve the pH optima.	[82-84] Tomschy., *et al.* 2000; Tomschy., *et al*. 2000a; Tomschy., *et al.* 2002.

Table 1: *Modification of microbial phytases for increasing their catalytic efficiency through genetic engineering.*

Phytases in Human Nutrition

Compared to intestinal mucosal phytase activity in pigs, humans phytases in small intestine has been reported with very low phytase activity therefore have limited potential to breakdown phytate. But the adaptive upregulation of intestinal phytase in mammals including humans offers advantage under P-inadequate diets [46]. In humans, however microbial diversity present in the colon help in degrading phytate [46].

Conclusion and Future Aspects

Depletion of rock phosphate reserve and unavailability of phosphorus from organic sources is a major area of concern across the world not only in undeveloped but also in developed countries. Therefore exploring microbes for P availability is a feasible and during the last two decades there has been an increase in the use of microbial phytases for potential biotechnological applications such as agriculture, environmental protection and as feed additives in diets for swine and poultry for efficient utilization of plant-based food products (Table 2). Phytases are also crucial for recycling phosphorus in ecosystem particularly from its organic form i.e. phytic acid and thus decreasing the demands of fertilizers to meet phosphorus deficiency. Already advancements in biotechnological techniques and use of genetic engineering such as codon optimization and site directed mutagenesis, attempts have been made for next-generation phytases, tailored for various applications either using fusion phytases or improving the catalytic efficiency, pH optima, thermostability, adequate gastric performance and resistances to proteolysis, acid, and heat. So far phytases of *Aspergillus*, *E coil* and *Bacillus* strains have been used for the production, purification and gene cloning. Eventhough microbial phytases are potential candidates in restoring the P balance in ecosystem but so far emphasis has been given on their role in feed additives. The reciprocal relation of calcium intake and phytate degradation in human gut is a concern for the inclusion of gut micro floral-derived phytase as a food additive [46,97]. Future emphasis on microbes capable of increasing the availability from inorganic or organic sources in agriculture can also help in combating P deficiency in a sustainable manner.

Application	Source	Transgenic lines	Description of study	References
Transgenic	*E. coli* periplasmic phytase	soybean line (CAPPA)	Improves phosphorus availability	[6] Bilyeu., *et al*. 2007
	Wheat endogenous 6-phytase [EC 3.1.3.26] and *Aspergillus* 3-phytase [EC 3.1.3.8]	Wheat	Improving phosphate and mineral bioavailability in food and feed.	[9-10] Brinch., *et al*. 2003; Brinch., *et al*. 2006
	Soybean phytase gene (GmPhy)	*Glycine max*	Ectopic expression of phytase for reducing phytate content in soybean seed	[16] Chiera., *et al*. 2005
	AtPAP15	*Arabidopsis thaliana* & *Nicotiana*	Tissue specific expression was observed which is likely to mobilize phosphorus reserves in plants during seed and pollen germination	[45] Kuang., *et al*. 2009
	Aspergillus niger (PhyA)	*Medicago truncatula*	A high level of enzymatically active, stable and recombinant phytase were produced and secreted into the culture medium	[62]Pires., *et al*. 2008
	Aspergillus phytase	Arabidopsis	Transgenic lines expressing phyA resulted improved phosphorus nutrition compared to plants supplied with inorganic phosphate	[69] Richardson., *et al*. 2001
	Aspergillus ficuum phyA	Potato and Alfa-alfa	The active and stable phytase has been produced in potato leaves for molecular biofarming.	[89-93] Ullah., *et al*. 2003; Ullah., *et al*. 2002
Plants	Wheat & barley	Wheat and *E.coli; wheat , mize, barley and rice*	Expression of MINPPs including Four TaPhyIIa and three HvPhyIIa was studied in plants and also overexpressed in *E. coli*. The differential expression was also studied which is driven by different promoters.	[22] Dionisio., *et al*. 2007
	Barley		Effect of temperature and seed germination on phytse production has been studied	[79] Sung., *et al*. 2005
	Nicotiana tabacum		A monomeric extracellular phytase release under phosphorus starvation has been purified and characterized from roots.	[53] Lung., *et al*. 2008
Plant growth promotion	*Sporotrichum thermophile*	Wheat	Role in growth promotion of plants	[77-78] Singh and Satyanarayana, 2010

Animal Feed	*E. coli*	Broiler chickens	Supplementation of the mixture of casein and IP6 with phytase improved the digestibility coefficients of amino acids and also excretion of endogenous minerals was increased	[19-21] Cowieson and Ravindran 2007; Cowieson., *et al*. 2006
	E. coli (appA)	poultry and swine	Thermal tolerance and gastric performance of a *E. coli* phytase engineered through site directed mutagenesis was improved and gastric stability assay was done	[29] Garret., *et al*. 2004
Environmental	Microbial phytase	Chicks	Combined effect of microbial phytase and citric acid has been studied	[55] Martinez., *et al*. 2006
Food industry	*P. anomala*		Permeabilized yeast cells were used mitigating phytate content of soymilk which has a potential application in food industry.	[96] Vohra., *et al*. 2003

Table 2: *Examples of using microbial and plant phytases for various applications.*

Acknowledgement

The author is thankful to Department of Science and Technology-New Delhi for providing funding under DST-FAST track young scientist scheme and Department of plant pathology, CSK-HPKV agriculture University-Palampur, India.

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