Low-cost phytase production methodology to enhance feed nutritional value for monogastric animals

D. Sarkar*, A.M. Haider, Md.I. Hossain, Md.A. Zihad, H.C. Bhoumik, A. Sutradhar

Department of Microbiology, Primeasia University, 12, Kemal Ataturk Avenue, Banani, Dhaka, Bangladesh
*Corresponding Author E-mail: sarkardayanidhi5@primeasia.edu.bd

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Abstract

Phytic acid consists of an inositol ring with six phosphate ester bonds. It is the primary phosphate storage compound in plant seeds. Phytic acid can bind with di- and tri-valent minerals and form very stable complexes, decreasing the availability of phytate phosphate to non-ruminant livestock. The salt form of phytic acid, phytate, also binds non-selectively to proteins and inhibits enzymes including trypsin and α-amylase, thus reducing protein digestibility in monogastric animals like pigs, poultry and fish. These protein-mineral-phytate complexes are insoluble. Phytase enzyme can reduce the antinutritional effect of phytate and improve the digestion of phosphorous, di- and tri-valent minerals and amino acids, as well as reduce the inorganic phosphate excretion to the environment. Phytase can catalyse the release of six phosphates from phytic acid. It is widely used as a feed supplement for monogastric animals in developed countries. In case of developing countries some large feed companies import phytase enzymes from developed countries to add their feed and sell at a high value. The terminal small farmers are unable to purchase high-value feed for their farms. Here we discuss a simple method to get phytase enzyme for feed additive. This methodology is applicable to the terminal farmers of developing countries. The supplemented phytase will increase phosphate uptake, mineral and nutrient absorption, reduce malnutrition, and will enhance animal growth and productivity.

Keywords: Carbon metabolism; antinutritional effect; Solid state fermentation; phosphorous pollution; Electroporation

Introduction

Cereal food is the main ingredient of monogastric and agastric animal feed. Feed for monogastric animals is formulated from legume and cereal seeds, mainly corn and soybean meal, which represents about 70% of the pig diet and substantially contributes to supply their energy, protein, mineral and vitamin needs (Pires et al., 2019). Phosphorus is an essential mineral in animal feeds. Phytic acid is a main reservoir of phosphorus (p) in plants and contributes to about 80% of the total P in cereal seeds (Vashishth et al., 2017). The phosphorus fraction stored as phytate range from 30% in roots up to 80% in seeds and cereals (Table 1). About 1-5% weight of oilseeds, legumes, nuts, pollen and grains is phytic acid, which chelates with divalent or trivalent metal cations (Fe^{+2} , Fe^{+3}, Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}). Due to the pronounced negative charge, phytic acid forms a complex with cation, amino acids (histidine), starch, proteins and enzymes, and disrupts their ability, solubility resulting bioavailability (Selle et al., 2006; Joudaki et al., 2023). Phytate is known to form complexes with proteins under both acidic and alkaline pH conditions (Vashishth et al., 2017). The diet of domestic farm animals is based on plant sources, grains and oilseeds, and therefore contains significant amounts of phytate that reduce the mineral uptake by monogastric animals (Singh, 2014). The phytase are a group of enzymes that hydrolyze the phospho-monoester bonds of phytic acid (Jatuwong et al., 2020). It has been long established that some feed ingredients have endogenous phytase activity (Ravindran et al.,1995). But their activities are not enough. Wheat, wheat bran, barley, rye contain high levels of phytase activity, whereas corn, soybean meal, peanut meal, sorghum, and cassava roots contain little or none of the enzyme (Table 2). The digestive system of monogastric animals, like humans, lacks the enzyme phytase, and therefore the accumulated phytate prevents the uptake of minerals from the animal diet (Gessler et al., 2018). Moreover, these non-absorbed minerals are released into the environment in large quantities through animal faeces, leading to environmental pollution (Joudaki et al., 2023). Reducing the amount of phytic acid by chemical and physical methods affects other food constituents, and generally reduces the nutritional value of food products (Joudaki et al., 2023). The use of phytase enzymes in feed as additive to reduce phytic acid in food can overcome these problems (Jongbloed et al 2013; Butani& Parnekar 2015). Phytase enzymes release mineral phosphate (P) reducing the anti-nutritional properties and preventing protein or enzyme complex formation with phytic acid, and also the chelation of metal ions (Fig.1). Therefore, microbial phytases extracted from yeasts, fungi, or bacteria are principally used for commercial purposes (Rizwanuddin et al., 2023). The higher cost of this enzyme limits their use in developing countries. The importation cost also raises the expenditure of the feed cost. *Aspergillus niger* is a known fungus with probiotic properties that contains several bioactive compounds and enzymes, such as tannase, phytase, α-galactosidase, L-asparaginase, xylanase, α-amylase, proteases, and cellulose, which provide benefits to poultry animals (Hong et al., 2004; Saleh et al., 2017).

In this review, we search for a low-cost phytase production platform and straightforward methodology to enhance feed nutrition for small-scale farmers. Here we suggest whole solid-state fermented media including *Aspergillus niger*, the probiotic, and its phytase enzyme as feed additive. This review also provides the production of low-cost phytase using simple instruments to improve feed nutrition for small-scale terminal farmers in developing countries. This also generates an awareness of phosphorous pollution in a bioeconomy.

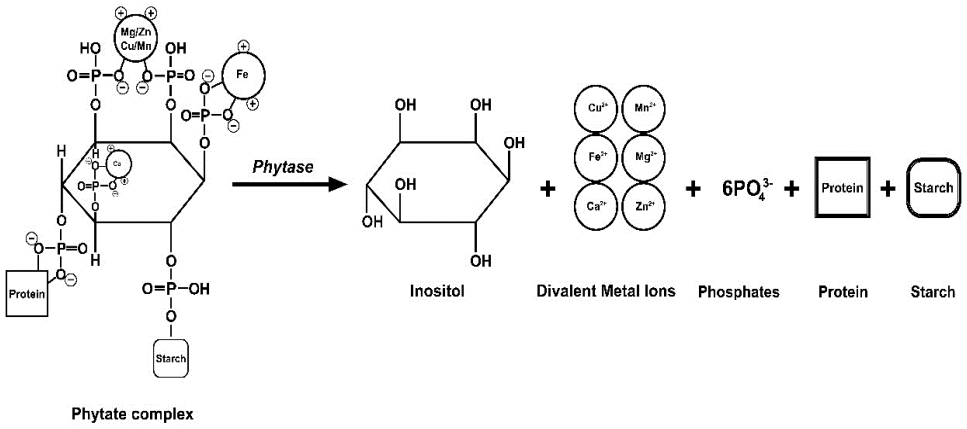

Fig 1. The degradation of phytate complex by phytase enzyme

Table 1. Phytate phosphorus contents of feed ingredients (Ravindran V. et al., 1995)

Ingredient	Phytate phosphorus, g/100g DM	Phytate P, as % of total P
Cereals		
Corn (Zea mays)	0.24	72
Barley (Hordeum vulgare)	0.27	64
Wheat (Triticum aestivum)	0.27	69
Oats (Avena sativa)	0.29	67
Sorghum (Sorghum vulgare)	0.24	66
Foxtail millet (Setaria italica)	0.19	70
Finger millet (Eleusine coracana)	0.14	58
Rice (Oryza sativa), unpolished	0.27	77
Rice, polished	0.09	51
Cereal by-products		
Rice bran	1.31	80
Wheat bran	0.92	71
Rice polishings	2.42	89
Roots and tubers		
Cassava (Manihot esculenta) root meal	0.04	28
Sweet potato (Ipomea batatas) tuber meal	0.05	24
Taro (Colocasia esculenta) corn meal	0.09	24
Grain legumes		
Field peas (Pisum sativum)	0.24	50
Cowpeas (Vigna unguiculata)	0.26	79
Green gram (Vigna radiata)	0.22	63
Pigeon peas (Cajanus cajan)	0.24	75
Chickpeas (Cicer arietinum)	0.21	51
Oilseed meals		
Soybean (Glycine max) meal	0.39	60
Cottonseed (Gossypium spp0.) meal	0.84	70
Peanut (Arachis hypogaea) meal	0.48	80
Rapeseed (Brassica napus) mea	0.70	59
Coconut (Cocos nucifera) meal	0.29	49
Sesame (Sesamum indicum) meal	1.18	81

Table 2. Phytase activity of some feed ingredients (Ravindran V. et al., 1995)

Ingredient	Phytase activity, units/kg[a]
Cereals and by-products	
Wheat	1193
Barley	582
Rye	5130
Corn	15
Sorghum	24
Wheat bran	2957
Rice bran	122
Roots and tubers	
Cassava roots	6
Sweet potato tubers	26
Grain legumes	
Field peas	116
Oilseed meals	
Soybean meal, 48%	8
Peanut meal	3
Rapeseed meal	16
Miscellaneous	
Alfalfa meal, dehydrated	60
Soybean hulls	99

[a]One unit is defined as the amount of phytase which liberates inorganic phosphorus from a 0.0015M sodium phytate solution at a rate of 1 µmol/min at pH 5.5 and 37°C.

Present perspective

Once upon a time, the village economy like Bangladesh depended only on rice planting. Nowadays they are turning to livestock cultivation as the protein consumption rate rising day by day rather than carbohydrate. Therefore, so many small poultry and fish farms are rising daily. Most of them cannot buy high-quality feed due to its higher cost. They use an indigenous feed mixture. Rice bran, wheat bran, intact rice, broken rice, broken maize, rapeseed oil cake, low-cost pulse etc. used for poultry and fish feed. As a result, most of the poultry suffer from malnutrition. They lose their weight and lay a lower number of eggs. The eggshell becomes very thin and the other hens eat the egg instantly. Some big feed companies buy phytase from developed countries and add it to their feed. Therefore, their feed contains a high nutrient value and is sold at a higher price. Small farmers are unable to buy such feed. Now they are struggling for survival.

Solid state fermentation

Solid-state fermentation has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products (Pandey, A., 2003). It is simpler, the moisture content is lower, and the protein production yield is higher. Filtration is generally unnecessary as the product is concentrated and may be used directly. Aseptic conditions need not follow for all time due to less risk of contamination. Temperature, pH, aeration, and agitation control are not required for tropical and sub-tropical countries. Simple cotton cloth and water are required to control incubation room humidity. This process can be operated by less skilled people and by very simple instruments. The capital investment and energy expenditure are also very low as compared with submerged culture.

Strain Selection

Very few organisms are suitable for solid-state fermentation. The microbiological process of solid-state fermentation has generated great interest in recent years due to the numerous advantages over submerged fermentation. Filamentous fungi are mostly used in such fermentation. Industrial valuable *Aspergillus niger* would be the best strain for enzyme production.

Strain Screening

A potential product-yielding strain should be screened first for industrial cultivation. Screening strategies are divided into two basic types: non-selective random screening, in which randomly picked isolates are tested for the desired qualities; and rational selection, a method based on prior knowledge of the metabolism and regulation pathways of microorganisms, so the identification is carried out in a targeted manner (Parekh et al., 2000; Heerd et al., 2014).

Strain engineering

This part should be done in a sophisticated laboratory by highly expert personnel. If it is not possible the natural isolate is enough for enzyme production. Microbial strain improvement for the overproduction of desired products has been the hallmark of all commercial fermentation processes. Successful development of improved strains requires a deep knowledge of physiology, metabolic pathway distribution and regulation procedures. Classical and genetic engineering are two approaches used for strain development.

Classical strain improvement

Classical strain improvement has long been regarded as the gold standard for fungal strain improvement in the industry because it can be applied even when there is limited knowledge about the genetic basis or biosynthetic pathways of the production organisms (Sonia et al., 2023). Organisms obtained by classical mutagenesis are not subject to GMO legislation and can be used in the industry in the short-term (Cadière et al., 2011). Physical and chemical mutagenesis and screening of the high-secreting mutants provide suitable strains for specific industrial goals (Steensels et al., 2014), such as the overproduction of penicillin (Kardos and Demain, 2011; Barreiro, 2012), increased production of lignocellulolytic enzymes (Ribeiro, et al., 2013; Bischof, et al., 2016; Dillon, et al., 2006), lipase (Karanam and Medicherla, 2008), citric acid (Javed et al., 2010) and bioethanol (Mobini-Dehkordi, et al., 2008; Zhao et al., 2022).

Strain improvement by random mutagenesis is a successful method, but it is mainly a trial-and-error process, which requires screening of large numbers of strains for the desired traits (Sonia et al, 2023). Random mutagenesis has been applied in a large number of fungal species for many industrial purposes, such as improved cellulase production in *Aspergillus* sp. (Vu et al., 2009), lipase production by *Aspergillus japonicus* (Karanam and Medicherla, 2008) or citric acid overproduction by the industrial workhorse *Aspergillus niger* (Lotfy, et al., 2007). Moreover, UV-derived mutations were reported in *Aspergillus niger* to increase Filter Paper activity (FPase) and carboxymethyl cellulase (CMCase) production (Irfan et al., 2011).

Whole cell-directed adaptive evolution relies on the basic principles of genetic variation and subsequent strain selection. A microbial population is cultivated under a selective pressure for several generations to get desired traits. Natural selection optimizes the strain without requiring prior knowledge of genetic modification. Adaptive evolution

can also be combined with other methods such as random mutagenesis in order to generate more genetic diversity for selection (Steensels et al., 2014, Winkler and Kao, 2014).

Protoplast fusion is the fusion between two cells with different genetic traits, which leads to a stable hybrid strain with the combination of the genetic traits of both parents. Protoplast fusion can be used to produce interspecific or even intergeneric hybrids (Verma et al., 2008). Strains resulting from protoplast fusion of *T. reesei* and *A. niger* showed a three-flod increase of citric acid production in comparison with the parent *A. niger* stran (El-Bondkly, 2006). Genome shuffling is time-consuming, but its application does not require expensive facilities (Gong et al., 2009). It is also recombination between multiple parents of each generation and several rounds of genome fusion. As a result, the final improved strains inherit the genetic traits from multiple initial strains (Leja, 2011).

Genetic regulation and metabolic pathway engineering

The cell growth and catabolic rates are modulated by the multi-level regulation machinery consisting of gene expression (transcriptional regulation), post-transcriptional regulation, translation, and post-translational regulation for ultimately modulating the metabolic fluxes (or enzymatic reaction rates) (Shimizu and Matsuoka, 2018). The main metabolism is primarily modulated for energy generation (catabolism) and biomass synthesis (anabolism), where such regulation system may be constrained by the more important upper regulation systems (including oxidative stress regulation) in the hierarchical regulation system for the cell survival (Shimizu and Matsuoka, 2019). The metabolic intermediates of the central carbon metabolism (CCM) must be converted to monomers such as nucleotides, amino acids, and lipids for biomass synthesis (Shimizu and Matsuoka, 2022). The engineer should know the ins and outs of cellular metabolism based on environmental stimuli. The target pathway should be optimized by cutting the branch pathways. Genetic engineering renders a higher level of strain construction. Over the last decades, GMOs have revolutionized many fields, including medicine, agriculture, food and pharmaceutical industries (Hug, 2008). Metabolic pathway engineering is applied for the improvement of the desired metabolite through the modification of specific pathways by insertion or deletion of genes. Metabolic engineering is considered as a combination of multidisciplinary subjects built on principles from chemical engineering, computational sciences, biochemistry and molecular biology (Yang, et al., 1998). *Agrobacterium tumefaciens*, a soil-dwelling bacterium can infect plants that induce tumors. It has been successfully applied in a range of filamentous fungi, such as the economically important *Aspergillus* sp. (Meyer et al., 2003; De Groot, et al., 1998).

Genetic engineering by electroporation

Electroporation is a fast and efficient transformation method that can be directly applied to both sporulating and non-sporulating fungal species (Chakraborty et al., 1991). Purified DNA fragments containing a target gene with a marker can be inserted into the cell by electric pulse and later the transformed cell can be selected in a specific media. Electroporation-mediated transformation has been applied in several fungal species such *as N. crassa, Penicillium urticae* (Chakraborty et al., 1991), *Pseudogymnoascus verrucosus* (Diaz et al., 2019), *Monascus purpureus* (Lakrod et al., 2003) or *T. harzianum* (Wang et al., 2022), and in some other filamentous fungi of industrial relevance such as *A. niger* (Ozeki et al., 1994), *A. oryzae* (Chakraborty et al., 1991), or *T. reesei* (Benocci et al., 2018).

CRISPR/Cas9 genome editing

CRISPR/Cas9 is a gene editing technology which turns on or off genes in cells and organisms rapidly and cheaply. The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated nuclease 9) technology has dramatically changed the field of genome engineering since its first discovery in bacteria and archaea (Jinek et al., 2012). The first application of the CRISPR/Cas9 technology in filamentous fungi was in the industrially relevant *T. reesei* (Liu et al., 2015) and in six different *Aspergillus species*, including the industrial workhorse *A. niger* (Nødvig et al., 2015). Nowadays, the CRISPR/Cas system enables the genetic improvement of a wide variety of filamentous fungal species, including *P. oryzae* (Arazoe et al., 2015), *N. crassa* (Matsu-Ura et al., 2015), *A. oryzae* (Katayama et al., 2016), *Aspergillus fumigatus* (Zhang et al., 2016), *P. chrysogenum* (Pohl et al., 2016), *Alternaria alternate* (Wenderoth et al., 2016), *Beauveria bassiana* (Chen et al., 2017), *F.oxysporum* (Wang et al., 2018), *Fusarium fujikuroi* (Shi et al., 2019), *A. niger* (Kun et al., 2020), *Penicillium subrubescens* (Salazar-Cerezo et al., 2020), *P. expansum*, *P. digitatum* (Garrigues et al., 2022) and the polykaryotic industrial fungus *Monascus purpureus* (Liu et al., 2020), among others.

Droplet-based microfluidics technology

Recently, droplet-based microfluidics technology has allowed major advances for the screening of microorganisms by significantly increasing the throughput and enlarging the range of systems that can be selected (Beneyton et al., 2016). The platform allowed (i) compartmentalization of single spores in 10 nl droplets, (ii) germination and mycelium growth and (iii) high-throughput sorting of fungi based on enzymatic activity (Beneyton et al., 2016).

Cost-effective Indigenous substrate Selection

Raw materials play an important role in the production of industrial products. Substrate should be available and at a low cost for cultivating desired organisms. Wheat bran, rice bran, soya bean meal, chickpea coarse powder, corn steep powder and urea, as a nitrogen source, would be the best selection for cultivation. Potato dextrose agar and broth are usually used for seed culture cultivation.

Cost-effective Industrial Production Method

Pure culture of *Aspergillus niger* would be cultivated in submerged fermentation for seed culture. The main cultivation would be solid-state fermentation. The solid-state media contains 50% water. The sterilized rectangular Stainless Steel (SS) tray contains the final culture. The humidity of the incubation room would be 90%. Wet muslin cloth inside the incubation room can maintain such types of moisture. The organism releases extracellular enzymes including phytase during the growth. The highest enzyme secretion time would be selected by checking the enzyme activity during the whole process. After the cultivation, the culture should dry for 48 hours at 60ºC. The enzyme activity should be checked at the final stage. This simple method can be operated by any person.

Inoculum Preparation

The stock culture needs to be cultivated in Potato Dextrose Broth. The broth flask would be incubated at 30ºC for 48 hrs under a shaking incubator.

Seed Preparation

The seed media contains wheat bran, rice bran, peptone, corn steep liquor/powder, chickpea coarse powder, dextrose, calcium phosphate and phytic acid. The medium pH should be 4.5 adjusted with phosphoric acid. The cultivation time is 48 hrs at 30ºC with agitation at 200 RPM.

Main fermentation at solid state

The fermentation media contains wheat bran, soya grits, chickpea ground, calcium phosphate and urea (modified method of Awad et al., 2014; Sabu et al., 3002; Bala et al., 2014; Berikten and Kivanc, 2014; Buddhiwant et al., 2016; Bhavsar et al., 2011). The media should contain 50% moisture and humidity at 90%. A rectangular SS tray with a cover lid would be used for solid-state fermentation at 30ºC without shaking. The cultivation time should be optimized by checking the enzyme activity. The wet muslin cloth should be placed in the incubation room to maintain 90% humidity. The whole culture should dry at 60ºC for 48 hrs or more and should be checked for phytase enzyme activity. Phosphoric acid (0.5%) powder needs to be mixed well before final packing. The whole culture should be added with the feed as the organism is probiotic for the animal. We did work on this procedure. We also checked enzyme activity and compared with best product of market. Our enzyme activity was better than the marketed product (data not published).

Feeding Process

During the feed pellet-making process the temperature reaches more than 90ºC. Most of the enzymes denature at this temperature. Thermo-stable phytase enzyme is desirable but rare. As a result, to maintain optimum phytase activity in feed it is needed to mix enzymes at the cooling stage of the feed-making process or during feeding instantly. The addition of microbial phytases to feed and food (up to 2200 FTU/kg, where 1 FTU is the activity of the enzyme which releases one micromolar orthophosphate from phytate per minute at pH 5.3) significantly enhances the release of phosphate and minerals from phytate (Kornegay 2001; Troesch et al., 2009).

Continuous process improvement

The enzyme activity needs to be checked with other company products available in the market. Strain and process development is a continuous process. Genetic engineering and biotechnology need to be applied to get more robust strains. The substrate selection and media composition as well as instrument optimization should be developed continuously.

Conclusion

Phytase supplementation to feed has become common practice in poultry and swine farms and made this enzyme to global sales in the feed sector (Sales at USD 580.56 million in 2023, Global market insights). Phytase reduces the chelating effect of phytate with the improvement of phosphorous utilization, increasing the production yield of either meat or egg. Major phytase research is the screening for thermo-stable enzymes. Very few phytases have been reported to have temperature stability. The fungal phytase from *A. fumigatus* was reported to withstand temperatures up to 100ºC over a period of 20 min (Pasamontes et al., 1997), but a later report from Ullah et al., 2000 did not confirm these results (Haefner et al., 2005). A transgenic pig has been developed that produced the *E. coli* phytase in its saliva with an average of 2000-3000 U/ml (Golovan et al., 2001b). However, this direction might be limited by public acceptance.

In this review, we discuss the enrichment of poultry feed adding supplemental phytase to enhance growth and reproduction. The production strategy of phytase enzyme will help the feed manufacturer to lower the feed price. As a consequence, the terminal farmers will able to purchase low-cost feed for their farm to survive.

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