Present Scenario and Future Prospects of Phytase in Aquafeed - Review -

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ABSTRACT : Aquaculture pollution is a major concern among the entrepreneurs, farmers and researchers. Excess discharge of phosphorus and nitrogen into the water bodies is the principal pollutant responsible for this. Plant-based feed ingredients due to its high phytic acid content enhances both nitrogen and phosphorus discharge thereby increasing the pollution level. Dietary phytase treatment is probably the best answer to address this problem. This review explains the nature and properties of phytate, its interactions with other nutrients and the application of phytase in aquafeed to reduce the pollution. This review also covers the different biotechnological aspects for lowering the phytic acid level in the common aquafeed ingredients, as an alternate approach to controlling the pollution level. Some of future research needs have also been highlighted to attract the attention of more researchers to this area. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1800-1812)***

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INTRODUCTION

Plant protein sources are more in use as alternatives to costly and scarce fishmeal in the aquafeed. A major concern with the plant ingredients is the presence of a wide variety of anti-nutritional substances. Phytic acid (PA) is one such anti-nutritional factor found in most of the feedstuffs like barley, rice, sorghum, wheat, maize, gram, groundnut, rapeseed, soybean, cottonseed and sesame. High protein content, low phosphorus level (compared to fishmeal) and ready availability make soybean meal a promising alternative protein source in aquafeed. However, about two-thirds of phosphorus in soybean meal is present as phytate, which is not efficiently utilized by fish (NRC, 1993). Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is the major phosphorus (P) storage compound in plant seeds and can account for up to 80% of total P. The remaining P is represented by soluble inorganic phosphate and cellular-P (P bound in nucleic acids, phosphorylated proteins, phospholipids, phospho-sugars). Because of its high density of negatively charged phosphate groups, phytate forms mixed salts with mineral cations called phytin. During germination, phytin is degraded by the action of phytases, which provides the growing seedlings with phosphate, mineral cations and myo-inositol. Apart from its storage function, phytate has also been assumed to play an important role in P homeostasis, buffering cellular P levels (Lopez et al., 2002). Phytate phosphorus is not available to fish (Lall, 1991; Riche and Brown, 1996) and it makes complexes with other nutrients like minerals, protein, carbohydrate and lipids thereby rendering them unavailable to the fish. Hence, phytate-rich ingredients have become major concern among the researchers and nutritionists while formulating aquafeed. Present review covers the inhibitory actions of phytate on nutrients and their corrective measures to enhance its use in aquafeed.

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PHYTIC ACID

Phytic acid (PA) is found in most of the ingredients commonly used in fish feed like barley, rice, sorghum, wheat, maize, gram, groundnut, rapeseed, soyabean, cottonseed and sesame (Halver, 1989; De Silva and Anderson, 1995). Phytic acid isolated from plants belongs to the group of organic phosphates and is a mixture of calcium-magnesium salt of inositol hexaphosphoric acid, also known as phytin. Salts of phytic acid are also called phytate. It is an abundant plant constituent comprising 1 to 5% by weight of the legumes, cereals, oil seeds, pollens and nuts (Vohra and Satyanarayan, 2003). It is an organic form of phosphorus, which is chemically a myo-inositol hexakis-dihydrogen-phosphate (IP_6). Phytic acid is found widely in eukaryotic cells (Sasakawa et al., 1995). Its molecular structure was determined by Johnson and Tate (1969).

The occurrence of phytates in plant foodstuffs is well documented. It constitutes between 0.7 and 2% of most cereal grains and oilseeds (Adeola and Sands, 2003) and is the primary source of inositol and storage form of phosphorus and other minerals in plant seeds that are used as animal feed ingredients (Hardy, 1998; Taiz and Zeiger,

1998; Powell, 2003). Most foods of plant origin contain 50 to 80% of their total phosphorus, or even higher in selected varieties, as phytate (Lall, 1991; Eeckhout and dePaepe, 1994; Harland and Morris, 1995; Ravindran et al., 1995; Pallauf and Rimbach, 1997; Raboy, 1997). On an average, the phytate-phosphorus content of cereals (like maize, rice, wheat, sorghum and barley) and oilseed meals (like groundnut, soybean, cottonseed and sunflower meals) varies from 51 to 82% of the total phosphorus present in them (Tyagi et al., 1998).

Effect of phytate on nutrient bioavailability

Mineral bioavailability : One of the major problems associated with the use of grain and oilseed products in feeds for monogastric animals is the presence of phytate. Phytate phosphorus is excreted in the faeces, which can contribute to nutrient enrichment of aquatic ecosystems. Microorganisms degrade the excreted phytate, thus releasing the bound phosphorus that contributes to algal growth and subsequently oxygen depletion in ponds, thereby causing aquaculture pollution. Phytate has also strong affinity to interact directly and/or indirectly with other minerals to reduce their bioavailability to animals. For example, calcium-bound phytate increases formation of co-precipitates of zinc and other trace minerals through the process of chelation, thus making them unavailable to fish (Hardy, 1998).

Interestingly, zinc availability in juvenile chinook salmon was greatly reduced when sodium phytate was added to their feed, and fish developed cataracts as a result (Richardson et al., 1985). Supplemental phytate (1.1%) in the feed of channel catfish increased the amount of dietary zinc necessary to prevent deficiency signs by 10 folds over the amount needed in a semi-purified diet (Gatlin and Wilson, 1984). Increasing the dietary phytate level from 1.1% to 2.2% decreased weight gain, feed efficiency and zinc content in vertebrae of channel catfish fed practical diets containing 50 mg zinc kg^{-1} (Satoh et al., 1989). Addition of sodium phytate (25.8 g kg^{-1} diet) resulted in decreased growth, feed and protein conversion and thyroid function in case of juvenile Chinook salmon (Richardson et al., 1985). Hossain and Jauncey (1990) also reported negative effect of sodium phytate on growth and feed utilization of common carp. Increased dietary calcium and magnesium levels in the presence of phytic acid significantly reduced the bioavailability of calcium, magnesium, zinc, iron and copper and resulted in abnormal changes in the epithelial layer of carp intestine. This reduced bioavailability was attributed to the formation of insoluble phytate-mineral complexes (Hossain and Jauncey, 1991).

Protein utilization : The association between phytate and protein begins in the seeds during ripening, when phytate accumulates primarily in the protein-rich aleurone layers on monocotyledons seeds and in the protein bodies of dicotyledonous seeds (Hidvegi and Lasztity, 2002). The interaction between phytic acid and proteins is believed to be an ionic type and is dependent on pH (Cosgrove, 1966). At low pH, phytic acid forms electrostatic linkages with the basic arginine, lysine and histidine residues resulting in insoluble complexes. As the pH approaches the isoelectric point, the charge on the protein is neutralized and the phytate is no longer bound and becomes soluble. In this soluble state, phytate complexes with protein because of the presence of divalent cations. These cations, usually Ca, Mg or Zn, act as a bridge between negatively charged protein, carboxyl groups and the phytate (Anderson, 1985).

In vivo studies have shown that phytate-protein complexes are insoluble and less subject to attack by proteolytic enzymes than the same protein alone. The reduced solubility of proteins because of such complexing can adversely affect certain functional properties of proteins, which are dependent on their hydration and solubility. Phytate is known to inhibit a number of digestive enzymes such as pepsin, α-amylase and trypsin (Ravindran et al., 1995). In protein-phytate interaction, the amino group present on the side chain of the amino acids is one of functional groups involved in the interaction, thereby decreasing the digestibility of proteins. Even the action of certain enzymes such as amylase, trypsin, acid phosphatase and tyrosinase has been shown to be inhibited by phytic acid and also by inositol pentaphosphate (Harland and Morris, 1995). Jongbloed et al. (1997) reported that phytate-protein complexes may be formed post feeding in the gut at pH 2 to 3. At this pH, it was found that soluble proteins in casein, corn, rice polish, soybean meal and sunflower meal were substantially precipitated in the presence of phytic acid.

Spinelli et al. (1983) reported that rainbow trout fed purified diets containing 0.5% phytic acid suffered reduction in protein digestibility and about 10% reduction in growth and feed conversion. Increasing Ca and Mg content of the diet in the presence of phytic acid did not affect growth and feed conversion. Fish fed diets containing over 1% Ca without phytic acid had a 5% reduction in growth and feed conversion. Therefore, they concluded that reduced growth was due to reduced protein bioavailability and not due to reduction in Zn, Fe, or Cu availability.

Starch utilization : Phytate may also reduce the solubility of starch by binding it, reducing its absorption and hence lowering glucose utilization. Starch binding occurs because of hydrogen bond formation (Thompson, 1986). The effect of phytic acid on starch digestibility was studied *in vitro* and correlated with the blood glucose response (glycemic index) in healthy volunteers (Yoon et al., 1983). They found that the glycemic index was correlated

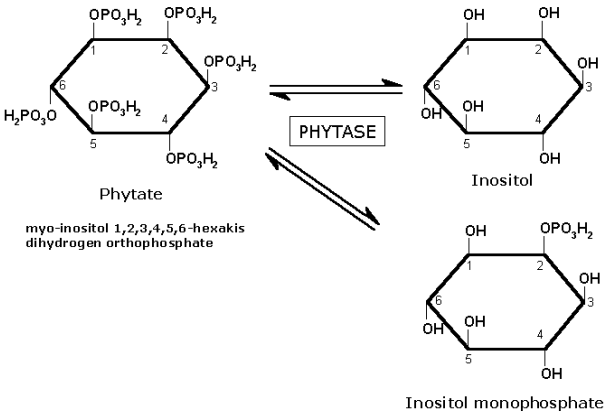

Figure 1. The enzyme phytase releases these phosphates from the inositol ring

negatively with the phytic acid content of the food tested. *In vitro* digestion studies involving human saliva at physiological pH and temperature showed that in the presence of sodium phytate, the rate of digestion of raw wheat starch was reduced by 50%. This was reversed by the addition of calcium that is known to complex phytic acid.

Lipid utilization : Phytate is a powerful chelator. It complexes with lipids along with other nutrients thereby reducing their digestibility (Vohra and Satyanarayana, 2003). *Cirrhinus mrigala* fed diets with phytic acid was found to contain lower fat than that without phytic acid (Usmani and Jafri, 2002).

PHYTASE

Phytase (myo-inositol hexaphosphate hydrolase) are phosphatase, enzymes that sequentially cleave orthophosphate groups from the inositol ring of phytic acid to yield available free inorganic phosphorus, a series of lower phosphoric esters (inositol pentaphosphate to inositol monophosphate) as intermediates, thereby decreasing phytates' affinity for different cations (Lie et al., 1993). The reaction ultimately leads to the production of free myo-inositol (Harland and Morris, 1995). In recent years, several phytases with different biochemical properties have been characterized and their effectiveness in animal feeding has been demonstrated (Han et al., 1998; Stahl et al., 1999). Several microbial phytase either as a dry powder or as liquid is available commercially. Natuphos® was the first commercially available phytase from a genetically modified *Aspergillus niger* strain. The enzyme phytase belongs to the Class "Hydrolases" and Family "histidine acid phosphatases" (Mitchell et al. 1997). Based on the type of reactions catalyzed, they are classified as 3-Phytase (EC 3.1.3.8) and 6-Phytase (EC 3.1.3.26). Phytate-degrading enzymes from microorganisms are considered to be 3-Phytases, whereas seeds of higher plants are said to contain 6-Phytases (Reddy et al., 1982; Nayini and Markakis, 1986). Microbial phytase have pH optima in the range of 2 to 6, while plant phytase tend to have a pH optimum at 5 (Wodzinski and Ullah, 1996). In one molecule of phytate six phosphate (PO_4) groups are covalently bound to a sugar called myo-inositol. The enzyme phytase releases these phosphates from the inositol ring as shown in Figure 1 below.

Phytases are widespread in nature, occurring in plants, microorganisms as well as in some animal tissues. Multiple forms of phytases have been reported in pumpkin (Goel and Sharma, 1979), lily (Baldi et al., 1988), rice (Hayakawa et al., 1989), rape seed (Houde et al., 1990), *Escherichia coli* (Greiner et al., 1993), *Aspergillus niger* (Hamada, 1994), spelt (Konietzny et al., 1995), *Saccharomyces cerevisiae* (Moore et al., 1995), soybean (Hamada, 1996), maize (Maugenest et al., 1999), wheat (Nakano et al., 1999) and barley (Greiner et al., 2000). Microbial phytase is detected in numerous microorganisms. Phytate-degrading activity has been detected in the mucosal extracts of the small intestine of rats, rabbits, guinea pigs, chicken, calves and humans (Bitar and Reinhold, 1971; Cooper and Gowing, 1983; Iqbal et al., 1994). Although, intestinal phytate-degrading activity does not play a significant role in digestion of dietary phytate in monogastrics (Iqbal et al., 1994), but dietary phytases have been shown to be an important factor (Lantsch et al., 1992). Degradation of phytate in monogastrics is also caused by the microbial flora of the large intestine. An increased degradation of dietary phytate during digestion in monogastrics could be obtained by supplementing food and feed with phytases (Simons et al., 1990; Cromwell et al., 1995).

Two main types of phytases identified are acid phytases and alkaline phytases with a pH optima around 5 and 8, respectively. Most of the phytases belong to the acidic ones and their pH optima range from 4.5 to 6.0 (Yamada et al., 1968; Ullah and Gibson, 1987; Pasamontes et al., 1997; Wyss et al., 1999a; Rodriguez et al., 2000). The temperature optima of phytases vary from 35 to 77°C. Phytase isolated from *Aspergillus niger* showed activity at temperatures between 25 and 65°C with an optimum at 55°C (Dvorakova et al., 1997).

APPLICATION OF PHYTASE

Animal feeds

Improving the nutrient digestibility and growth performance has been one of the most important nutritional aspects in animal farming, be it in poultry, piggery or pisciculture. Use of exogenous enzymes in animal diet has attracted considerable interest over last 10-15 years, the effects of which depended on several factors like type and composition of enzyme supplements, characteristics of feed

ingredients used, species, age and physiological status of the animal (Kim et al., 2004). Microbial phytase is one of the most commonly used enzymes in monogastric animal diets (Shim et al., 2004). Feedstuffs of plant origin form the major constituents of poultry diets. About two-thirds of P of those feedstuffs is present as phytate. Phytate-P is unavailable to poultry (Nelson, 1967). In addition, phytate-P chelates several important minerals and thereby reduces their bioavailability. Ruminants inhabit microbes that can enzymatically release inorganic-P from phytic acid. However, monogastric animals produce little or no phytase in the intestine for phytate degradation. Hence, supplementation of such diets with phosphates is a must to provide the animal with necessary nutrient. The unutilized phytin-P is disposed of in the animal's faeces (Mullaney et al., 2000). The availability of P from plant ingredients can be improved by addition of microbial phytase to the feed or by using phytase-rich cereal diets (Nelson, 1967). Enzyme treatment reduces the need for inorganic P supplementation in the diet due to the improvement in the utilization of P from feed, thus reducing P excretion in the manure (Mohanna and Nys, 1999). Diet manipulation for management of environmental pollution has been reviewed by Lenis and Jongbloed (1999) that emphasized the importance of dietary phytase. Addition of 250 to 1,000 U phytase/kg diet can fully replace P supplementation in poultry feed (Golovan et al., 2001). Phytase supplementation significantly increased serum concentrations of Ca, P, Mg, Zn, Fe, and Cu, and the weight and length of tibia; contents of crude ash, Ca, P, Mg, and Zn were adversely affected by lowering non-phytate phosphorus level in the diet of broiler chickens, but partially recovered by enzyme supplementation from *Aspergillus ficcum* (Paik et al., 2000). Um et al. (2000) reported that with the adjustment of non-phytate phosphorus level and phytase supplementation in the diets of broiler chicken could reduce the phosphorus excretion by 50%. Phytase supplementation of diets for growing-finishing pigs had improved growth performance and nutrient availability (Hong et al., 2001). Addition of phytase increased digestibility and decreased excretion of phytic P in broilers (Peng et al., 2003). Paik (2003) conducted series of experiments in broilers and layers to evaluate the effects of microbial phytase on several minerals such as N, P, Cu, Zn and K and showed that the dietary treatment could reduce P excretion enormously. He had also recommended the use of selected brands of wheat bran as a source of phytase in broiler feeding. Supplementation of phytase in low non-phytate phosphorus diets improved growth performance, relative retention of nutrients and minerals in blood and bone of broilers (Singh et al., 2003). Selle et al. (2003a) demonstrated the feasibility of reducing protein, amino acids, energy and phosphorus levels in broiler diets with appropriate level of phytase supplementation to formulate least-cost rations. Phytase is also being used in combination with other enzymes to improve growth performance and nutrient digestibility in pigs fed corn-soybean meal based diets (Shim et al., 2004). Addition of phytase in isolation or in combination with xylanase replacing 0.08% dietary inorganic P increased body weight gain and feed utilization efficiency of broilers fed wheat-based diets and decreased overall mortality (Peng et al., 2003). Simultaneous addition of phytase and carbohydrases improved feed efficiency ratio, nutrient digestibility and nutritional value of soybean meal, rapeseed meal and cottonseed meal by improving ileal amino acid digestibilities in growing pigs (Shim et al., 2003). Phytase and xylanase were reported to have a synergistic effect for enhancing amino acid digestibility in broilers, which was attributed to their complementary modes of action (Selle et al., 2003b). However, Combination of phytase and glucanase had no positive effects on laying performance of leghorn hens and excretion of nitrogen and phosphorus (Jacob et al., 2000).

Aquafeeds

The degradation of phytate (*myo*-inositol hexakis phosphate, $InsP_6$) is of nutritional importance because the mineral binding strength of phytate decreases and the solubility increases when phosphate groups are removed from the inositol ring resulting in an increased bioavailability of essential dietary minerals (Lonnerdal et al., 1989; Brune et al., 1992; Sandstrom and Sandberg, 1992; Sandberg et al., 1999).

Phytate of plants are also unavailable to fish like other monogastric animals that lack intestinal phytase (Pointillart et al., 1987). Thus, primary source of phosphorus found in fish ponds is of dietary origin. The addition of microbial phytase to the diet has been reported to improve the utilization of phytate phosphorus in rainbow trout *Oncorhynchus mykiss* (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995) and common carp *Cyprinus carpio* (Schafer et al., 1995). Jackson et al. (1996) supplemented practical diets of channel catfish (*Ictalurus punctatus*) with different levels of microbial phytase and reported that bone ash, bone phosphorus, weight gain and feed consumption were higher and feed conversion ratio lower for fish fed diets supplemented with phytase as compared to control group. They also showed that the concentration of faecal phosphorus decreased linearly as phytase supplementation level increased, clearly demonstrating the efficacy of phytase in improving bioavailability of phytate phosphorus in channel catfish.

Addition of 250 units of microbial phytase per kg of diet effectively improved bioavailability of phytate phosphorus in channel catfish. Besides, fish fed diet containing dicalcium phosphate had lower bone phosphorus

content than fish fed diets containing microbial phytase justifying the possible elimination of inorganic phosphorus supplement in channel catfish diets (Li et al., 1997). A phytase of 250 FTU/kg diet applied post-pelleting could effectively replace the dicalcium phosphate supplement in channel catfish diets without affecting growth, feed efficiency or bone phosphorus deposition (Robinson et al., 2002). They also reported that use of phytase in catfish diets was more economical than inorganic phosphorus supplements.

Effects of dietary supplementation of fungal phytase at different levels on the utilization of dietary protein and minerals by channel catfish *Ictalurus punctatus* fed an all-plant-protein diet composed of soybean meal, corn and wheat middlings was studied by Yan et al. (2002). They reported that contents of ash, calcium, phosphorus and manganese were significantly higher in bone of fish fed 500 FTU or more per kg than in bone of control group, but there was no difference in dry matter digestibility or crude protein digestibility among treatment groups. They also showed that dephosphorylation of phytate occurred primarily in the stomach within 2-8 h after diet ingestion, depending on the level of phytase supplementation.

Dietary supplementation of phytase significantly improved the availability of Ca, Mg, Mn, total phosphorus, phytate phosphorus and gross energy in rainbow trout. It was found that phytase supplementation significantly increased the bioavailability of Ca, K, Mg, Cu, Mn and Zn from barley; improved the digestibility of gross energy, Ca, Mg, S, total phosphorus, Cu, Mn and Zn from canola meal; but for wheat, supplementing phytase only increased the digestibility of Mg and total phosphorus, whereas the digestibilities of K, Cu and Zn were reduced (Cheng and Hardy, 2002). The apparent digestibility coefficient of phosphorus from soybean meal based diet was significantly improved when the diet was supplemented with microbial phytase at the rate of 1,000 and 2,000 FTU/kg diet (Oliva-Teles et al., 1998).

Influence of dietary inclusion of phytase and high levels of cholecalciferol on phytate phosphorus utilization of rainbow trout was investigated by Vielma et al. (1998). It was found that phytase supplementation had a positive effect on weight gain; but higher cholecalciferol concentrations reduced the weight gain. Inclusion of phytase improved phosphorus availability as indicated by significantly higher apparent availability of phosphorus, higher bone ash and higher plasma and body phosphorus concentrations. In the same study, it was reported that dietary cholecalciferol content did not influence phosphorus utilization, instead caused higher deposition of Ca, Mg and Zn in kidney.

Phytase supplementation increased the apparent absorption of phosphorus, nitrogen (protein), ash, calcium, magnesium, copper, iron, strontium and zinc in low-ash diets containing soybean meal, but had little effect in high-ash diets containing both soybean and fishmeal (Sugiura et al., 2001). It was also reported that in high-ash diets, dietary acidification with citric acid decreased the effect of phytase, whereas in low-ash diets, acidification markedly increased the effect of the enzyme.

Vielma et al. (2000) evaluated the influence of partial replacement of fishmeal protein for soy-derived protein in large rainbow trout fed practical, high-energy diet with and without supplemental phytase. They reported that phytase supplementation did not increase the bone ash of fish fed soy diets significantly.

Hughes and Soares (1998) determined the effects of phytase on dietary phosphorus utilization by striped bass *Morone saxatilis* fed high phytate diets. Phytase supplementation was found to improve dietary phosphorus absorption and utilization; however, dry matter digestibility was not improved with added phytase, suggesting that the enzyme did not have a significant effect on protein or other organic components of the diet. Scale, vertebral and serum concentrations of phosphorus significantly differed in phytase and no-phytase groups. A clear positive effect of phytase treatment on phosphorus digestibility and retention in case of African catfish *Clarias gariepinus* fed soybean meal-based diets was seen (Van Weerd et al., 1999).

The potential for using dietary phytase to improve the nutritive value of canola protein concentrate, rich in phytic acid, was assessed by Forster et al. (1999) for rainbow trout. A positive dose-response of phytase on dietary phytate digestibility and phosphorus availability was observed and it was concluded that dietary phytase had potential to improve the nutritive value of canola protein concentrate and the availability of phytate phosphorus. Pre-treatment of soybean meal with phytase for *Oncorhynchus mykiss* diets significantly reduced the phosphorus concentrations in hatchery effluents (Cain and Garling, 1995).

The effect of phytase supplementation on apparent digestibility of four practical plant feedstuffs fed to striped bass (*Morone saxatilis*) was investigated by Papatryphon and Soares (2001). They determined the effect of the enzyme on apparent dry matter, crude protein and phosphorus digestibility of isolated soya protein, soybean meal, corn gluten meal and wheat middlings. Dry matter and crude protein digestibilities were not influenced by phytase supplementation, but phosphorus digestibility was improved by approximately 23% with the addition of 1000 units of phytase per kg dry diet for all the four feedstuffs, though it was significantly lower in case of wheat middlings.

The effects of dietary phytase treatment (0 or 1,000 U phytase/kg diet) and increasing feeding rates (0.5, 1.0, 1.5, 2.0, 2.5% BW and satiation) on growth, body composition,

nutrient digestibility, retention and phosphorus release in effluent water were studied in rainbow trout (Lanari et al., 1998) with diets containing 33% soybean meal. The apparent digestibility of phosphorus increased from 58.6 to 68.1% ($p<0.01$) in the treatment group. Daily weight gain and feed conversion ratio improved in fish fed the phytase supplemented diet with feeding rates greater than 1.5%. Body protein, ash and phosphorus contents tended to be lower in fish fed no-phytase diet, and they were shown to decrease with increasing feeding rates upto 2.0% BW/day and then reached a plateau. The phosphorus released into the environment was higher for fish fed no-phytase diet and increased with increasing feeding level.

Availability of protein, phosphorus and other elements from fishmeal, soy-protein concentrate and phytase-treated soy-protein concentrate-based diets to Atlantic salmon, *Salmo salar* was found to be different (Storebakken et al., 1998). Phytase treatment of the soy concentrate resulted in a P-solubility of 64% (10 min) and 70% (60 min), which was only 5% (10 min) and 7% (60 min) for the untreated diet. Phytase treatment reduced the concentration of phytic acid from 8 g kg^{-1} to less than 0.5 g kg^{-1}. Protein digestibility and retention were improved by the phytase treatment. Replacement of fishmeal with untreated soy concentrate resulted in lowered whole body ash and Ca, Mg, P, Sr and Zn, and a higher concentration of Mn.

Supplementation of phytase at 500 FTU/kg significantly improved weight gain, protein efficiency, apparent net protein utilization and feed conversion efficiency in *Pangasius pangasius* fingerlings (Debnath et al., 2005a). Apparent dry matter and protein digestibility were also significantly ($p<0.01$) higher at a minimum supplementation of 500 FTU/kg or higher. Liver alkaline phosphatase activity increased significantly in phytase-supplemented groups, which could be correlated, with the increase in available phosphorus concentration.

Studies on the mineral status in *P. pangasius* fingerlings with respect to dietary phytase (Debnath et al., 2005b) showed improved ($p<0.05$) apparent absorption of calcium (Ca), phosphorus (P), magnesium (Mg), manganese (Mn), zinc (Zn), iron (Fe), potassium (K), copper (Cu) and cobalt (Co) in the phytase-supplemented groups compared to the control group. Faecal ash and P contents were significantly ($p<0.05$) higher in the control than the phytase-supplemented groups. Whole body contents of Ca, P, Zn, Fe, Cu and Co were significantly ($p<0.05$) improved by phytase supplementation. Concentrations of bone Ca, P, K, Cu and Co were significantly higher in phytase-supplemented groups. Bone ash also showed increasing trend upto a level of 500 FTU/kg diet.

Phytase in feed can reduce or sometimes eliminate the necessity of mineral supplementation, which in turn can reduce the cost of feeds (Baruah et al., 2004). 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 this enzyme. Addition of organic acid along with phytase, especially in agastric fishes, is of special interest, and needs serious attention by the aquaculture nutritionist. As in animal farming, phytase has also been used in combination with other enzymes like cellulase in the diet of *Labeo rohita* fingerlings (Xavier, 2005).

EFFECT OF DIETARY PHYTASE ON

Bioavailability of phosphorus

Phosphorus is an important mineral for fish. Although fish can absorb soluble phosphorus through the skin, fins and gills, the concentration of phosphorus in fresh and seawater is low (Tacon, 1990; NRC, 1993). Therefore, the phosphorus requirement for fish is dependent on the feed.

Hughes and Soares (1998) conducted an experiment to study the effect of phytase on dietary phosphorus utilization by striped bass *Morone saxalitilis* fed plant based diets containing inorganic P (positive control), phytase at 600, 1,200 and 2,400 FTU/kg, and control diets with no phytase or inorganic P (negative control). A significant difference ($p<0.05$) in scale-P was observed in 2,400 FTU/kg treated group when compared with the negative control and 600 FTU/kg group. There was also significant difference in vertebral-P between the 2,400 FTU/kg treatment and the negative control group. Similar changes were observed in vertebral-P concentrations. No significant difference was detected in vertebral-P concentration between the positive control and 2,400 FTU/kg treatment groups. Again, apparent P digestibility coefficients were significantly higher in phytase treated groups than no phytase or positive control groups.

Forster et al. (1999) reported that dietary phytase improved the nutritive value of canola protein concentrate and decreased P output in rainbow trout reared at 11°C. Similar reports have been reported in different species like rainbow trout (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995), channel catfish (Jackson et al., 1996; Li et al., 1997), African catfish (Van Weerd et al., 1999), common carp (Schafer et al., 1995) and *Pangasius pangasius* (Debnath et al., 2005b). Robinson et al. (2002) reported that 250 FTU per kg diet effectively replaced 0.75% dicalcium phosphate supplement in channel catfish diets without affecting growth, feed efficiency or bone P deposition. In all above experiments, it was found that microbial phytase was effective in enhancing the bioavailability of P considerably, thereby reducing the faecal-P.

Bioavailability of other nutrients

Phytate-mineral complexes may be formed with various di- and trivalent cations, as well as with proteins (Wise,

1980). For example, Ca-bound phytate increases chelation with trace minerals especially with zinc to form co-precipitates that make the Zn unavailable to animals. Phytase added to diets improves the bioavailability of copper and zinc in pigs (Pallauf et al., 1992; Lei et al., 1993; Adeola, 1995; Adeola et al., 1995) and poultry (Thiel et al., 1993; Yi et al., 1996). Microbial phytase also improves the apparent absorption of magnesium, zinc, copper and iron in pigs. Similar results were also reported in fishes.

Cheng and Hardy (2002) conducted an experiment by supplementing microbial phytase in barley, canola meal, wheat and wheat middlings at 500 FTU/kg diet. Each of the four ingredients was added at 30% in a reference diet. It was observed that the phytase supplementation significantly increased the digestibility of Ca, K, Mg, Cu, Mn and Zn in barley. It also improved the digestibility of GE (gross energy), Ca, Mg, S, Cu, Mn, total-P and Zn in Canola meal. For wheat, supplementing phytase only increased the digestibility of Mg and total-P, whereas the digestibility of K, Cu and Zn were reduced. However, for the wheat middling by products, wheat middlings, phytase supplementation significantly increased the digestibilities of K, Mg, S and total-P.

Yan et al. (2002) conducted a feeding trial to quantify the effects of phytase at 0, 500, 1,000, 2,000, 4,000 and 8,000 FTU/ kg diet on utilization of dietary protein and minerals by fingerlings Channel catfish, *Ictalurus punctatus* fed an all plant protein diet composed of soybean meal, corn and wheat middlings. After 14 week, it was found that fish fed phytase supplements had higher ($p \leq 0.05$) concentration of ash, Ca, P and Mn in bone than fish fed the unsupplemented diet. Concentration of these minerals in bone did not differ significantly in phytase supplemented groups. Bone Mg levels did not differ among fish fed ≥500 FTU/kg. The amount of Zn in bone of fish fed 8,000 FTU/kg was significantly higher than that in 0 or 500 FTU/kg.

Oliva-Teles et al. (1998) had reported that fish fed phytase supplemented diets had higher concentration of ash, Ca, P and Mg in their bone than the fish fed control diet. They further delineated that phytase supplementation at 500 units/kg diet was sufficient to improve the retention of Ca, P and Mg significantly by catfishes fed an all-plant-protein diet. Vielma et al. (1998) had also found an increase in the concentration of minerals like Mg, P, Ca, Mn and Zn both in plasma**,** bone and whole body. These results are in agreement with those reported by Sugiura et al. (2001), Cheng and Hardy (2002), Debnath et al. (2005b).

Protein digestibility

As mentioned earlier, phytase chelates with protein beside other nutrients. Phytase treatment of soy-protein concentrate was found to improve the protein digestibility and retention (Storebakken et al., 1998). Microbial phytase supplementation in the diet of *Pangasius pangasius* also increases the apparent net protein utilization (Debnath et al., 2005a). He further concluded that apparent protein digestibility of the diets was significantly ($p<0.01$) improved by enzyme supplementation, while without enzyme supplemented groups showed a low protein digestibility confirming the established properties of phytate to form phytate protein complexes that are resistant to proteolytic digestion (Cheryan, 1980). In addition, phytate binds trypsin *in vitro* and thus reduces protein digestibility as reported by Singh and Krikorian (1982). Some authors had also reported improved digestibility of dry matter (Papatryphon et al., 1999) beside crude protein (Storebakken et al., 1998) in species other than catfish. The negative effect of phytate on protein utilization in fish has been observed by many workers (Singh and Krikorian, 1982; Spinelli et al., 1983). Phytase supplementation in plant based practical diets has been reported to increase (Storebakken et al., 1998; Vielma et al., 1998), remain unchanged (Lanari et al., 1998) or even decrease (Teskeredzic et al., 1995) the protein digestibility. In poultry also phytase improves the protein and amino acid utilization through breakdown of phytin-protein complexes (Kornegay, 1995). But in fish, the situation is somewhat ambiguous. This may be due to the presence or absence of a stomach in some species as phytase activity is pH specific. However, Yan et al. (2002) reported that phytase supplementation at levels of 8,000 FTU/kg diet did not increase weight gain or improve dietary protein utilization of channel catfish fed an all-plant-protein diet. In tilapia (*Oreochromis niloticus*) fed diets based on plant protein (soybean meal and canola meal) as the only protein source and supplemented with phytase, digestibility of CP was improved from 89.6% in the control diet to an average of 93% in phytase treated groups. These shows that phytase has a positive effect on protein digestibility (Heindl, 2002).

Growth performance of fish

Alvi (1994) concluded that both live weight gain % and specific growth rate of Indian major carp *Labeo rohita* significantly decreased when dietary phytic acid was included above 1%. Similar effect of phytic acid on growth performance and body composition of *Cirrhina mrigala* fry was observed by Usmani and Jafri (2002). Richardson et al. (1985) reported that Chinook salmon, *Oncorhynchus tshawytscha* fed semi-purified diets containing various levels of Ca, P, Zn and sodium phosphate with a high dietary phytic acid (2.58%) exhibited depressed growth. But the growth performance was increased when microbial phytase was incorporated in the diets. There was an increase in weight gain of channel catfish fed phytase supplemented

diets containing only plant protein or plant and animal protein sources (Jackson et al., 1996). The weight gain and feed consumption were found to be increased by 23.52 and 11.59%, respectively compared to the control. Similar performance was also observed in *Pangasius pangasius* (Debnath et al., 2005a), African catfish *Clarias gariepinus* (Van Weerd et al., 1999). Beside these, several researchers also found the effect of phytase treatment on weight gain but in most of these experiments fish were mostly fed *ad libitum* (Channel catfish, Robinson et al., 1996; Li and Robinson, 1997; Rainbow trout, Rodehutscord, 1995; Rodehutscord and Pfeffer, 1995) and part of the improved weight gain can be explained by an increase feed intake. Conversely, Cain and Garling (1995) and Schafer et al. (1995) found a phytase induced increase in growth rate of rainbow trout and common carp respectively, fed restricted rations. Better performance of fish fed phytase supplemented feed implies that either the P requirement along with other nutrients was met or there is another positive effect of phytase on performance.

Water quality

Pollution due to animal farming and production has become the major concern of environmentalists throughout the world. Paik (2001) critically reviewed the various aspects of pollution and emphasized on dietary phytase supplementation for curtailing nitrogen and phosphorus enrichment of the ecosystems. The environmental impact of aquacultural operations is increasingly under review and environmental lobbyists as well as governments are putting restrictions on this industry. From freshwater aquaculture to coastal marine operations, many farmers are facing increasing pressure concerning the discharges into the surrounding ecosystems. Eutrophication occurs because of these discharges in general or because of loading of phosphorus into the environment in particular. Phosphorus in the feed ingredients occurs in a number of forms. In ingredients of animal origin, it occurs in the inorganic form as well as phosphate complexes of protein, lipid and carbohydrate. These forms are usable by fish. In the contrary, plant feedstuffs contain phophorus in the form phytate, which is generally unavailable to finfish and monogastric animals as mentioned earlier. It comes out through the excreta to the water which is enzymatically cleaved by soil and water-borne microorganisms thereby releasing P into the water bodies causing eutrophication (Bali and Satyanarayana, 2001), which in turn results in oxygen depletion due to excessive algal growth. Microbial phytase supplemented into the diet can overcome this problem. It makes the chelated-P available to fish and hence there is less faecal-P excretion and thereby reduces environmental pollution. Li and Robinson (1997) reported in juvenile catfish, *Ictaurus punctatus* that microbial phytase supplementation in diets reduces the excretion of faecal-P by about 60%. Beside these, many studies suggests potential environmental benefits through 30 to 40% reduction in P excretion (Omogbenigum et al., 2003).

This was first used in Netherlands around 1994, following legislation, which called for drastic cuts in the levels of phosphorus (phosphates) in animal manure. If phytate is not digested, the only other way to provide the animal including fish with phosphate is by supplementation of minerals (calcium phosphate). This itself is poorly absorbed and a lot of it passes into the faeces. The environmental benefits of using this enzyme are: i) less mineral supplement is required and therefore less inorganic phosphate is required in the diet, ii) less organic phosphate (phytic acid) is excreted thereby less phosphate loads on the environment in areas of intensive animal production.

Synergistic effects of phytase and organic acids

Numerous experiments have shown that microbial phytase can partly render phytate-P available to animals (Cromwell et al., 1995; Jongbloed et al., 1996). With supplemental phytase, the availability of P present in soybean products is in the range of 50-60% (Vielma et al., 1998). This implies that 40-50% of the phytate-P is still unavailable. Jongbloed (1987) reviewed that lowered intestinal pH increases the solubility of P and phytate and improves P absorption in the small intestine. In addition to their effect on intestinal pH, supplementary organic acids can also bind various cations along the intestine and may act as chelating agents (Ravindran and Kornegay, 1993), resulting in increased intestinal absorption of minerals

Several studies have shown that the optimum pH of microbial phytase occurs at two peaks: the highest being at pH 5.0 to 5.5 and the second highest being at 2.5 (Simons et al., 1990). Gastrointestinal acidity in case of stomach less fish is also not favourable for efficient hydrolysis of phytates by phytases. But addition of acidifiers, such as citric acid, fumaric acid, formic acid etc is known to lower diet acidity. So, lowering the dietary pH might reduce the pH of the stomach digesta and thereby increase the effectiveness of microbial phytase. Improving the efficiency of phytase could lead to reduced feed costs and to a greater use of phytase, which would be of environmental importance.

In a recent study by Baruah (2004), it was evident that supplementation of citric acid (3%) and/or phytase (500 FTU/kg) improved weight gain, feed and protein efficiency ratio, and mineral bioavailability in *Labeo rohita* fingerlings.

LIMITATIONS IN THE USE OF EXOGENOUS PHYTASE IN FEED

There is lack of information on the site at which the

enzyme acts on the substrate within the gastrointestinal tract. Phytase is reported to be have optimum activity in two pH ranges i.e., alkaline and acidic pH. The pH of the gastrointestinal tract of fish depends on the presence or absence of a stomach. Hence, phytase activity is species specific. The use of phytase as a feed additive is limited due to several factors like cost, inactivation at high temperatures required for pelleting (>80°C), loss of activity during storage and narrow optimum pH range. The inherent phytase content of various plant ingredients needs to be studied in detail. Activity of natural phytase in certain plant feedstuffs is high enough to be considered in feed formulation (Paik, 2003). This phytase may not hydrolyse phytate in the gastrointestinal tract of fish. Moreover, wide diversity in the feeding habits, presence or absence of functional stomach in fish can greatly affect the dietary requirement of phytase. Hence, lot of research data are required to decide the optimum dose of dietary phytase for different species.

PRESENT NEED

Beneficial effects of feed enzymes cannot be denied, but usefulness of such enzymes will increase when new forms of enzymes with the following properties are available: (i) higher activities under normal conditions (ii) high heat resistance, (iii) cheap to produce, (iv) long storage life under ambient conditions, (v) resistant to proteolysis, (vi) activity at broad pH range. Nature of interactions of phytase with different dietary constituents need to be established. For example, whether enhancement of phosphorus digestibility due to phytase supplementation has any effect on other minerals and nutrients is to be clearly understood. No studies have been carried out on the effects of enzyme supplementation on various physiological and endocrine parameters like secretion of other enzymes, bile salts, on the immune response, hormone levels including growth hormone, thyroid hormone, insulin etc. Comprehensive studies have to be carried out to characterize these effects.

FUTURE RESEARCH THRUST

Monogastric animals cannot produce phytase endogenously. Development of transgenic monogastric animals which are able to produce phytase and hydrolyse phytate would be of immense benefit to livestock and fish farmers. In the line of production of canola seed with improved phytase activity, research should be able to develop transgenic soybean, cotton, sunflower and other grain and cereal plants, which have potential uses in fish feed. Development of genetically modified grains and cereals with reduced phytic acid content can also serve the purpose. Biotechnological tools should be applied to develop thermo-resistant phytase by genetically modified microorganisms. Similarly, phytases of broad pH optima should be developed, which will be effective for all types of fishes.

CONCLUSION

Expanded aquaculture production will require more fish feed, which will in turn require higher quantities of alternate protein sources to substitute fishmeal. These proteins will be supplied from a variety of sources, most of which will require special processing or enzyme supplementation to explore their full potential. Phytases are being recognized for their beneficial environmental role in reducing the phosphorus levels in manure and minimizing the need to supplement phosphorus in diets. It improves the nutritional value of feeds by hydrolysing phytic acid, which acts as an anti-nutritional factor present in several plant based feed ingredients. Increasing the use of phytase in aquaculture offers a tremendous opportunity in order to allow the use of low-cost plant meals. The research on phytase is now being directed to improve thermostability, pH optima and substrate specificity. Industrial scale production of phytase should be focussed for its cost-effective usage.

From our experience, it seems that use of phytase in herbivorous fish diet is very encouraging. However, studies are needed to establish optimal dose of phytase for several other species of fish with varying feeding habits and different life stages. At present phytase constitute about 20% of the total enzyme use in the livestock or allied sector, which is expected to increase many fold in near future due to increasing research output. Intensification of livestock or aquaculture farming without considering the phosphorus discharge may threaten the environment in long run. Hence, dietary phytase may be the right approach in this regard.

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