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AN OVERVIEW ON MICROBIAL PHYTASE AND ITS BIOTECHNOLOGICAL APPLICATIONS

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ABSTRACT:

Phytic acid, the main storage form of phosphorous in plants cannot be digested by monogastric animals and is excreted into environment. This compound also has antinutritional effect due to its negative charge which makes it able to chelate minerals and organic elements like calcium, iron, zinc and amino acids to provoke malnutrition. Alarming increase in eutrophication and cost of supplementation of feed has necessitated a need to look for alternative solution to overcome these problems. Phytase enzymes have been considered for degradation of phytate content in feed stuff. Another approach is expression of microbial phytase gene in crops to reduce the phytate content without increasing fodder cost. The aim of this paper is to provide recent development with regard to phytic acid, its structure and biosynthesis, antinutritional and environmental effects, microbial phytase enzyme as a bioresource for food supplementation of non-ruminants and expression of microbial phytase gene in plants.

Keywords: Phytic Acid, Microbial Phytase, Biotechnological application, Transgenic Plants, Environmental Pollution, Malnutrition

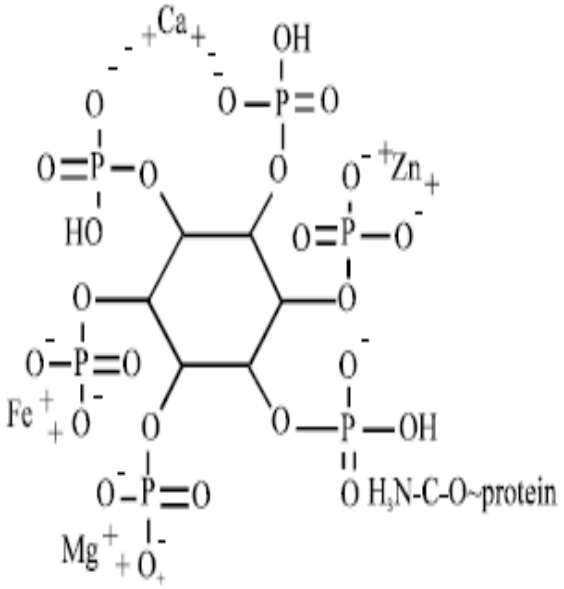
1. INTRODUCTION

Phytic acid (*Myo*-inositol (1,2,3,4,5,6) hexaphosphoric acid, IP6) is the main storage form of phosphate as energy source and antioxidant for the germinating seed in all cereals, legumes, nuts and oil seeds which accounts for 60-90% of the total phosphorus [1]. Table 1 shows total phosphorous and proportion of phosphate phytate in plants or plant byproducts [2].

Phytate, the salt form of phytic acid, is stored in protein storage vacuoles in the embryos or seed aleuronic cell layer [3, 4]. In addition to its role as storage form of phosphorous, it acts as an antinutrient agent by chelating divalent cations (Zn^{2+}, Fe^{2+}, and Mg^{2+}) due to its negative charge preventing the uptake of these minerals [5, 6]. Figure 1 shows structure and interaction possibilities of phytic acid with cation and proteins. Lower inositol phosphates are also involved in stress responses and intracellular signaling. It has been reported that the amount of phosphorous in plant phytates might be sufficient

to meet the requirements of feeding animals [7]. However, non-ruminants animals such as poultry, fish and human have very low or no phytase activities in their digestive tracts so they are not able to utilize the phosphorus from phytate [8]. This type of phosphorous is then excreted into the environment causing serious threat of phosphorous pollution in regions where animal production is intensive. Phosphorous pollution accelerates the process of eutrophication. The outcome of this event would be the lake's biological death, cyanobacterial blooms, hypoxia and death of aquatic animals due to depleted bioavailable oxygen and production of nitrous oxide, a potential greenhouse gas [9]. To escape from antinutrient behavior of phytic acid and improvement of feed quality of crops, several approaches have been investigated and suggested.

Figure: 1. Structure of phytic acid and its interaction with Cation and proteins [5].



Here we review the structure and biosynthetic pathway of phytic acid followed by evaluating the potential of different strategies for reducing the phytate content from food stuff and nutritional aspect of microbial phytase for feeding of poultry and fishes.

[Table-1]

[2].STRUCTURE AND BIOSYNTHESIS

Inositol phosphates consist of an inositol ring and at least one phosphate group (Fig 1). Six groups of phosphates in *Myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate have been attached to the inositol ring. Using the prefix "hexa*kis*" instead of "hexa" indicates that the phosphates are not internally connected and the compound is consequently a polydentate ligand, which is a chelator that can bind to more than one coordination site of the metal atom. Each of the phosphate groups is esterified to the inositol ring and together they can bind up to 12 protons in total [10, 11].

Generally, three main features of phytic acid are responsible for its involvement in a number of metabolic processes in eukaryotes: its chelating property; its ability to function as a phosphate donor/acceptor which makes it ubiquitous/abundant in numerous cell systems; the lower inositol phosphates are also involved in a number of cell signaling pathways and it may act as a precursor for such compounds [10].

The chelating property of the phosphate groups causes phytic acid to bind readily to cations. The degree of the mineral cations to form complexes in vitro with inositol phosphates has been found to be of the order: Cu^{2+}>Zn^{2+}>Cd^{2+} for all InsP3-InsP6 at pH 3 to 7, however, binding strength is weaker for the lower inositol phosphates [12]. Similar binding assays using only phytic acid and none of the lower inositol phosphates showed the order Cu^{2+}> Zn^{2+}>Ni^{2+}>Co^{2+}>Mn^{2+}>Fe^{3+}>Ca^{2+} and Zn^{2+}>Cu^{2+}>Co^{2+}>Mn^{2+}>Ca^{2+} in two independent studies [10].

Two pathways are considered for biosynthesis of phytate; lipid dependent and lipid independent. In the former route, phytate is obtained from the successive phosphorylation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3. Later compound is released from PtdIns(4,5)P_2 (phosphatidylinositol 4,5-biphosphate) by the action of a specific phospholipase C. The phosphorylation steps are catalyzed by two enzymes: an Ins(1,4,5)P_3 3-/5-

/6-kinase, which has been isolated from *Arabidopsis thaliana* encoded by two genes, *AtIPK2α* and*AtIPK2β*; and an inositol polyphosphate 2-kinase, encoded by the *Arabidopsis AtIPK1* gene. All these enzymes can catalyze the conversion of several inositol phosphates [13].

Second pathway relies on the phosphorylation of *myo*-inositol or Ins(3)P. The biosynthesis of phytate following this pathway has only been reported in the aquatic monocotyledonous plant *Spirodela polyrhiza* [14]. It differs from that reported in the slime mould *Dictyostelium discoideum* which goes via Ins(3,4,5)P_3 instead of Ins(3,4,6)P_3 in this pathway. Ins(3)P_1 can be obtained from two different routes: either the conversion of glucose-6-phosphate or *myo*-inositol. The initial substrate of the first reaction is glucose 6- phosphate that is obtained either from photosynthesis or carbohydrate storage products. This reaction is performed by an Ins (3) P1 synthase (MIPS) [15] and The second reaction is catalyzed by a *myo*-inositol kinase [16].

2. Phytases

The term phytase (*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate phosphohydrolase) is defined as a class of phosphatases with the *in vitro* capability to release at least one phosphate from PA, thereby releasing phosphate and lowering inositol phosphates and potentially chelated minerals. The earliest report of a phytase activity is from the blood of calves [17].

Three classes of phytase enzymes have been acknowledged by IUPAC-IUBMB (the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology) [18]. Initiation of phytic acid dephosphorylation at different positions on the inositol ring is done by these enzymes, and it produces different isomers of the lower inositol phosphates [19].

2.1. EC 3.1.3.8: the 3-phytases

3-phytases (EC 3.1.3.8) are the largest group of phytases to date and generaly are found in microorganisms, fungi and bacteria. The structure of most 3-phytases shows homology to histidine acid phosphatases (HAP) or β-propeller phosphatase (BPP). The latter is tightly bound to three Ca ions and needs two adjacent phosphate groups to bind to the “affinity site” and “cleavage site” before hydrolysis and produce **t**he end products inositol-triphosphate-either Ins(1,3,5)P3 or Ins(2,4,6)P3 [20, 21], however, Oh (2006) reported that Ins(2,4,6)P3 is the sole end product [22]. Most phytases isolated from plant, bacteria and fungi belong to the HAPs which contains two phytase subgroups: some of them show broad substrate specificity and low specific activity for PA, whereas second group has narrow substrate specificities and high specific activity for PA. All members of the histidine acid phosphatases class share two conserved active site motifs, RHGXRXP and HD, and can hydrolyse metal-free phytate in the acidic range of pH [23].

2.2. EC 3.1.3.72: the 5-phytase

The 5-phytase (EC 3.1.3.72) has been reported till now is only from lily pollen. The amino acid sequence homology of its active site is higher towards multiple inositol polyphosphate phosphatase (MINPP) from mammals like human and rat [24].

2.3. EC 3.1.3.26: the 4/6-phytases

This group of phytase act on the carbon atoms next to C5 of the inositol ring. Purple acid phosphatase (PAP), the ADP phosphoglycerate phosphatase (related to EC 3.1.3.28), and as HAP-class is also involved in this group. Generally, the 4/6-phytases are most active in acidic environments (pH 4-6) with a temperature optimum in the range 40-60°C [23].

3. Strategies for reducing Phytate effect

The strategies for reducing the effect of phytate can be divided into two main categories; transgenic approach and non-transgenic approach.

As it has been mentioned earlier monogasteric animals are not able to utilize the phosphorous from phytic acid. To meet the nutritional needs of animals, farmers often supplement the feed either with an available form of phosphorus derived from rock phosphate or with phytase, which degrades feed phytate after ingestion to release phosphorus for uptake. However, as supplementation is costly, an attractive alternative is to deal with the problem at its source by developing low phytate crops. Phytase enzyme can be expressed in plants to produce functional recombinant proteins at high level [25]. Transgenic tobacco, wheat, corn, soybean, alfalfa, canola, rice and Arabidopsis have been generated by expression of exogenous phytase gene. Efforts for developing seeds by expression of different microbial phytase genes have been shown in table 2. The first report of expression of the microbial phytase gene in plants was reported by Pen et al [26]. The β-propeller phytase from *Bacillus subtilis* fused to carrot extension signal peptide was expressed in tobacco for root-specific secretion [27]. A minimal linear phytase gene cassette with T-DNA borders without selection marker was introduced into soybean through the pollen tube pathway [28].

The highest activity of *phyA* which had been amplified from *Aspergillus awamori* was 150 U/mg protein, compared to 56 U/mg protein in untransformed controls. In another study, *AfPhyA* gene from *Asperigillus ficuum* was transformed into soybean under control of *Arabidopsis Pky*10 gene promoter. In this study, the carrot extension signal peptide was used for root-specific secretory expression of *AfPhyA* gene [29].

Seed-specific cruciferin (*cru*A) promoter was used to derive phytase gene fused to cruciferin signal peptide sequence for specific secretion into the seeds [30]. The phytase activity was 600 U/g per mass of seed. A modified *phyA* gene according to the codon usage of *Brassica napus* was overexpressed in canola. CaMV 35S promoter was used to derive phytase gene, and tobacco PR-S signal peptide and KDEL sequence for retention of secreted proteins in the endoplasmic reticulum (ER). Phytase accumulation in transgenic seed was 2.6 % of the total soluble proteins [31]. Barley α-amylase signal peptide has been fused to *phyA* gene for localization of phytase enzyme in apoplast of transgenic wheat [32, 33]. Expression of *phyA* from *Aspergillus ficuum* in alfalfa resulted in production of phytase at concentrations of 1.5 % of the soluble protein [34]. In addition to introduction of phytase gene in plants, another strategy was used by Shi and his colleagues for decreasing the phytate content of maize and soybean seeds. In this strategy, the ATP-binding cassette (ABC) transporter which is a key contributor to phytic-acid accumulation was silenced and high-Pi transgenic maize seeds were generated [70]. Creation of transgenic livestock has been as an attractive strategy to the degradation of phytic acid by monogastric animals. Up to 75% reduction in phosphorous excretion was observed in transgenic pigs that constitutively secreted microbial phytase from their salivary glands [35]. The need for inorganic phosphorous supplementation for growing these animals was decreased to almost zero. Generation of transgenic chickens also has been experimented using expression of avian MINPP phytase. These approaches would overcome public scepticism towards “foreign” proteins in the food [10, 36]. Expression of salivary *appA* phytase gene derived from *E. coli* reduced fecal phosphorus by 11% [37].

Apart from gene manipulation for improving the phytate content in plant materials, some other techniques also have been investigated to reduce the phytic acid content. As phytate is water soluble so a significant phytate reduction can occur after soaking the seeds followed by discarding water. This process may last for a few minutes or for long period overnight. Since the endogenous phytase also contributes to phytate

reduction, temperature and pH value have significant effect on phytate degradation during soaking [38]. When the soaking step was carried out at temperatures between 45- 65 °C and pH values between pH=5.0 and 6.0, 26–100 % of phytate was enzymatically hydrolysed [39, 40]. Phytic acid is heat stable so destruction of phytate during cooking is not expected to occur [38]. The use of plants containing heat stable endogenous phytases or addition of exogenous one may improve phytate dephosphorylation during cooking.

Germination can breakdown antinutrients such as phytic acid in legumes and cereals because during this process phytate degrading activity is promoted [41]. However, long time period is needed for improvment of minerals bioavailability during germination, so this approach can be applied in household applications and does not seem to be a useful method for reducing the phytate at industrial level [38].

Lactic acid bacteria, moulds and yeast which are the major fermentation microorganisms are beneficial in elimination of phytic acid and other antinutrient compounds. Some studies have shown that lactic acid bacteria, *Rhizopus oligosporus* and *Aspergillus oryzae* which are used in fermentation process produce phytate-degrading enzymes and contribute significantly to phytate degradation [42, 43, and 44].

4. Dietary phytase supplementation for poultry and fishes and impact on environment

Supplementing the diets with extrinsic microbial phytases has become a promising alternative all over the world to enable dephosphorylation of the dietary phytate [45]. Supplementation of microbial phytase to diet of monogasteric animals including poultry and fishes not only reduces phosphorous excretion and substantially environmental pollution but also improves minerals and amino acid availability, performance and energy [46]. Addition of phytase supplement of 500 FTU reduced the total phosphorus content in rations for pigs and poultry by 1 to 1.5 g/kg [47, 48]. In maize/soya-based diets that are low in P even this concentration of phytase may be much lower than the potential optimum [49]. If the corresponding amount of P is reduced in the ration the corresponding phosphorus amount under these conditions will not be excreted in the feces [50]. It has been demonstrated that phytase can significantly reduce the endogenous secretions, measured as sialic acid (SA), from broiler chickens [51, 52]. Other researches show that increased concentration of SA is associated with health problems like bacterial infections, cellular senescence, and osmotic fragility [52]. Poultry species have different ability to utilize plant based P because their intestinal absorption characteristics are not same [53]. Studies have shown that phytate hydrolysis in the intestine depends on several factors such as phytase activity of the feed ingredients [54], intestinal pH [55], microflora [56], and endogenous phytase activity [57]. Cowieson and V. Ravindran reported that the flow of endogenous amino acid markedly increases with the ingestion of phytic acid; however, microbial phytase reduces this adverse effect on endogenous investment [58]. The modern broiler strains are genetically potent to deposit lean tissue compared to fat, so the phytase supplementation would enhance the ability of these broilers to meet this potential [59].

Diets fortified with phytase could increase bone phosphorous, bone calcium and bone ash compared with pigs which were fed with unsupplemented phytase diets [60, 61]. It has been reported that 30- 50% of dietary phytate P can be released by exogenous phytase supplementation allowing reductions in dietary non-phytate phosphorous of 0.1-0.2%, and decreasing fecal P concentrations from 35 to 50% [61]. Although there is a potential possibility of releasing the phosphorus in the soil if phytase enzyme is excreted in the feces but it has been

demonstrated that like any other protein source exogenous phytases are completely inactivated during digestion processes. So such excrements can be used as fertilizer even by tenfold overdose because they do not increase the solubilization of phosphorus in the soil [50].

As has been mentioned earlier release of phosphorous from livestock to the ecosystem causes eutrophication. The impact of phosphorous excreted from fishes is even more important than other animals because it is released to the water bodies directly causing algal bloom. On the other hand the plant byproducts are considered as a promising source of protein and energy which can be used for the formulation of economical and environment friendly aquafeeds. However, anti-nutritional factors like phytate disturb the physiology of digestive tract, limiting the overall fish growth [62]. Among an array of methods which were made to liberate phosphorous from phytic acid [63], enzymatic hydrolysis of phytate by phytase supplementation showed the best result [64]. Supplementation of corn gluten meal based diets with 750 FTU/Kg of phytase may prove highly beneficial in developing cost effective and environment friendly aqua feed for major carps [62]. Gao *et al*. studied the impact of phytase supplementation in the diet of grass carp and concluded that 500-1000 FTU/Kg levels of phytase supplementation enhances the phosphorous availability to fish and can reduce the phosphorous excretion through feces [65]. The improved feed intake and feed conversion ratio using supplemented exogenous phytase on *Labeo rohita* Fingerlings also has been reported [66].

Enrichment of aquafeed with microbial phytase increases the bio-availability of phosphorus and decreases its discharge into the aquatic environment, hence causing less pollution [58]. Yan and Reigh delineated higher concentration of ash, calcium, phosphorus and manganese in bones of channel catfish fed fungal phytase-supplemented diets than the fish fed on a control diet [67]. In Atlantic salmon, soy-protein concentrate treated with phytase improved protein digestibility and retention [68]. Net protein utilization also increased when microbial phytase supplementation in the diet of *Pangasius pangasius* was added. Furthermore, digestibility of dry matter, crude protein and phytate-protein complexes were improved by dietary phytase supplementation [69].

5. CONCLUSION

Increasing world population needs more and more food which should be obtained from plants directly or from livestock who also depend on plants. In both cases intensive cultivation of crops and animal farming is required to meet the need of human. Owing to application of phosphate fertilizers and feed supplements for these purposes, high phosphorous accumulation takes place in cultivated and animal production land. Phosphorus present in most grains and plant by-products is generally unavailable to monogastric animals. Fish excrete phosphorus in soluble and particulate forms. The soluble forms, organic phosphorus and phosphates affect water quality directly. The particulate forms accumulate in the sludge and the phosphorus is released slowly to the water. Dissolved reactive phosphorus is usually regarded as the most important factor affecting water quality, because it is easily available for phytoplankton growth. This describes global efforts and policy for equilibrium fertilization; reduction of acid deposition and protection of surface and ground water quality. Microbial phytase supplementation in the diet of fish can overcome this problem. It makes the chelated phosphorus available to fish and hence there is less fecal excretion, leading to less environmental pollution. Reduced requirement of mineral supplements, thereby reduce chances of excess inorganic phosphorus getting into the aquatic system. Use of phytase in feed reduces or sometimes eliminates the necessity of mineral supplementation, which also decreases the cost of feed. Moreover, phytic acid

has anti nutrient effects and can increase malnutrition particularly in developing countries. Although phytase was first used for environmental reasons, it has now been discovered that there are a range of other nutritional and health benefits from using these enzymes. Phytases have been used to generate transgenic crops in order to reduce the phytate content. These transgenic fodder can be used as additives for improving of digestibility of phytic acid in animal feed, and thereby phosphate excretion from non-ruminants would reduce phosphates in manure. These enzymes also have been produced commercially as feed supplements. According to the previous experience regarding animal feed supplementation with phytases, it has been concluded that feeding the same phytase concentrations can show different responses among species, so, phytase concentration has to be optimized for the individual species.

Phytate also limits the protein availability to the fish. However, it is evident that phytase supplementation improves the bioavailability of the phosphorus and nitrogen (protein), which are the main culprits of aquaculture pollution. The increased bioavailability of nitrogen and phosphorus in the diet leads to reductions in feed costs. Though the role of phytase supplementation has been well proven and documented in poultry, its use in fish feed is less known, which is due to the pH specificity of phytase.

Like transgenic pigs, generating transgenic fish and poultry to produce their own digestive endogenous phytase in the digestive tract may be inevitable in future.

As a final point environmental and antinutritional effects of phytic acid are the subjects that need to be seriously researched.

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TABLES:

	Total P (g/kg)	Phytate-P (g/kg)	Proportion (%)
Cereals			
Wheat grain	3.07	2.19	71.6
Oat	3.60	2.10	59.0
Corn grain	2.62	1.88	71.6
Barley grain	3.21	1.96	61.0
Sorghum grain	3.01	2.18	72.6
Rye	3.05	1.95	63.9
Oilseed meals			
Canola meal	9.72	6.45	66.4
Cottonseed meal	10.02	7.72	77.1
Corn glutton meal	4.24	2.67	63.0
Rapeseed meal	9.60	6.34	66.0
Soybean meal	6.49	3.88	59.9
By-products			
Rice bran	17.82	14.17	79.5
Wheat bran	10.96	8.36	76.3

Table: 1. Phytat content in plants and their byproducts

Name of Transgenic crop	*phy* gene used	Organism Source of *phy* gene	Ref.	Name of Transgenic crop	*phy* gene used	Organism Source of *phy* gene	Ref.
tobacco	*phyA*	*A. ficuum*	26	Rice	*phyA*	*A. fumigatus*	41
	b-propeller phytase (*168phyA*)	*B. subtilis*	27		*SrPf6*	*Selenomonas ruminantium*	42
					appA	*E. coli*	42
alfalfa	*phyA*	*A. ficuum*	44		*phyI*	*A. niger*	43
Arabidopsis	propeller phytase (*168phyA*)	*Bacillus subtilis*	28	soybean	*phyA*	*A. niger*	10
					phyA	*E. coli*	36
	phyA	*A. niger*	46		*AfphyA*	*A. ficuum*	37
canola	*phyA*	*A. niger*	33		*phyA*	Aspergillus awamori	28
maize	*phyA*	*A. niger*	47	wheat	*phy A*	*A. niger*	39
	phy2A	*A. niger*	48		*phyA*	*A. fumigatus*	40

Table: 2. Transgenic plants expressing microbial phytase gene