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Review

Application of microbial phytase in fish feed

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Abstract

Phytate is the main storage form of phosphorus (P) in many plants, but phytate-bound P is not available to monogastric or agastric fish animals. Phytase, an enzyme specific to hydrolyze indigestible phytate, has been increasingly used in fish feed during the past two decades, mainly in response to heightened concerns over P pollution to the aquatic environment. Since global phosphate reserves are not renewable, phytate-P as an alternative and economical P source can be effectively converted to available-P by phytase. The capability of this enzyme to enhance bioavailability of P and reduce P load is well documented. Phytase supplementation also leads to improved availability of other minerals and trace elements. Nevertheless, there is still no consistent conclusion that phytase could enhance protein and energy utilization. Studies in amino acid digestibility after phytase supplement are mutative and the underlying mechanisms have not been fully understood. Because phytase is very sensitive to pH and temperature, the utilization of phytase in fish feed is still on its first stage compared with that of in poultry and swine feed. A wide variety of phytases were discovered and characterized in order to find the optimum enzyme which is stable in application, resistant against high temperatures, dust-free, and easy to handle. Initial steps to produce phytase in transgenic plants and fish animals are also undertaken. In this review, the authors focus on comparing properties of phytase from different sources, examining the effects of phytase on P utilization and aquatic environment pollution, meanwhile providing commercial potentiality and impact factors of phytase utilization in fish feed.

Keywords: Microbial phytase; Fish feed; Phytate; Phosphorus

Contents

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1. Introduction

To developing sustainable and environmentally friendly aquaculture, various plant proteins such as soybean meal and canola meal are considered promising protein sources as substitutes for fishmeal in fish feed [1]. However, one of the major problems associated with the use of plant proteins in fish feed is the presence of anti-nutritional factors, such as phytate (*myo*-inositol-1,2,3,4,5,6-hexakisphosphates), which is the main storage form of P. Up to 80% of the total P content in plants may be present in the form of phytate and is practically not available for monogastric or agastric aquatic animals [1] due to lack of intestinal phytases for efficient phytate hydrolysis during digestion (Table 1) [2]. Therefore, most of the phytate-P ends up being excreted into the water which may cause pollution in terms of algal growth [3]. Besides, the absorption and bioavailability of indispensable minerals such as calcium, zinc, magnesium, and iron may also be negatively affected by forming insoluble chelate complexes with phytate [4]. Phytate can also combine protein and vitamin as insoluble complexes to reduce their utilization efficiency, activity, and digestibility [5,6]. In addition, some vitro studies have shown that phytate–protein complexes are less attacked by proteolytic enzymes [7], even some enzymes such as pepsin, amylopsin, and amylase would be inhibited by phytate. Furthermore, phytate may interfere with the digestibility of lipid and starch [8]. It was also reported that the growth and feed conversion efficiency in commonly cultured fish species, such as carps, tilapias, trout and salmons, were negatively affected by the phytate in the diets [9].

Phytases, a group of enzymes chemically known as *myo*-inositol-hexaphosphate phosphohydrolase, are ideal approaches specific to hydrolyze undigestable phytate in plants. Although phytase activity was first detected in rice bran nearly a century ago, attempts to develop a phytase feed enzyme did not initiate until 1962 in North America [10]. Warden and Schaible were the first to show that exogenous phytase enhances phytate-P utilisation and bone mineralisation in broiler chicks [11]. However, before 1990s, addition of phytase has been mainly reported to improve the utilization of plant P in poultry and swine, while less used in fish diets because of lacking research information and manufacturing restraints [12].

The first commercial phytase products derived from *Aspergillus niger* with the capacity to release phytate-bound P and reduce P excretion, was introduced into market in 1991 [13]. After mid-1990s, more and more studies about the effects of supplemental phytase on nutrient utilization or growth of fish have been started in common aquaculture species such as rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio* L.), channel catfish (*Ictalurus punctatus*), salmon (*Salmo salar*), stripped bass (*Morone saxatilis*), Nile tilapia (*Oreochromis niloticus*) [3,14–19]. Phytase has been utilized by spraying onto pellets [20], pre-treating or dephytinizing feedstuffs before pelleted [21,22]. Various parameters to evaluate phytase effects have been used including nutrients digestibility, nutrients retention and fish growth performance.

Currently, researches are mainly focus on phytase effects on digestive systems in different fish growth phases, the dose–response study, specific kinds of phytase for distinct fish species and the most efficient ways of supplement [23–25]. Besides, the addition of organic acid along with phytase, especially in agastric fishes, is of special interest, and gains serious attention [3,26]. It is well documented that the use of microbial phytase in fish feed can enhance the bio-availability of phytate-bound P and nitrogen and thus less P discharged into the aquatic environment [25,27]. Therefore, phytase is increasingly considered as the dispensable additive for a cost-effective and environmentally friendly fish feed formula.

Though the role of phytase supplementation has been well proved in pigs [28], its use in fish feed is still in an early stage. Many fundamental issues relating to phytate and phytase remain to be elucidated. Phytase activity is largely dependent on many factors such as appropriate pH and temperature during processing. Since fish are either monogastric or agastric, the effects of phytase on fish growth and nutrient utilization vary from species to species [3]. In this article, the authors focus on reviewing the available literatures on the use of microbial phytase in fish feed in relation to P utilization and aquatic environment pollution. The specific objective of the current review is to clarify the current understanding of phytase in fish feed, locate gaps and constraints of recent research, and provide topics for potentially instructive studies.

Table 1
Phytate contents in plants or plant products (adjusted from [1,13])

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

2. Phytase enzyme

Phytases are widespread in nature because they can be found in animals, plants, and microorganisms. Generally, phytase activity of animals is negligible compared to their plant and microbial counterparts [29]. Most of the scientific work has been done on microbial phytases, especially on those originating from filamentous fungi such as *Aspergillus ficuum*, *Mucor piriformis* and *Cladosporium* species [30]. Although some plants such as wheat, and barley are rich in intrinsic phytase, because of a narrower pH spectrum of activity, their phytase activity is less effective than microbial phytases. The stability of most plant phytases decreased dramatically at pH values below 4 and above 7.5, while the majority of the corresponding microbial enzymes are stable even at pH values above 8.0 and below 3.0 [31]. Besides, plant phytase is more heat labile and its activity is reduced or even eliminated in steam-pelleted diets [32]. The majority of the plant phytases are irreversibly inactivated at temperatures above 70 °C within minutes, whereas most of the corresponding microbial enzymes retain significant activity even after prolonged incubation times [33]. Additionally, the bio-efficacy of plant phytases was only 40% compared to microbial phytases [34]. Therefore, broad pH optima, thermal stability as well as higher specific activity of microbial phytases make it more favorable for an application in fish feed.

Natuphos was the first commercially available phytase in 1991, from a genetically modified *A. niger* strain. Since then, phytase activity is defined as *fytase* units (FTU or U), where one FTU is defined as the quantity of enzyme that liberates 1 micromol of inorganic-P per minute from 0.0015 mol/l sodium phytate at pH 5.5, and 37 °C [35]. This definition provides a useful measure of quantity of phytase activity and represents a simple bench mark measurement. In the past 15 years, phytate-degrading enzymes of yeasts [36] such as *Schwanniomyces occidentalis* [37], *Pichia anomala* [38], *Arxula adeninivorans* [39], gram-negative bacteria such as *Escherichia coli* [40], *Pseudomonas* species [41], *Klebsiella* species [42,43], and gram-positive bacteria such as various *Bacillus* species [44,45] were identified and characterized (Table 2). According to the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB), phytase feed enzymes fall into two categories depending on the site where the hydrolysis of the phytate molecule is initiated. 3-Phytase (EC 3.1.3.8) preferentially liberates the P moiety at position C3, whereas 6-phytase (EC 3.1.3.26) commences at position C6 of the *myo*-inositol hexaphosphate ring [13]. The phytate-degrading enzymes also can be divided into two types based upon their optimal pH. These are the acid phytate-degrading enzymes with a pH optimum around 5.0, and the alkaline phytate-degrading enzymes with a pH optimum around 8.0 [46]. Most of the phytate-degrading enzymes belong to acid type. However, it has to be taken into account that microbial phytases of different source can differ in their bio-efficacy per unit.

Several distinct microbial phytase products are now commercially available. Phytase feed enzymes may be included in fish feed as powder, granulate or liquids, via post-pelleting or pre-treatment to avoid thermo-stability problems at high pelleting temperatures (>80 °C). Phytases produced on commercial scale are either derived from fungal strains mutated or by using recombinant DNA technology. The three commonly used phytase feed enzymes are derived from *A. niger* which is a 3-phytase, *Peniophora lycii* and *E. coli*, which are 6-phytases. The fungal phytase has the higher thermo-stability and lower optimum pH range than the bacterial phytase [47]. In general, different

Table 2
Comparison of microbial phytases from different sources[a]

Phytase source	Phytase activity (U/mg) (37 °C)	PH optimum	Temperature optimum (°C)
Fungi			
Aspergillus caespitosus	NA[b]	5.5	80
Aspergillus fumigatus	23–28	5.0–6.0	60
Aspergillus niger	50–103	5.0–5.5	55–58
Aspergillus oryzae	11	5.5	50
Aspergillus terreus	142–196	5.0–5.5	70
Penicillium simplicissimum	3	4	55
Peniophora lycii	1080	5.5	58
Thermomyces lanuginosus	110	6	65
Bacteria			
Bacillus amyloliquefaciens	20	7.0–8.0	70
Bacillus subtilis	9.0–15	6.5–7.5	55–60
Citrobacter braakii	3457	4	50
Escherichia coli	811–1800	4.5	55–60
Klebsiella terrigena	205	5	58
Lactobacillus sanfranciscensis	NA	4	50
Pantoea agglomerans	23	4.5	60
Pseudomonas syringae	769	5.5	40
Yeasts			
Candida krusei	1210	4.6	40
Pichia anomala	NA[b]	4	60

[a] *Source*: [31].
[b] NA: not available.

Table 3
Commercial production information of microbial phytases[a]

Company	Country	Phytase source	Production strain	Trademark
AB Enzymes	Germany	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lycii*	*A. oryzae*	Bio-Feed Phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*P. simplicissimum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*Aspergillus awamori*	*T. reesei*	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme® Roxazyme®

[a] *Source*: [30,48].

sources of phytases have different characteristics, which must be considered before applied in fish diets.

Table 3 summarizes published phytase properties and commercial information from different authorized phytase companies. Phytase activities are determined on the basis of inorganic-P released from phytate. Due to obvious differences with respect to cultivation conditions and slight differences with respect to phytase assay conditions, a comprehensive comparison and evaluation of the production strains is difficult. According to the market research report [48], phytases from Europe and North America are more competitive than phytases produced by Asian companies. The reason is that the former has higher activity per unit. Usually, the activity of powder phytase from Europe and North America can reach 40,000–4,000,000 U/g. Their lipid phytase activity is over than 40,000,000 U/ml. In addition, these phytases have wider pH range and temperature tolerance than those from Asian companies [26]. For example, Natupohs enzyme produced by BASF Company can maintain 75% activity under 75 °C for 15 min [44]. Allzyme phytase produced by Alltech Company can keep more than 60% of activity when pH reaches above 6.5 or below 2.5. The market prices of phytases from the former companies (approximately $12.5–15 per kilogram) are also more stable than those from the later [26]. In contrast, the phytases from many Asian companies are newly developed, such as in China, Japan and South Korea. Their formulation methods are still immature. The phytase activity is usually around 500–5000 U/g or 5000–50,000 U/ml. And the prices of these phytase fluctuate around $3–10 per kilogram [44].

3. Effects of phytase application in fish feed

3.1. Effect of phytase on bioavailability of P

P is an important constituent of nucleic acids and cell membranes, a major constituent of the structural components of skeletal tissues, and is directly involved in all energy-producing cellular reactions [1]. Thus, it is an essential nutrient for growth, skeletal development [49] and reproduction of fish [50]. However, the phosphate uptake from water is negligible in fish and dietary sources are more important than water to fulfill the P requirement of fish. Meanwhile, P is a critical pollutant in the aquatic environment. Excessive P concentrations are the most common cause of eutrophication of rivers, lakes and reservoirs [51]. The inclusion of microbial phytases in fish diets was prompted by the need to reduce P excretion and its loss into the environment, where P pollution is a hazard to water quality.

Many studies have demonstrated that phytase supplementation makes the chelated phytate-P available to fish [20,52,53]. Schafer and Koppe [14] reported that 20% and 40% of phytate-P can be released by the phytase addition of 500 and 1000 U/kg respectively in carps fed with soybean meal based diet. Yu and Wang [54] found that in soybean meal based diet for crucian carp, 60% and 80% of phytate-P can be released by the phytase addition of 500 and 1000 U/kg, respectively. Phytate-P is converted to available-P by phytase which can be utilized directly by aquatic animals. Thus, the utilization rate of P can be considerably enhanced by phytase. Apparent P digestibility and bone mineralization are considered as the most sensitive criteria for assessing the influence of phytase on P utilization. The capacity of phytase to increase total-P digestibility in fish has been frequently demonstrated. Sugiura et al. [6] observed that in rainbow trout fed diet containing 50% soybean meal with 4.21 g/kg diet total-P, the apparent digestibility coefficients (ACD) of P in diet containing pretreated soybean meal (200 U phytase/kg dry soybean meal) reached 93%. However, when the total-P level increased to 14.7 g/kg diet, the ADC of P in diet containing 1000 U phytase/kg diet was only 62%. Sajjadi and Carter [55] reported that after phytase supplementation, the P digestibility was significantly higher than the phytase control in Atlantic salmon study, similar to other salmonid studies [20,27]. However, it should be known that using an excess amount of P leads to lower digestibility. P digestibility peaks at approximately the dietary requirement level and then declines with increasing dietary P. The positive effect of phytase supplementation on ADC of P has also been observed in tilapias [3] fed soybean meal based diets.

Since supplementation of phytase can improve the ADC of P in soybean meal or canola meal-based diets, then it is possible to improve the P retention of diets and reduce the P discharge into water that was considered as one of the main pollution elements in water environment. Jackson et al. [2] reported that phytase

could promote the P deposition in fish bone and lead to 33% decrease of P load in channel catfish. Sugiura [6] reported that in low-ash diets of rainbow trout, the apparent absorption of P increased accord with the level of phytase added into to the diets, from 27% (no phytase added) up to 90–93% (phytase added, 4000 U/kg diet). The similar results that addition of phytase to plant-based fish diets improves the utilization of phytate-P by 25–55% and decreases the total-P load to the environment by 30–50% were found in other fish species such as tilapias [57], salmons [21], rainbow trout [27,56] and carps [14,58].

Many resent studies are designed to establish the P equivalency or replacement value of microbial phytases in fish diets. As global phosphate reserves are not renewable, phytase can save P resources by converting phytate P into available P and substituting for the inorganic-P in fish feed [59,60]. Graded amounts of an inorganic P source or graded phytase inclusion levels are incorporated into available P-deficient basal diets and P replacement values are calculated from regression equations best describing responses of selected parameters. Frequently, the parameters monitored are body weight gain and percentage bone ash, as both are considered to be sensitive indicators of P availability [61,62]. Schafer and Koppe [14] concluded that in common carp diets mainly based on plant proteins, supplementation with phytase at levels of either 500 or 1000 U/kg diet can replace 1.9 g P from dicalcium phosphate. It was reported that the effect of 1000 U/kg phytase addition is equivalent to that of 0.85–1.28% monocalcium phosphate [63]; also phytase replacement can avoid the toxin compromise of fluorin brought by inorganic-P input, thus enhancing the security of feed. However, these data must be verified in trials conducted in ponds, prior to recommending removal of supplemental P.

3.2. Impact of exogenous phytase on fish growth performance

Investigations into the effects of various microbial phytases on growth performance of different fish species have been conducted. Generally, growth improvements were observed in the studies that used diets entirely or almost entirely based on plant protein sources. However, growth performance responses to phytase supplementation showed somewhat inconsistent.

Many studies reported that the addition of phytase to P inadequate diets has been shown to enhance growth performance. An increase of weight gain has been reported in channel catfish fed phytase supplemented diets containing only plant protein or a combination of plant and animal protein sources [2]. In the study of Li and Robinson [15], fish fed the diets containing 250 units of microbial phytase per kg or above consumed more feed, gained more weight, and had a lower feed conversion ratio in comparison to fish fed the basal diet containing no microbial phytase. Yu and Wang [54] reported that phytase addition of 1000 units per kg diet improved average weight gain of 25%. Positive results of phytase addition were also reported in common carp [14], African catfish [16], stripped bass [4], rainbow trout [22], Atlantic salmon [55] and korean rockfish *Sebastes schlegeli* [60]. A significant increase in energy retention was observed by Debnath [25] because of phytase supplementation. This indicated better utilization of nutrients in the presence of dietary microbial phytase, in agreement with Forster [17], who reported an increased apparent energy digestibility from canola protein concentrate with phytase supplementation. Increase in growth because of enzyme supplementation can explain the improved energy retention. Survival was not significantly affected by enzyme supplementation, indicating no adverse effect of supplementary enzyme, in agreement with Robinson et al. [19].

In contrast, no significant differences in feed intake and growth performance were observed in other studies. It was found that no improvement on growth was observed between diets with and without phytase in pond-raised channel catfish [19]. Further research needs to confirm whether this conclusion indicates that the function of phytase relates to diet formulation, fish size, development status of fish digestive system, or the content of endogenous phytase in fish digestive system.

3.3. Impact of phytase on protein availability

Phytase effectively increases phosphorus availability of soybean meal, but less information is available on protein and amino acid utilization. Better protein economy of plant-based aquafeeds by phytase would increase the interest of the feed industry towards this relatively new feed supplement. In pigs, phytase was reported to improve protein and amino acid utilization through breakdown of phytin–protein complexes [64]. In fish, however, the results are somewhat controversial. Variations in the outcome of different authors may be attributed to variation in phytic acid content in different feedstuffs, species used and various other inherent characteristics of feed ingredients, or probably due to the presence or absence of the stomach in different fish species, as phytase activity is pH specific [53].

Phytase supplementation in plant-based practical diets has been reported to increase protein digestibility in some studies. Vielma [65] reported that positive effects of supplemented phytase on protein digestibility in in rainbow trout fed with semi-purified diet based on soybean meal; however, lysine utilization was not significantly increased. Cheng and Hardy [66] found that phytase supplementation in expelled soybeans increased ADC of crude protein significantly compared to ADC in raw soybeans. Debnath [25] reported that apparent protein digestibility of the diets was significantly improved by enzyme supplementation, while the control group showed a low digestibility, confirming the established properties of phytate to form phytate–protein complexes that are resistant to proteolytic digestion. In his study, the increase in protein digestibility compared with the control was maximum to 500 FTU/kg, after which no further improvement was evident. Similar results were also found fish species such as carps [23] and rainbow trout [6,17].

In contrast, Papatryphon and Soares [4] could not report any improvement in apparent protein digestibility in striped bass even up to a level of 1000 FTU/kg. Similarly, Riche et al. [67] reported that no differences were detected in ACD of protein between tilapia diets with and without phytase, neither by Storebakken et al. [21] and Lanari et al. [56] in salmonids. Riche et al. [67] further concluded that the available methionine and lysine

decreased with increasing incorporation of phytase pretreated soybean meal due to the removal of phytate which may increase the efficiency of other anti-nutritional factors and protect amino acids from degradation, or decrease leaching of water soluble components. Thus, at present, the mechanisms underlying the protein-associated responses to added phytase remain largely speculative and further research is required.

3.4. Effect of phytase on bioavailability of other nutrients

Phytate also can chelate with other minerals to decrease their bioavailability to fish. Phytase supplementation can hydrolyze phytate and increase the concentration of minerals like magnesium, calcium, manganese, and zinc in plasma, bone and the whole body [68]. Channel catfish fed phytase-supplemented diets had higher concentrations of ash, calcium, phosphorus and manganese in their bones than the fish fed on a control diet [69]. Supplement of phytase in the diets of rainbow trout could improve the apparent digestibility coefficient of minerals except copper and iron [24]. Similar results were reported that treatment of plant products with phytase can increase the availability of minerals and the enzyme has been used to improve dietary mineral retention in salmonids [20,21,68], common carp [14], stripped bass [4,70], and Nile tilapia [3].

Thus, the addition of phytase to a plant-based diet increased the bioavailability of minerals, thereby increasing bone mineralization. However, there was a further increase in the effectiveness of phytase as a result of the addition of citric acid in low protein diet. Radcliffe et al. [71] discovered that using citric acid or citrate buffer to dissolve phytase in the pretreatment could strengthen the activity of phytase in swine feed. Since citrate can release calcium from the phytate or decrease the combination of phytate with Ca, phytate become more vulnerable for phytase to hydrolyze. According to Baruah et al. [12], dietary supplementation of microbial phytase (500 U/kg) in *Labeo rohita* (Hamilton) juveniles' diet significantly increased bone Na, Ca, K, Mn and Fe content by 15, 12.1, 17.4, 20.4 and 40.7%, respectively. Moreover, the increase because of phytase supplementation was more prominent in groups containing 3% level of citric acid, resulting in a significant interaction between citric acid and microbial phytase. The above results showed that phytase increased the bioavailability of some minerals by breaking down the bonds between minerals and phytate and mineral absorption increases at acidic pH thereby increasing the retention in bone. However, absorption and utilization of particular minerals may be species specific and also depend on the feed ingredients used. This area needs further research.

3.5. Dose–response studies

In dose–response studies, phytase addition of 250–1500 U/kg is usually considered feasible in many fish species (Table 4). The optimum dose changes along with many factors such as fish species, different phytase sources, diet formulation (amount of substrate for phytase) and selected response parameters. Thus, phytase addition dose in each fish diet should be adjusted based on consideration of former impactors. However, there are no comparative studies on phytase addition in different fish diets. Only limited data are available for different sources of microbial phytase, mainly in plant based tilapia diets. Liebert and Portz [3] compared nutrient utilization of Nile tilapia fed plant based low phosphorous diets supplemented with graded levels of different sources of microbial phytase. Two different sources of phytase were used: phytase A (SP 1002 CT) and phytase B (Ronozyme P5000). It was found that phytase A supplementation of at least 750 U/kg diet was adequate to improve growth, feed conversion, protein deposition, while supplementation of at least 1000 U/kg from phytase B resulted in intermediate growth results as compared to addition of phytase A. This result indicated that different phytase sources might lead to different effects on zootechnical parameters and nutrient deposition. Furuya et al. [57] observed that phytase supplementation between 500 and 1500 U/kg diet was enough to maintain growth of Nile tilapia fed with plant-based diets. 250–500 U/kg phytase is adequate to maximize the phytate P utilization and may possibly eliminate the use of an inorganic P supplement in channel catfish [2,15]. Robinson et al. [19] reported that phytase of 250 U/kg diet could effectively replace dicalcium phosphate supplement in the diet of channel catfish without affecting growth, feed efficiency or bone P deposition. For stripped bass, the phytase supplementation of 1000 U/kg is adequate to maintain growth rate and health comparable to an inorganic-P supplemented diet [4]. Phytase supplementation of 500 U/kg in Crucian carp diet could improve 9.6% of growth rate, 8.7% of minerals utilization, 32% of phytate-P utilization, 6.6% of crude protein digestibility, respectively [72]. Bai et al. [73] discovered that phytase supplementation of 800 U/kg in diets of common carp could efficiently release enough available P for its growth. Yoo et al. [60] reported that 1000 U/kg of phytase in the diet in Korean rockfish could gain better growth rate than the control. The dietary microbial phytase supplementation at 500 U/kg diet improves growth in *Pangasius pangasius* fingerlings [25]. Variation in the optimum dose of phytase largely depends upon the ingredients and composition of feed formulation and species under study. Accordingly, conclusive studies dealing with the mechanism of phytate degradation of different fish species depending on different diet formulation, specific characteristics of digestive tract and varying activity from different supplemental phytase sources are needed. Further investigations have to decide the optimal level of phytase supplementation for practical application.

Table 4
Optimum dose of phytase addition in diets of different fish species

Fish species	Optimam dose of phytase addition (U/kg)	Reference
Channel catfish	250–500	[2,15]
African catfish	250–500	[16]
Stripped bass	1000	[4]
Nile tilapia	500–1500	[3,57]
Crucian carp	500	[72]
Common carp	800–1000	[73]
Korean rockfish	1000	[60]
Pangasius pangasius	500	[25]

4. Impact factors of phytase activity

4.1. PH value and temperature

The phytase activity usually shows two wave crests: the highest activity around pH 5.0–5.5 and second highest around pH 2.5. Within the pH range of 2.5–5.5, microbial phytase can gain optimum activity. Phytase shows different efficiency in different fish species because of the diversity in their digestive systems. Fish can be classified into two big types with totally different pH value in the digestive systems: gastric and agastric fish. Ji [74] reported that the pH value was 6.8–7.3 in the digestive systems of agastric fish which showed poor efficacy of phytase, while the gastric fish with lower pH value in their digestive systems gained much better results of phytase addition. Phytase cannot be fully used by the agastric fish like carps whose digestive tract's pH is about 6.5–8.4 [75]. To solve this problem, phytase pre-treatment of feed [6,20] or producing neutral phytase corresponding to agastric fish is applicable. Yu and Wang [54] reported that in vitro of the carps, the phytate-P was already hydrolyzed effectively and converted to available-P by the acidified phytase. Fu and Sun [76] found that neutral phytase had better effect than acidic phytase on carps. Zeng et al. [58] reported that neutral phytase supplementation of 300 U/kg could gain the same result as that of 1000 U/kg supplementation of acidic phytase and neutral phytase supplementation of 1000 U/kg could replace the inorganic P supplement.

Phytase, sensitive to high temperature and pressure, is not heat stable and should be applied by avoiding excess heat during extrusion which may destroy the phytase effect. Heat treatment at 100°C for 10 min resulted in loss of all phytase activity. Similarly high temperatures (>70°C) caused partial or total inactivation of native phytase. Most phytases have an optimal pH in the range of 4.5–6.0 and a temperature range of 45–60 °C. Outside the optimal range of pH and temperatures the action of phytase is reduced [77]. Temperature specificity of phytase is not compatible with fish feed manufacturing which usually needs processing temperature higher than 85 °C. Pre-treating feedstuff with phytase could avoid these heat and pressure concerns [20]. Dissolving phytase as liquid suspension to spray onto the feed pellet after feed processing could be a solution to this problem as well [70].

4.2. Ratios of calcium (Ca) to P

Dietary levels of Ca (Ca:P ratios) are crucial to phytase efficacy, as reviewed by Angel et al. [78] in poultry. Data on this aspect in fish are limited. High dietary Ca or a high ratio of Ca: P interferes with P absorption and reduces the effectiveness of phytase activity. High concentration of Ca in fish feed will chelate with phytate to become insoluble complex, or compete with phytase to change the phytase activity center site as an inhibitor, or increase the pH value to inhibit the activity of phytase. High concentration of P in fish feed would also repress the activity of phytase [73]. The phytase activity reduces when the concentration of non-phytate P which is also called available-P in diets increases. Only when the available-P level in the diets is less than the P requirement of fish, the positive result of phytase can be achieved. Nevertheless, appropriate dietary Ca levels and Ca:P ratios, in phytase-supplemented fish diets still require proper definition; although, there is consensus that 'narrow' Ca:P ratios should be adopted. When Ca:P ratios is in the range of 1.1–1.4:1, phytase can perform most efficiently; otherwise, the activity of phytase would decline [58].

4.3. Feed additives

Various feed additives may complement the efficacy of phytase in fish feed where Vitamin D analogs (cholecalciferol and ergocalciferol) and citric acid have probably received the most attention, especially in agastric fish. Vitamin D analogs may indirectly improve utilization of phytate P digestion by increasing absorption of the hydrolyzed P. Vitamin D3 can promote the absorption of Ca, thus reducing the chance of Ca chelated with phytate, which indirectly boost the activity of phytase. There is evidence that vitamin D analog supplementation of vitamin D-adequate diets could stimulate the hydrolysis of phytate and in combination with phytase supplement, further improve phytate P absorption in poultry [79]. It was reported that low dietary level (2500 IU/kg) of cholecalciferol with supplemental phytase at 1500 U/kg in rainbow trout fed a diet based on soy protein concentrate could increase weight gain [68].

Phytase activity changes along the digestive tract, with most efficient phytate hydrolysis occur in the stomach. In carnivorous fish like rainbow trout, production of acids assist in lowering the dietary pH but in stomachless fish, no such mechanism exists and hence addition of organic acids or acidifiers may reduce the dietary pH and help in mineral utilization by lowering the pH of the intestine. Baruah et al. [12] reported that *in Labeo rohita* (Hamilton) juveniles fed plant-based ingredients, the result showed that the addition of microbial phytase or citric acid enhanced the availability of various minerals from plant sources, improved their absorption and hence bone mineralization. The increase in bone minerals was more prominent because of interaction between citric acid and microbial phytase. However, absorption and utilization of nutrients may be species specific and also depend on the feed ingredients used. This area needs further research. Organic acid such as citric acid could also strengthen the activity of phytase in pigs [71]. The pH value of plant-based diets is around, while the optimum pH values of microbial phytase are 2.5 and 5.5, respectively. Therefore, organic acid could regulate the pH value to maximize the phytase activity. However, Yi [80] found that citrate acid supplementation of 1.3 and 3% in turkey poults diets resulted in the decrease of phytase activity from 370 to 269 and 250 U/kg, respectively. This was probably because the organic acid supplementation reduced the pH value below the optimum level of phytase. Thus, appropriate supplementation of organic acid could facilitate the phytase activity. Otherwise, the activity of phytase would be reduced. It is recommended that the supplementation of citrate acid should be less than 1.5% [81].

Besides, the incorporation of mineral chelating agents into fish diets has the potential to enhance phytate degradation by microbial phytase. In contrast, it has been shown that other feed

additives may have deleterious effects on phytase efficacy. For example, high levels of zinc and copper have been shown to have negative influences. Further investigations into the combined use of phytase and feed additives in fish feed are merited, although the inclusion cost of feed additives is clearly a critical consideration [13].

4.4. Processing methods

Extrusion methods including dry and wet extrusion are commonly used in fish feed. Extrusion processing of feed ingredients results in a non-perishable meal that can be incorporated into the diets for a variety of fishes. The use of this relatively new method for aqua feed could lower production costs and reduce the nutrient contribution of these agricultural by-products to environmental pollution [82]. During extrusion processing, physical and chemical changes occur in fish feed. Cheng and Hardy [66] reported that reported that availabilities of copper, phosphorus and zinc in barley, corn gluten meal and wheat-based rainbow trout feeds were significantly reduced after extrusion; this was due partially to the destruction of intrinsic phytase in feed ingredients by the high temperatures used in extrusion processing. Phytase is not heat stable and the enzyme should be applied by avoiding excess heat during extrusion and other steps in diet manufacture. To avoid inactivation of the enzymes, various phytase processing methods have been developed. Phytate levels can be decreased to some extent by taking advantage of the endogenous enzyme phytase during processing, such as soaking or fermentation at a suitable temperature and pH. Several phytases have been applied both prior to pelletizing (pre-treatment of feedstuffs; dephytinization) and onto pellets. The effects of phytase application onto the surface of pellets have been more extensively studied and benefits have been shown in common aquaculture species such as rainbow trout [68], common carp [14] and channel catfish [2]. The easiest process is to mix the enzyme concentrate with a stabilizer and to spray dry the solution [80]. Typical stabilizers are inorganic salts with a bivalent cation, such as $MgSO_4$. The desired enzyme concentration is achieved by down blending the enzyme with a carrier. However, the pelleting stability of products obtained from this technology is limited. An alternative to the use of dry enzyme formulations is the addition of liquid enzyme formulations post-pelleting on the cooled feedstuff pellets. With this method, the enzymes can bypass heat inactivation that would occur during the pelleting process. Dephytinization of feed ingredients with phytase, could become a practical alternative, particularly with oilseed meals. However, the effects of dephytinization are still somewhat inconsistent. In Cain and Garling [20], the pre-treatment of soybean meal with phytase increased weight gain and P utilization in juvenile rainbow trout. Similarly, dephytinization of soy protein concentrate increased protein and P utilization in Atlantic salmon in seawater [18], whereas a decrease in rapeseed protein quality by dephytinization was noted by Teskeredzic et al. [82] in rainbow trout. Vielma et al. [22] reported dephytinization had a positive effect on weight gain, feed efficiency and on protein, P, calcium, magnesium and zinc utilization.

5. Outlook

Along with the environmental impact assessment of aquaculture is getting increasing concerns in relation to P pollution, rigorous restrictions are being set by governments and environmentalists. A better appreciation of the application of microbial phytases and their decreasing inclusion costs in fish feed are the central goals of future research. During the past 15 years, research on evaluation of microbial phytases in diets for different fish species has rapidly expanded, but much focus of these researches has been on the assessing of various phytases from different sources rather than the investigation of the underlying factors causing variability in phytase responses [30].

Fundamental information in respect of phytate and phytase is lacking in many aspects. There is an urgent need to clarify and define the P requirements of fish accurately and to develop appropriate terminology to express these requirements uniformly [13]. Dietary manipulations to facilitate the activity of exogenous phytases should be considered and applied appropriately. Enhancement of thermal tolerance and increase in specific activity are two important issues for fish feed. A major trend is to produce the ideal enzyme which has high specific catalytic activity (per unit of protein), good thermo-stability during feed processing, high activity under wide ranges of gut pH, resistance to proteolysis and good stability under ambient temperatures. Different strategies have been used to obtain an enzyme capable of withstanding higher temperatures. The original phytase feed enzymes were produced mainly from fungi. Recently, new exogenous phytases have been derived from other forms of microorganisms, such as bacteria and yeast. A synthetic enzyme, deduced from several fungal phytases and subsequent refinements by site-directed mutagenesis, resulted in unfolding temperatures up to 90.4 °C [83]. In addition, phytase from B. amyloliquefaciens exhibited optimal activity at 70 °C and stability at 90 °C during 10 min incubation [84]. In pelleting experiments, this enzyme activity retained higher than 85% at temperatures ranging from 60 to 90 °C. A shift in temperature optimum of the *E. coli* phytase from 55 to 65 °C and a significant enhancement in its thermal stability at 80 and 90 °C were achieved by expression of the enzyme in the yeast *Pichia pastoris* after introduction of three glycosylation sites into the amino acid sequence of the *E. coli* phytase by site-directed mutagenesis. Gene site saturation mutagenesis technology was a further approach used to optimize the performance of the *E. coli* phytase. However, there are still very few phytases reported with temperature stability or sustaining temperature higher than 70 °C [30].

Protein engineering may deal with the pH profile of phytases. The pH range for phytase activity of the *A. niger* phytase [85] and the *E. coli* phytase [30] were broadened at acidic pH by mutagenesis. The future researches should been focus on using gene project to find the phytase which can suffer high temperature and more stable, or raising transgenic fish which may produce their own digestive phytase. Most successful feeding trials were performed with acidic phytase. Preliminary data suggests that phytase with neutral pH optimum also show relevant biological activity [13]. More data for neutral and alkaline

phytases are required to evaluate the potential of these enzymes for commercial applications. A relatively new field is the use of transgenic animals to produce active endogenic phytase directly in the digestive tract, though several past attempts to express a fungal phytase in a transgenic animal ended unsuccessfully [86,87]. Besides, several attempts were made to use transgenic plants as expression hosts for phytases. Transgenic plants might contain sufficient phytase activity to replace phytase supplementation in feed. Alternatively, transgenic plants could be used as bioreactors for the production of phytase as a supplement. Future developments in molecular biology may increase phytase efficacy, reduce phytate concentration in plants, or enhance endogenous phytase synthesis in both transgenic plants and fish animals.

In addition, the optimum supplement doses of phytase in diets of different fish species also vary greatly and needs further research. It is difficult to determine the standard dose of phytases addition for each species due to their different formulation and sources such as microbes or plants. The including methods of phytase into fish diets also needs further study to find out an ideal way to maximize the phytase efficacy and keep lost at the minimum level.

6. Conclusions

Due to the decreasing fishmeal production, the use of plant-based feed in aquaculture is inevitable in the near future. However, plant ingredients have their own limitations because of the presence of phytate. Phytate-rich plant ingredients restrict the bioavailability of P along with other minerals thereby increasing discharge into aquatic environment. It is clear that supplemental phytase can enhance the digestibility and bio-availability of P, nitrogen and other minerals, reduce the amount of inorganic-P supplement to maximize growth and bone mineralization, and markedly decrease P load to aquatic environment. However, there are still many results on protein utilization and growth rate not consistent. Data concerning the hydrolysis conditions for phytase in the gut of fish species are very limited. The optimum doses for phytase to replace inorganic P have not been evaluated in fish diets. Accordingly, further investigations about phytase application in fish feed are largely needed. The use of phytase in fish feed will expand along with the need of cost-effective feed and environmental protection concern increasing.

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