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Biotechnological Approach to Improve the Nutritional Availability in Livestock and Consequence for Reduction the Environment Pollution by Implement of Transgenic Phytase in Animal Feed

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ABSTRACT

Phytases are hydrolytic enzymes that initiate the release of phosphate from phytate In recent years the application of the phytase enzyme have been studied intensively. Phytase enzymes have a wide distribution in plants, microorganisms, and in some animal tissues. Many strategies have been developed for improving phosphate and mineral availability in feed. However in recent years, expression of transgenic microbial phytase in plants can be successfully applied as animal feed supplementation for innovative means of delivering phytases to non-ruminants to inflation of bioavailability of mineral such as calcium, magnesium, zinc copper, enhance the utilization of phytate bounded phosphorus and reduce P pollution of animal excreta. Moreover, improved biotechnological processes in the production of transgeneic plant contain microbial phytase can eliminate the costs associated with phytase production, purification, and supplementation for commercial use. In current study we discussed about the comprehensive descriptions on source of phytase, expression profiling and their potential application in animal feed. Hence it is concluded that molecular farming in the production from microbial sources of stable phytase in feed could open a new venture for commercial purposes.

Keywords: Phytate, phytase, transgenics, livestock feed.

Over the past decade expanding demand for livestock products in developing countries was translated into rapid growth in meat products. The large-scale commercial livestock can provide a steady stream of food and supports the livelihoods of an estimated 675 million rural poor (Steinfeld, 2003). Indeed it

requires the use of vast amounts of nutritionally balanced animal feed. However large majority of the world's suffer with low-input mixed farming systems, from either permanent or seasonal nutritional stress. Therefore the world-wide researches efforts have been made to develop sustain a wide range of high-quality leguminous feeds for commercial feedstock additives. Strategic feeding with key supplementation ingredients maximize the bioavailability of nutritionally important elements, that can improve the overall utilization of low-quality diets in common animal feedstocks.

Plant seeds such as oilseed meals, cereal grains and legumes are usually classed as bring primarily a source of energy or protein and other nutrient elements in animal feed ingredients. In the seeds of higher plants majority of the phosphorus c. 60–90% of the total phosphorus content in plants is stored as phytic acid or phytate (Reddy *et al*., 1982; Gontia *et al.,* 2012). Phytate complex consist with several different types of proteins and multivalent cations such as calcium, iron, manganese, and zinc, which are essential for proper growth and maturation (Figure 1). In the digestive tract the ability of a phytase to hydrolyse phytate is depending on the acidic pH optimum and high resistance to pepsin. It was reported that mainly two main types of phytases have been identified acid phytases with an

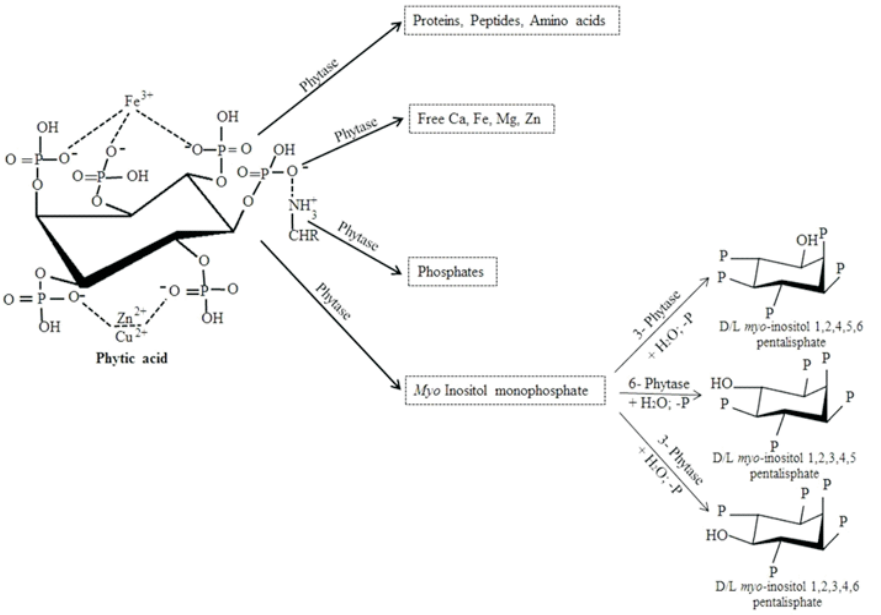

Figure 1. Systematic diagram of the breakdown of Phytate (phytic acid) by phytase enzyme, to produce free phosphate, metals (Fe^{3+}, Zn^{2+}, Ca^{2+}, and Mn^{2+}), myo-inositol (3- , 4/6-, and 5-phytases) and the intermediate metabolites (Kerovuo, *et. al*., 2000; Lei and Stahl, 2001; Yu, *et al*., 2012).

pH optimum around pH 5.0 and alkaline phytases with an pH optimum around pH 8.0 (Konietzny *et al.,* 2002).

The enzymes like phytases catalyzed by phytate into inorganic phosphorus and myo-inositol. However the monogastric animals like poultry, pigs and fish have very low or no phytase activities in their digestive tracts, which would not allow degrading phytate (Huff *et al*., 1998; Singh *et al*., 2003). They are unable to efficiently utilize phosphorus from the feed present in the form of phytate, as a result supplements end up in their excretion in the animal's feces or urine cause serious pollution and ecological problems known as eutrophication. The increasing public concern regarding the environmental impact of high P levels in animal excreta was derived the biotechnological development of phytase and its application in animal nutrition. Therefore the economic interest in phytase containing animal feeds is due to its nutritional quality by increase the bio-availability of inorganic phosphorous.

Nowadays, genetic manipulation, transgenic plants which express nutritionally can improve the applicability of the bio-compounds. The microbial phytase produced by the fermentation process gets added or socked to the feed. However, this was high cost of production and the special care at the time of feed formulation. Further the approaches involving recombinant and transgenic plants having phytase expression. The produce high expression phytase transgenic crops have been developed for improving phosphate and mineral availability in food/feed instead of direct supplementation of microbial phytase to animal feed.

The biotechnological applications of phytase in the livestock feed area was taken into the economical consideration (Konietzny *et al.,* 2003). The present review emphasized on the comprehensive descriptions on source of phytase, expression profiling, impact and their potential application in animal feed.

Sources of phytase enzyme use for feed stock

According to Sebastian *et al.* (1998), mainly four possible sources for phytase, i.e., animal origin from intestinal phytase found in digestive secretions; phytase produced by exogenous micro-organisms; in digestive tract of ruminants phytase originating from microbes and endogenous phytase from plant feed-stuffs. However, the phytase activity varies between and among of the sources. Two types of phytase, EC 3.1.3.8 or 3-phytase consist of hydrolyzes the ester bond at the third position of myo inositol hexakis phosphate to produce d-myo-Ins-1, 2, 4, 5, 6-pentakisphosphate and orthophosphate mainly found in microorganisms The second type is EC 3.1.3.26 or 6-phytase consist of hydrolyzes the ester bond at the 6th position of myo inositol hexakisphosphate to produce d-myoIns-1, 2, 3, 4, 5-pentakisphosphate and orthophosphate reported in plant (Khalid *et al*., 2013).

In addition only a single 5-phytase (EC 3.1.3.72), the alkaline phytase is from lily pollen. This enzyme hydrolyses phytic acid initiates on C5 of the inositol ring.

A wide range of plant, bacterial, fungal and animal tissues are reported as the source for phytase, but most extensively studied in the filamentous fungi (Gibson *et al.,* 1990) such as *Aspergillus fumigatus, Talaromyces thermophilus* (Pasamontes *et al*., 1997), *Aspergillus ficuum* (Ullah, 1988), *Aspergillus niger* (Piddington *et al*., 1993), *Mucor piriformis* (Howson and Davis, 1983), *Rhizopus oligosporus*, (Casey and Walsh, 2004), *Emericella nidulans*, *Thermomyces lanuginosus* (Berka *et al.,* 1998), *Peniophora lycii, Trametes pubescens, Ceriporia sp.* and *Agrocybe pediades* (Lassen *et al*., 2001). Among the filamentous fungi, *A. ficuum* produces a phytase which has a particularly high specific activity and thermostability. Several studies have already investigated that yeast can also used as expression system platform. However, *P. pastoris* that expressed the modified phytase gene (phy-pl-sh) from *D. castellii* of 10,540 U/ml) was reported.

Apart from the fungal, some bacterial have also been reported to produce phytase enzyme. Two phytases isolated from *Pedobacter nyackensis* and *Erwinia carotovora* were reported for their potential application. Expression of phytases gene also reported in *Klebsiella pneumoniae* (Huang *et al*., 2009) *Bacillus subtilis, Yersinia kristeensenii, Pseudomonas syringae* (Yao, *et al*., 2011), *Lactobacillus sanfranciscensis* (De Angelis *et al.,* 2003), *Lactobacillus pentosus* (Palacios *et al.,* 2005), *Citrobacter braakii* (Kim *et al.,* 2003). In addition, some of the anaerobic rumen bacteria, mainly *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Mitsuokella multiacidus*, and *Mitsuokella jalaludinii* are also reported in the expression of the phytase gene (Konietzny *et al*., 2004). The expressed of *E. coli* phytase with transformants was produced up to 600 U of phytase/ml (~0.3 g/l) of minimal media after 30 h growth (Golovan *et al*., 2000). The increase in intensive application of the *E. coli* based phytase in the yeast expression system would be the potential cost effective way to produce the additive for poultry and swine.

The extracellular phytase structural gene was also reported from the marine yeast, *Kodamaea ohmeri* BG3 (Li *et al.,* 2009). The ORF coding of phytase gene from *Debaryomyces castellii* phytase was isolated and overexpression reported in the methylotrophic yeast *Pichia pastoris*. Maximum production level obtained were found at 107 U/mL, i.e. 1340 U/g DCW and 16.5 U/mL i.e. 300 U/g DCW with the AOX1 and GAP expression system respectively. The cold active yeast phytase was also reported in *Issatchenkia orientalis* strain PA4 isolated from Himalaya. The maximum phytase activity showed 31.26 U/ mg at wet biomass after 48 h of incubation period. Therefore this cold adaptive phytase could be used effectively as a low cost bioinoculant in Himalayan livestock nutrition (Suyal *et al*., 2013).

Expression profiling of the phytase gene in plant

Expression of transgeneic phytase gene in plants is a convenient approach to eliminate costs associated with phytase production, purification, and supplementation (Pen *et al*., 1993) and thus can be used for animal feed supplements. Expression of phytase gene from microbial origin was reported in various plants such as soybean, canola, tobacco, potato, maize, alfalfa, sesame, wheat, Arabidopsis, rice, *Trifolium repens* and *Medicago truncatula* (Gontia *et al*., 2012).

The studies have been reported on the successful transformed of the *phy* gene in soybean cells (Li *et al*., 1997). The supplementation of transgenic phytase soybeans to the poultry and pig feed at 1200U/kg and transgenic canola at 250, 500 and 2500 U/kg per day significantly improved the nutritional values and remarkably reduced phosphorus excretion (Gontia *et al*., 2012). The highest activity of phyA from *Aspergillus awamori* was 150 U/mg compared to 56 U/mg protein in control. Over-expressed microbial phytase in soybean or canola seed was reported the improving phytate-P utilization by broilers and pigs (Zhang *et al*., 2000). The expression of *phy* gene in *Arabidopsis thaliana* PAPs (AtPAP15) in transgenic soybean plants exhibited enhanced bioavailability of phosphorus. Overexpression of phyA in transgenic Arabidopsis with the Pht1:2 promoter increased extracellular phytase activity.

Phytase gene from *Brassica napus* activities in transgenic canola seeds ranged from 1,138 to 1,605 U/ kg whereas no phytase activity was detected in wild type seeds. Therefore, production of transgenic phytase plant for monogastric animals can open the new era for animal feed industry (Wang *et al.,* 2013). Maize seeds are the major ingredient of commercial pig and poultry feed. Chen (2008) reported the phytase activity in transgenic maize seeds reached approximately 2,200 units per kg seed, about a 50-fold increase compared to non-transgenic maize seeds. The overexpression of heterologous phytases in transgenic potato offers an ideal feed additive for improving phytate-P digestibility in monogastric animals (Hong *et al*., 2008).

Profiling the conditions for processing of phytase in feed industry

The used of microbial phytase have two limitations, i.e., the high production cost and inactivation at the high temperatures during pelleting feed and storage (Greiner and Farouk, 2007). However, increase heat tolerance of the phytase enzyme can be reduced the production cost of animal feed. Therefore thermal stability of phytases is relevant in animal feed applications. Recently new industrial enzymes have focused on novel extremophiles microbial sources. The enzyme is normally incorporated into the grains prior to pelletization and the feed briefly reaches

processing temperatures of 85 to 90 °C. The research demonstrated that phytase from *T. lanuginosus* was a stable enzyme activity at elevated temperatures with performance advantages over the conventional *A. niger* enzyme (Berka *et al.,* 1998). The increase plant production and for P-nutrition for monogastric animals from a single transgenic plant can significant implications for the improvement of phytate-P bioavailability in soil and seeds (Wang *et al.,* 2013). It was reported that body temperature of mature broilers ranges between 41 and 42 °C. Therefore phytases from *A. niger* and *S. cerevisae* presented 92.87 and 76.79% of their maximum activity at 41 °C, respectively might be considered as phytate contained in broiler feed.

The pH tolerance and proteolytic resistance phytase are also the crucial criteria for the efficiently utilized as animal feed supplements. Phytases from different origins may have different optimal pH. The recombinant phytase *Aspergillus japonicus* BCC18313 (TR86) and *Aspergillus niger* BCC18081 (TR170) reported by Promdonkoy *et al.* (2009) exhibited pH stability from 2.0 to 8.0 and >50% activity was retained after heating at 100 °C for 10 min, suggested the efficiency as commercial phytase for hydrolyzing phytate in corn-based animal feed supplement. Moreover, some bacterial phytases, exhibit a pH optimum in the range from 6.0 to 8.0, thus it would be beneficial as feed additives for livestock crop (Konietzny *et al.,* 2004). In other study, *Enteroccus faecium* and *Lactobacillus plantarum* have shown 0.74 U/ml and 0.71 U/ml of extracellular phytase activity respectively (Anastasio *et al*., 2010). The chicken trial on the efficacy of transgenic canola-derived Aspergillus phytase showed effective as the commercial microbial phytase (Peng *et al*., 2006).

Consequence of phytase supplementation on livestock feed on nutritional impact

The approach to produce transgenic crops with high phytase expression is becoming a prerequisite to improve the bioavailability of phosphorus in food/feed instead of direct supplementation of animal feed with microbial phytase. During the past decade, the inclusion of microbial phytase in animal diets has increased remarkably. The first commercial phytase products have been started in earlier 90s at Netherlands, followed by various improvements for commercial production. Nowadays annual world market of phytase as an animal feed additive is estimated to be $500 million.

The studies have been carried out to evaluate the influence of phytate on the utilization of nutrients, not only lowers the bioavailability of phosphorus, calcium and other minerals, but also protein, fat, and starch (Pallauf and Rimbach, 1997; Coon and Leske, 1999). In addition phytase corn-soy diets supplemented enhanced the P level in blood and plasma (Perne, *et al.,* 1993; Yi, *et al.,* 1994;

Khalid, *et al.,* 2013). Phytase supplementation might increase the tibial ash in broilers as compared to the control. Wheat-sorghumsoy based diets with 2.9, 3.7 and 4.4 g kg^{-1} phytate P concentrations or high energy feed supplemented with phytase (1000 FYT/kg^{1}) in broilers fed can improved tibial ash contents (Cabahug *et al.,* 1999; Zanini and Sazzad, 1999). It did not only reduce total phosphorus excretion, it has the potential to reduce manure P lability and thus, reduced the environmental impact of land application of manure.The present observation is in the agreement with the earlier studies. Moreover, improvements in biologically utilization of Ca, Mg, Zn, Mn and Cu in response to phytase supplementation have also been reported. Phytase supplementation also results in increased amino acid digestibility especially histidine, arginine, leucine, threonine and valine (Yi, *et al.,* 1996; Khalid, *et al.,* 2013).

However, no reproducible data have existed to show that transgene DNA in commercialized transgenic crops has unique behavior relative to native plant DNA (Lemaux *et al*., 2008). Gao *et al.* (2014) demonstrated that recombinant *phy*A2 gene was not detected in muscle tissues and reproductive organs. The European Food Safety Authority (EFSA) in 2007 has released the statement of the genes and proteins in food and feed, which suggested that rDNA fragments or proteins derived from genetically modified plants have not been detected in tissues, fluids or edible products of farm animals.

Research into discovering new phytases, engineering better phytases and developing more cost-effective expression systems using low-phytic acid corn, along with microbial or plant phytase supplementation, may be the right direction to pursue the solution to meet its enormous nutritional and environmental demand.

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