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PRODUCTION, PURIFICATION AND APPLICATION OF MICROBIAL PHYTASE: AN OVERVIEW

Ali Nawaz, Sheeba Shakoor, Syeda Quratulain Gillani, Hamid Mukhtar* and Ikram ul Haq

Institute of Industrial Biotechnology, GC University Lahore Pakistan 54000
***Corresponding author: hamidwaseer@yahoo.com**

ABSTRACT

Phosphorus is stored in the form of phytate in cereal grains, legumes and oil seeds. Usually this phytate is not digested by the animals and animal manure results in phosphorus pollution. Phytase (myo-inositol hexakisphosphate phosphohydrolases, EC 3.1.3.8) catalyze the hydrolysis of phytate (myoinositolhexakisphosphate), phytic acid and results in the release of phosphorus. There are many sources of phytase production like plants, animals and microbes (bacteria, fungus, yeast) but on industrial scale microbial source is preferred. On industrial level phytase is produced by microbes by solid state and submerged fermentation. Phytase is purified by different methods like ammonium sulfate precipitation, dialysis and size exclusion chromatography. The applications of phytase with respect to animal feed supplement, bread making, aquaculture feed, degradation of pesticides and transgenic crops are emphasized in this paper.

Key words: Phytate, phytic acid, Anti-nutritional factor, Micronutrient bioavailability, Animal feed, fermentation, Chromatography

INTRODUCTION

Phytase (EC 3.1.3.8) also known as myo-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate phosphohydrolases is an enzyme which have ability to hydrolyse phytic acid, the storage form of phosphorus in legumes and cereals to inorganic phosphate and myoinositol phosphate derivatives (Olajuyigbe, 2016). Phytic acid has ubiquitous nature and it constitutes 1-3% by weight of many oil seeds and cereals. It is principal source of phosphorus in cereals and oil seeds. Phytate is considered as a food inhibitor and is known to chelate minerals like Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}(Shobirin, 2010). Phytate form complexes with carbohydrates and other proteins which makes it difficult for humans to digest and have less nutritional value (Dahiya *et al.,* 2009) Phosphorus (P) amount present in oilseed meal and cereal grain is enough for the optimal growth of animal if the entire phosphorus amount from phytate is available (Gontia *et al.,* 2012).

Monogastric animal's e.g., humans, poultry, pigs and fishes cannot use this form of phytate due to the absence of enzymes. As a result of this absorption of phytic acid doesn't occur in the animal's digestive tract and phosphorus is released into the animal faeces causing P pollution (Inostroza *et al.,* 2016). To fulfill the requirement of P addition of micronutrients and phosphorus is done in the diet of animal, which is expensive process. To reduce the cost, enzymes like phytases are used for the reduction of phytic acid content in animal feed. It improves the nutritional value of food along with the removal of anti-nutritional properties and reduces the P pollution in environment (Kaur *et al.,* 2017).

For the last few decades considerable research has been carried out on phytase because of its numerous applications in the field of nutrition, animal feed and environmental pollution. Phytases can be obtained from different sources including animals, plants and microorganisms. For the commercial production of phytase microbial source is the most promising one. Phytases can be divided into acidic or alkaline form. Industrial application of phytase are usually based on histidine acid phytases produced from *Aspergillus* species but on the basis of substrate specificity, pH, calcium dependency and high thermal stability alkaline phytases are an alternative source of fungal enzymes. Alkaline phytases are produced by Bacillus species (Rocky-salimi and Hashemi, 2016). Since 1991, phytases are used as food additive in USA. The annual sale of phytases was approximately US $350 million at the end of 20^{th} century (Supreeth *et al.,* 2015).

Phytases produced from microbial sources by fermentation is used as animal feed additive to overcome the nutritional and environmental pollution problems caused by phytate. In this process high cost is involved for diet formulation which is a limiting factor for its commercial use. Research is going to make the process economical. Transgenic crops are also produced having phytase gene in them, which can improve phosphorus bioavailability in food instead of supplementing microbial phytase (Gontia *et al.,* 2012). This review summaries the production and purification of phytase from different sources and its applications in different fields.

Production Sources:

Different sources like plants, animals and microbes are being used nowadays for the production of phytases. Commercially microbial sources are considered more effective and useful. Fungi, bacteria and yeast all are effective in the production of phytases but different strains of fungus give better results (Panday *et al.,* 2001).

Bacterial source:

Bacillus phytases were being produced in wheat bran medium or in soil extract medium by inoculating a single strain of *Bacillus* (Elhadi *et al.,* 2011). Different bacterial strains like pseudomonas and *Klebsiella* were also used for phytase production through Submerged and Solid State fermentation. Bacterial phytases were easy to produce because of less generation time of bacteria. *Lactobacillus brevis* was used to produce phytase by using cheese as substrate through submerged fermentation (Sumengen *et al.,* 2012).

Fungal source:

Phytase was being produced by different fungal strains like *Aspergillus niger* and *Thermomyces lanuginosus* having different origins. Solid state as well as submerged fermentation was used for phytase production in different fermenting medium of varying pH. Mainly submerged fermentation was being used for the production of phytase from both of these fungal strains (Bujna, 2014). *Aspergillus niger CFR and Aspergillus fucuum SGA01*were used as a source for phytase production in wheat bran medium by solid state fermentation (Gunashree and Govindarajulu, 2015).

Table 1. Production of Phytase from different sources.

Source (organism)	**Substrate**	**Production technique**	**Reference**
Lactobacillus brevis	Cheese	Submerged fermentation	**(Sumengen *et al.,* 2012)**
Leuconostoc mesenteroides	Soyabeen	Submerged fermentation	(**Oh Namsoon *et al.,* 2009)**
Bacillus amyloliquefaciens PFB-02	Nill	Submerged fermentation	**(Olajuyigbe , 2016)**
Streptomyces luteogriseus	Wheat bran	Solid state and submerged fermentation	**(Aly Mohamed *etal.,* 2015)**
Klebsiella sp	Orange peel	Submerged fermentation	**(Mittal *et al.,* 2012)**
Rhizopus microspores	Soyamince , orange peel ,maltose	Solid state fermentation	**(Sato Sayuri *et al.,* 2014)**
Aspergillus niger	Wheat bran	Solid state fermentation	**(Banerjee and Purva, 2002)**
Schyzophyllum sp.	Wheat bran	Solid state fermentation	**(Salmon Naomii *et al.,* 2011)**
Aspergillus niger CFR and Aspergillus fucuum SGA01	Wheat bran	Submerged and solid state fermentation	**(Gunashree and Govindarajulu, 2015)**
Sporotrichum thermophile	Cane molasses	Submerged fermentation	**(Singh and Satyanarayana, 2008)**
Penicillium oxalicum	Wheat bran	Solid state fermentation	**(Kaur *et al.,* 2017)**

Down streaming:

Phytase enzyme is usually produced extra-cellularly and the enzyme is present in the culture medium. In submerged fermentation phytase is present in the solution while in the solid state fermentation distilled water is added , crude extract is then obtained by using filter paper.

Purification:

There are many methods used for the purification of phytase enzyme. Basically, all the purification methods were performed on the lower temperatures like 4°C. Usually the enzyme solution obtained is mixed with sodium acetate buffer ranging in acidic pH and after that eighty percent of saturation results by the addition of solid ammonium sulfate. In cold conditions, this solution is kept overnight to obtain precipitation. After centrifugation the solution, precipitates are collected. Dialysis is performed after suspending the protein pellet in sodium acetate buffer. After dialysis, the supernatant is further purified by column chromatography mostly commonly used is DEAE-Sephadex column chromatography. The chromatography column is washed with the sodium acetate buffer. The elution buffer is usually of NaCl solution. NaCl is removed from the eluted protein by dialysis so that it can be loaded in the next ion exchange column. To check the homogeneity High protein liquid chromatography can also be observed. The eluted fractions are lyophilized and their molecular masses are determined (Gunashree and Govindarajulu, 2015).

Phytase activity assay:

Phytase activity assay is used to determine the release of phosphorus from phytate or phytic acid. It involves the hydrolysis of phytate that results in the release of orthophosphate under controlled conditions. For phytase enzyme assay supernatant having phytase is mixed into sodium acetate buffer along with phytate as substrate. After different time intervals enzymatic reaction is stopped by using HCL or TCA solution. Then the absorbance is measured with a Spectrophotometer (Qvirist *et al.,* 2015). Another method of phytase activity assay involves the addition of supernatant (phytase) in the phytate solution that is dissolved in sodium acetate buffer. The hydrolysis reaction is then stopped by using ammonium molybedate reagent solution. After centrifugation of the reaction mixture, the absorbance is measured with a spectrophotometer (Idriss *et al*., 2002).

Applications of Phytase in different fields:

Animal feed:

Phytases have been used in the feed of monogastric animals for many years. Barely existing amount of phosphorus is produced in the intestine of animals (Nakagi *et al.,* 2013). So, the addition of Phytase is done in the diet of monogastric animals. In the poultry feed major amount of phosphorus is not available to poultry animals because phosphorus is bounded to phytate (Shobirin, 2010). In the seeds phytate is serving as a storage site for phosphorus. The poultry animals do not have ability to utilize phosphorus present in this form. Enzymes such as Phytases and Phosphatases have ability to free the phytate bounded phosphorus, make it available for poultry animals (Kathirvelan *et al.,* 2015)

Usually the requirement of phosphorus for poultry feed was fulfilled by giving rock phosphorus with Soya beans and other meals, by giving such type of feed the phosphorus demand is attained. In the manure of poultry feed phytic acid is present; it is cleaved by microorganisms present in soil and water. The released phosphorus makes its way to water bodies and cause eutrophication. In the result of this algal growth occur and consequently oxygen depletion occurs (Dahiya *et al.,* 2009). The phosphorus availability can be increased by the addition of microbial Phytase in the feed. Over the past 20 years Phytase enzyme is added in the feed of poultry animals to enhance the bioavailability of phosphorus from the phytate. Phytase decrease the need of inorganic phosphorus in the poultry feed and thus minimize the phosphorus excretion in manure (Silversides and Hruby, 2009).

Phytase present in the poultry feed enhances gut health of the animals by the reduction in gastrointestinal secretion which increases the energy utilization (Li *et al.,* 2011). After the addition of Phytase in poultry feed the amount of phosphorus is decreased in the manure so poultry manure can also be used as a fertilizer also it has appositive effect on the environment (Abdel-megeed and Tahir, 2015). The cost of poultry diet can be decreased by minimizing the amount of amino acids, fats and soya bean meals that were added earlier (Kiarie *et al.,* 2013). Research indicates that by the addition of Phytase to the feed there is positive effect on the growth of young chicken. The body weight increases after consuming Phytase (Józefiak, 2006). The availability of some trace elements like iron, copper, zinc and manganese can be increased by the action of phytase (Afify *et al.,* 2011).

Research indicates that through the supplementation of Phytase, digestion of certain minerals like protein, phosphorus, calcium and zinc can be increased. These positive effects can be achieved without any effect in the quality of meal (Kaur *et al.,* 2016). Recent studies also indicate that through the Phytase supplementation there is

positive effect on the bone growth and egg shell strength of broilers (Kathirvelan *et al.,* 2015). Current research reveals that through the supplementation of Phytase to phosphorus deficient broiler diet there is an increase in the mucosal IgA hence there is a boost in Immune system of the animal (Islam *et al.,* 2017).

Table 2. Different methods of Phytase purification.

Phytase source	**Method of purification**	**References**
Bacillus sp.KHU.10	Acetone precipitation DEAE-Sepharose column chromatography Phenyl-Sepharose column chromatography	**(Choi *et al.,* 2001)**
Bacillus nealsoniiZJ0702	Ammonium Sulphate precipitation Sepharose anion exchange Column chromatography Sephadex G-100 size exclusion chromatography	**(Yu Pinhg and Chen yirun, 2013)**
Lactobacillus plantarum	Ammonium Sulphate precipitation	**(Sanbuga Elif *et al.,* 2014)**
Alcaligens spp.	Ammonium Sulphate precipitation	**(Vijayaraghavan *et al.,* 2013)**
Bacillus subtilis	Ammonium Sulfate precipitation P.D-10 gel filtration column chromatography	**(Kerovuo *et al.,* 1998)**
Streptomyces Luteogriseus	Ammonium Sulphate precipitation Sephadex G75 column chromatography	**(Aly Mohamed *et al.,* 2015)**
Thermomyces Lanuginosus	Ammonium Sulphate precipitation Ion exchange chromatography Gel filtration Chromato focusing Hydrophobic interaction chromatography	**(Gunashree and Govindarajulu, 2015)**
Aspergillus niger CFR 335	Ammonium Sulphate precipitation Dialysis DEAE-Sephadex G-250 column chromatography	**(Bujna Erika, 2014)**
Aspergillus niger NCIM 563	Phenyl -Sepharose CL-4B column chromatography	**(Soni *et al.,* 2010)**
Aspergillus Tamari	Ammonium Sulphate precipitation DEAE-Sepharose column chromatography CM Sepharose column chromatography	**(Shah *et al.,* 2012)**

Table 3. Different methods of phytase activity assay.

Substrate	Stop reagent	Centrifugation	Colorimetric/Spectro-photometry	References
Sodium phytate	Trichloroacetic acid	No	Spectrophotometry	**(Qvirist *et al.*, 2015)**
Sodium phytate	Ammonium molybedate	Yes	Spectrophotometry	(**Idriss *et al.*, 2002)**
Sodium phytate	Trichloroacetic acid	Yes	Colorimetry	**(Kim and Lei, 2004)**
Sodium phytate	Trichloroacetic acid	No	Colorimetry	**(Boyce *et al*., 2004)**
Sodium phytate	Trichloroacetic acid	No	Colorimetry	**(Bae *et al*., 1999)**
Phytic acid	Nitric acid	No	Spectrophotometry	**(March *et al*., 1995)**

Aquaculture Feed:

Aquaculture feed industry is one of the fastest growing industry. Aquaculture contributes more than 19 million tons of fish and shell fish annually to the world's fish supply. Aquaculture feed industry depends upon fishmeal which is most preferred source of protein for fishes because fatty acids and amino acids are present in highest proportion (Hussain *et al.,* 2017). Due to limitation in its supply and high cost it is not easily available for the farmers. Plant proteins are getting attention now days instead of fish meal. Plant products are good source of energy and protein. Plant products are low in cost. The problem with plant protein is the presence of anti-nutrient compounds like phytate (Kumar, 2012).

In the plant derived fish feed phytate is an important constitutes which is formed during maturation of plant seeds (Cao *et al.,* 2007). Phytate bound phosphorus is not available for fishes. Phytate present in aqua feed has negative effect on mineral uptake, growth performance, energy and nutrient utilization. With the action of phytase the bound P is converted to available phosphorus (Khan and Ghosh, 2012). From the last few years phytase has been used in aqua feed industries to enhance the availability of minerals to enhance energy utilization and to control Phosphorus pollution (Baruah *et al.*, 2004). Researchers indicate that with the addition of phytase in the diet of fish the body weight and growth performance of fish's increases (Hussain *et al.,* 2017). The benefits of using microbial phytases on the environment is that it reduces the need of mineral supplements hence the chance of inorganic phosphorus to become part of aquatic system is also reduces. It also reduces the output of organic phosphate.

Baking Industry:

In worldwide health departments suggests the intake of whole grain cereal products. This recommendation is done to balance the diet. From the last few decades scientists recommend fiber intake. Whole wheat flour is a good source of fiber, minerals, proteins and carbohydrates. Bread is an important source of iron. Along these benefit compounds some undesirable compounds like phytate is also present in cereal flour (Azeke *et al.,* 2011). Phytate decreases the multivalent cation availability because of the formation of complexes in the gastrointestinal tract (Shobirin, 2010).

Research indicates that a decrease in phytate content is seen during bread making process. In cereal flour Phytase a naturally occurring enzyme do the hydrolysis of phytate. It is seen that phytase improves the process of bread making (Bohn *et al.,* 2008). Phytase decreases the formulation time of bread without affecting its pH (Kopjar *et al.,* 2009). Bread volume and texture also improved due to its addition. Improvement in shape was obtained (Greiner and Konietzny, 2006).

Degradation of Organophosphorus pesticides:

With the increase in World population food demand also increases and it leads to extensive use of pesticides and insecticides in the agricultural fields to get better crops. Organophosphorus pesticides (OPP) are used on a large scale to control variety of insects and pests. OPP cannot easily be removed from food items by simply washing with

tap water and it leads to accumulation in food chain (Vendan, 2016). It has adverse effects on the nervous system of animals and humans. There is a need to degrade OPP before harvesting so it cannot become the part of food chain. Instead of using physiochemical process using whole cell microorganisms is advantageous as it is safe, environmental friendly and economic. Research indicates that phytase enzyme produced from *Aspergillus niger* NCIM 563 is used to control OPP pollution. Studies show that phytase can degrade 72% of Chlorpyrifos (Shah *et al.,* 2017).

Transgenic crops:

There are methods for cloning and expression of phytase in plants, fungi bacteria and yeast. Through these experiments valuable information about difference in post translational modifications, secretion and stability of phytase level came to know (Shobirin, 2010). Transgenic plants which express microbial phytase are the best approach to deliver phytase to non-ruminants. This approach enhances the mineral and phytate phosphorus uptake. There is no difference in the phytase produced by fungi, bacteria and by recombinant plants. So phytase production from plant sources would open a new venture for commercial purposes. Through these transgenic crops there is improvement in the digestion of phytic animals. It is also seen that phytic content in the manure of animals also decreased (Duliński *et al.,* 2015).

For the development of transgenic crops there are few phytase genes used. Further research is carried out to find new genes. Researchers explained the unique features of genetically engineered Sweet potato. It is seen that there is increase in size and yield of potato tuber when organic fertilizers are supplemented to the genetically engineered plants. Test of animal feeding shows that phytase supplement produced from potato was as effective as commercially available microbial phytase. Hence overexpression of phytase gene in transgenic crops not only offers an ideal feed additive but also enhances the yield and has potential for phytoremediation (Hong *et al.,* 2008).

Table 4. Applications of Phytase in different Industries.

Industry	Applications	Reference
Animal feed Industry	Control Phosphorus pollution	**(Silversides and Hruby, 2009)** **(Kaur *et al.,* 2016)**
	Improve nutrient uptake Increase the availability of phosphorus, calcium, amino acids for animals	**(Afify *et al.,* 2011)**
	Produce energy in diet for growing pigs Helps to boost immune system	**(Kiarie *et al.,* 2013)**
	Improve gut health of animal and	**(Islam *et al.,* 2017)**
	Increase protein intake	**(Li *et al.,* 2011)**
Aquaculture feed Industry	Increase bioavailability of minerals in fish feed Reduce organic phosphorus outputs	**(Khan and Gosh, 2012)**
	Decrease water pollution and Increase body weight and growth performance of fishes	**(Kumar *et al.,* 2012)**
		(Hussain *et al.,* 2017)
Baking Industry	Decrease fermentation time	**(Kopjar *et al.,* 2009)**
	Increase bread volume and Activate alpha amylase	**(Bohn *et al.,* 2008)**
	Decrease phytate content in bread	**(Greiner and Konietzny, 2006)**.
Agriculture Industry	Helps to degrade Organophosphorus pesticides	**(Shah *et al.,* 2017)**
	Fractionation of cereal beans, Corn wet milling and Role in production of transgenic crops	**(Shobirin, 2010)**
	Role in phytoremediation and Production of plant protein isolates	
	Role in decrease of phytic content in animal manure	**(Hong *et al.,* 2008)**. **(Duliński *et al.,* 2015)**

Conclusion:

Phytase can be produced by different species of bacteria like *Bacillus, Pseudomonas* and *Klebsilla*. It can also be produced by different species of fungus like *Aspergillus* and *Penicillium*, Different substrates like wheat bran, cheese, soyamilk, sugar cane bagasse and orange peel are used in the production of phytase through solid state and submerged fermentation. Usually phytase production is preferred through bacterial source because of its less generation time and thermostability. Phytase can be purified by different methods but high yield up to 70% is obtained by Ammonium Sulphate precipitation that is followed by dialysis and size exclusion chromatography. Phosphorus pollution caused by animal manure can be reduced by using phytase and it is among the most promising applications of phytase.

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