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Microbial Phytase Supplementation in Rohu, *Labeo rohita*, Diets Enhances Growth Performance and Nutrient Digestibility

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Abstract.—A feeding trial was conducted for 60 d to study the effect of dietary microbial phytase on growth performance and nutrient digestibility of rohu, *Labeo rohita*, fingerlings. One hundred and twenty fingerlings (average initial weight: 9.17 g/fish) were equally distributed into five experimental tanks, each with four replicates. Five isonitrogenous (35%) and isocaloric (16.79 kJ/g) diets were prepared from plant-based ingredients, supplemented with microbial phytase at the level of 0, 250, 500, 750, and 1000 U/kg diets and fed to T_0, T_1, T_2, T_3, and T_4 groups, respectively. Weight gain %, food conversion ratio, protein efficiency ratio, and apparent net protein utilization were significantly ($P < 0.05$) improved in groups fed phytase-supplemented diets compared to control, the highest being observed in T_3 group. Maximum apparent digestibility of phosphorus and crude protein was recorded in T_3 group. Bone ash, phosphorus (P), and calcium (Ca) contents were also significantly ($P < 0.05$) increased in phytase-fed groups. However, maximum was recorded in T_3 group. Results from the present study indicated that addition of 750 U microbial phytase/kg diets effectively improved nutrient utilization, bone mineralization, and hence growth of *L. rohita* fingerlings.

Indian major carps (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*) contribute about 87% of the total freshwater production (ICLARM 2001). They occupy an important place in aquaculture owing to their high commercial value. They are mostly raised in a polyculture system either traditionally or semi-intensively. Plant-based diets like mixture of oil cake and rice bran (1:1) are generally used as a supplementary feed for these fish (Jhingran 1991). But the use of such plant ingredients in aquafeed is limited because of the presence of wide variety of anti-nutritional compounds (De Silva and Andersons 1995), which have deleterious effect on the physiology and morphology of the digestive tract (Grant 1989; Van den Ingh et al. 1991), thereby affecting the overall fish growth (Hendricks and Bailey 1989; Usmani and Jafri 2002). Phytic acid or phytate (myo-inositol hexakis phosphate) is one such compound that is a common constituent of plants, making up about two-thirds of the P. This phytate-P cannot be efficiently used by fish (Francis et al. 2001; Usmani and Jafri 2002) as they lack the intestinal phytase enzyme (Pointillart et al. 1987). This unutilized phyate-P gets excreted into the environment and causes eutrophication of water body. Moreover, phytic acid also forms complexes with proteins and amino acids (O'Dell and De Boland 1976; Knuckles et al. 1989) and inhibits proteolytic enzymes such as pepsin and trypsin (Singh and Krikorian 1982). In short, phytic acid can reduce the absorption of these important nutrients.

Microbial phytase is capable of releasing the inorganic phosphate group from phytate, thereby making them available for absorption and utilization by the animals. However, the

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efficacy of microbial phytase is found to be pH dependent, the highest activity being observed at two pH optima, that is, 5.0–5.5 and 2.5 (Simons et al. 1990). Phytase activity also changes along the digestive tract, with most efficient phytate hydrolysis occurring in the stomach (Yi and Kornegay 1996). In carnivorous fish, there is a true stomach secreting acids, but in stomach-less fish like carps, no such mechanism exists (Ogino et al. 1979).

Studies with monogastric fish like rainbow trout (Sugiura et al. 2001; Cheng and Hardy 2002; Vielma et al. 2002, 2004; Zongjia et al. 2003), Korean rockfish (Yoo et al. 2005), Nile tilapia (Furuya et al. 2001; Portz and Liebert 2004; Liebert and Portz 2005), *Pangasius pangasius* (Debnath et al. 2005a), channel catfish (Robinson et al. 2002; Yan and Reigh 2002), African catfish (Van Weerd et al. 1999), sea bass (Oliva-Teles et al. 1998), striped bass (Papatryphon and Soares 2001), Atlantic salmon (Sajjadi and Carter 2004), and Japanese flounder (Masumoto et al. 2001) had shown that dietary microbial phytase improved the bioavailability of phytate-bound P and thus growth. But little published information exists on the use of microbial phytase in stomach-less fish like *L. rohita.* However, Schäfer et al. (1995), in common carp, an agastric species, observed an improvement in use of phytate-P and thus weight gain with dietary microbial phytase. Moreover, Baruah et al. (2005), in *L. rohita* juveniles, observed an increased bone mineralization with dietary microbial phytase in combination with citric acid and different protein levels. But the effect of microbial phytase alone on growth and nutrient utilization in this fish species is still unknown. Therefore, the present study was carried out to investigate the effect of microbial phytase on growth performance and nutrient digestibility of *L. rohita* fingerlings.

Materials and Methods

Diet Preparation

A basal diet (Table 1) was formulated using plant-based ingredients to contain 35% crude protein (CP). Diets were prepared by blending all the ingredients, except the vitamin–mineral mixture, in a plastic bowl. Oil was added to the dry ingredients and dough prepared with the required amount of water. The dough was then kept for 1 h for proper conditioning, followed by steaming for 20 min in a pressure cooker. The vitamin–mineral mixture and vitamin C were mixed after cooling. Pellets (2 mm) were prepared using a hand pelletizer, air-dried for 30 min, and kept in an oven at 60 C until dry. The required amount of microbial phytase (Natuphos® 5000 G; BASF India, Mumbai, India) was dissolved in 50 mL of distilled water and sprayed over 1 kg of the basal diet as described by Robinson et al. (2002). The control diet was sprayed with a similar amount of distilled water to maintain an equal level of moisture. Thus, five experimental diets were prepared by

TABLE 1. *Ingredients and proximate composition of experimental diets.*

Ingredients	% inclusion
Soybean meal	40.85
Fish meal	5.00
Wheat flour	9.00
Mustard oil cake	14.00
Rice polish	8.00
De-oiled rice bran	15.00
Fish oil	6.00
CMC	1.00
Vitamin–mineral mix[a]	0.55
Vitamin C[b]	0.10
Chromic oxide	0.5
Proximate composition	(% Dry Matter ± SE)
CP	33.97 ± 0.53
Ether extract	8.96 ± 0.45
Ash	10.16 ± 0.06
Mineral contents	(%)
Phosphorus	0.57
Calcium	0.59

CP = crude protein, CMC = carboxymethyl cellulose.

[a] Composition of vitamin–mineral mix (Emix™ plus, Mumbai, Maharastra, India) (quantity/2.5 kg): vitamin A, 5,500,000 IU; vitamin D_3, 1,100,000 IU; vitamin B_2, 2000 mg; vitamin E, 750 mg; vitamin K, 1000 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 6 mg; calcium panthothenate, 2500 mg; niacinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; iodine, 1000 mg; Fe, 7500 mg; Cu, 2000 mg; Zn, 5000 mg; Co, 450 mg; Ca, 500 g; P, 300 g; Se, 50 ppm; L-lysine, 10 g; DL-methionine, 10 g; satawari, 2500 mg; Carrier, quantum sufficient; *Lactobacillus*, 120 million units, and yeast culture, 3000 crore units.

[b] Stay C (Hoffman La Roche, Inc., Nutley, NJ, USA), 15% ascorbic acid activity.

mixing graded levels of phytase to the basal diets at 0 (T_0), 250 (T_1), 500 (T_2), 750 (T_3), and 1000 (T_4) U/kg diet (1 U is defined as the amount of phytase that liberates 1 μmol of inorganic P from 0.0051 mol/L of sodium phytate per minute at 37 C and pH 5.5). The diets were stored at 4 C until use. During the last 20 d of the experiment, chromic oxide (0.5%) was mixed in all the test diets for digestibility study.

Fish and Feeding Trial

Prior to initiation of the experiment, *L. rohita* fingerlings, procured from Khopoli Fish Seed Farm, Maharashtra, India, were given a prophylactic dip in $KMnO_4$ solution (50 mg/L) and stocked in a circular tank (1000 L) with aeration for acclimatization, during which they were fed basal diet (35% CP) for 4 wk. The feeding trial was conducted in uniform size plastic containers (60 × 40 × 47 cm) of 100-L capacity.

At the start of the feeding trial, groups of six fingerlings (average weight: 9.17 g/fish) were stocked in 20 containers, which were randomly distributed in five treatments with four replicates each following a completely randomized design. Each diet was fed thrice daily (0700, 1200, and 1700 h) to approximate satiation for 60 d. Initially, diets were fed at 3% of the body weight and subsequently adjusted based on daily intake. Round-the-clock aeration was provided to all the containers from a compressed air pump, and manual water exchange was carried out daily. Water quality parameters were checked every week using the methods of APHA (1998).

Sampling and Analyses of Samples

Proximate analysis of all the diets was carried out following standard methods (AOAC 1995). Moisture content was determined by drying the samples at 105 C to a constant weight. Nitrogen content of the samples was measured by Kjeltec (2200 Kjeltec auto distillation; Foss Tecator, Höganäs Sweden), and CP was calculated by multiplying nitrogen by 6.25. Ether extract was measured by Soxtec (1045 Soxtec extraction unit; Tecator, Höganäs Sweden) using diethyl ether (boiling point of 40–60 C) as a solvent; ash content was estimated by incinerating the samples in a muffle furnace at 600 C for 6 h.

Growth Study

Fish in each container were bulk weighed every 15 d. Growth performance of fingerlings was evaluated in terms of weight gain %, food conversion ratio (FCR), protein efficiency ratio (PER), and apparent net protein utilization (ANPU) based on following standard formulae:

$$\text{Weight gain \%} = (\text{final weight} - \text{initial weight})/\text{initial weight} \times 100$$

$$\text{FCR} = \text{total dry feed intake (g)}/\text{wet weight gain (g)}$$

$$\text{PER} = \text{total wet weight gain (g)}/\text{CP fed (g)}$$

$$\text{ANPU} = 100(\text{final tissue protein} - \text{initial tissue protein})/\text{CP fed.}$$

Digestibility Study

Apparent digestibility coefficients of P (ADC_p) and CP (ADC_{CP}) of different diets were measured using 0.5% chromic oxide (Cr_2O_3) as a marker (Hardy and Barrows 2002). Wet ashing of diets and fecal matters was carried out according to the methods of AOAC (1995), and the chromium content of feed and fecal matters was determined using flame ionization atomic absorption spectrophotometer (AAS 4129; Electronics Corporation of India Ltd., Hyderabad, India). The fecal samples collected were also analyzed for CP by AOAC (1995) method. Apparent digestibility coefficients for P and CP were calculated as:

$$\text{Apparent digestibity (\%)} = 100 - 100\left(\frac{\%Cr_2O_3 \text{ in feed}}{\%Cr_2O_3 \text{ in feces}} \times \frac{\%\text{ nutrient in feces}}{\%\text{ nutrient in feed}}\right),$$

where nutrient is P and CP.

Mineral Analysis

At the end of the feeding trial, fish from each group were sacrificed using clove oil (50 μL/L). Intact trunk and caudal vertebrae were removed from each group of fish and pooled for bone ash and mineral analysis. The whole fish were

boiled for 20 min; the excess flesh was stripped off from the vertebrae and the adhering flesh removed by light brushing and rinsing in distilled water. The vertebrae were then dried for 2 h at 110 C and extracted with anhydrous ethyl ether for 7 h, then pulverized, dried again, and weighed. The dried samples were ashed at 550 C for 6 h. Ash weight was calculated as a percentage of dry, fat-free bone weight. For mineral estimation, the ash was digested in a boiling nitric acid and perchloric acid mixture (2:1) according to the method by AOAC (1995). After appropriate dilution, Ca content was estimated by atomic absorption spectrophotometer, while P was estimated spectrophotometrically using molybdovanadate method (AOAC 1995). The mineral content in the diets and fecal matter was also analyzed in the same way as described for bone.

Statistical Analysis

All the data were subjected to one-way ANOVA using statistical software Statistical Package for the Social Sciences (SPSS) version 11.0. Duncan's multiple range tests was used to determine the differences among treatment means at 5% level of significance (Duncan 1955).

Results

Growth Performance

Growth data (Table 2) showed that weight gain %, FCR, PER, and ANPU of *L. rohita* fingerlings were significantly ($P < 0.05$) improved by phytase supplementation. Highest weight gain was obtained when the fish were fed on T_3 diet. FCR, which ranged from 1.09 to 1.67, was found maximum in T_0 group and minimum in T_3 group. PER and ANPU were also recorded to be highest in T_3 group.

Nutrient Digestibility

Apparent digestibility coefficients of phosphorus (ADC_p) and CP (ADC_{CP}) of fish fed phytase-supplemented diet were significantly ($P < 0.05$) higher than of those fed control diet (Table 3). The highest level of ADC_p and ADC_{CP} was observed in fish fed T_3 diet and lowest in those fed T_0 diet.

Bone Ash, Ca, and P

Phytase-supplemented groups in general recorded significantly ($P < 0.05$) higher percentage of bone ash and also higher concentration of bone Ca and P compared with the control group (Table 4). Bone ash and bone P content were found to be highest in T_3 group, which did not differ significantly ($P > 0.05$) from T_2 group. Bone Ca content was also recorded to be maximum in T_3 group. No significant ($P > 0.05$) differences among T_1, T_2, and T_4 groups were observed for bone Ca content.

Discussion

Results from the present study demonstrated that incorporation of microbial phytase in basal diets improved overall growth performance and P availability in *L. rohita* fingerlings. A significant increase in growth performance was commensurate with the increase in phytase concentration to

TABLE 2. *Weight gain %, FCR, PER, and ANPU of* Labeo rohita *fingerlings fed experimental diets containing various levels of microbial phytase*[1] *for 60 d.*

Treatments	Weight gain %[2]	FCR	PER	ANPU
C	191.75 ± 2.66[a]	1.67 ± 0.02[d]	1.92 ± 0.07[a]	33.20 ± 0.40[a]
T_1	255.21 ± 2.54[b]	1.32 ± 0.02[c]	2.28 ± 0.02[b]	36.21 ± 0.21[b]
T_2	268.74 ± 2.81[c]	1.23 ± 0.02[b]	2.42 ± 0.02[c]	35.85 ± 1.04[b]
T_3	312.09 ± 2.75[d]	1.09 ± 0.02[a]	2.79 ± 0.03[d]	39.08 ± 0.28[c]
T_4	257.19 ± 2.65[b]	1.37 ± 0.03[c]	2.28 ± 0.01[b]	35.31 ± 0.41[b]

ANPU = apparent net protein utilization, FCR = food conversion ratio, PER = protein efficiency ratio. Means in the same column with same superscript are not different significantly ($P > 0.05$, Duncan's new multiple range test). Data are means of four replicates ± SE.

[1] Natuphos 5000 G produced from *Aspergillus niger* (BASF India Limited).

[2] Mean initial weight was 9.17 g/fish.

TABLE 3. *Apparent digestibility of P (ADC_p) and CP (ADC_{CP}) of* Labeo rohita *fingerlings fed experimental diets containing various levels of microbial phytase*[1] *for 60 d.*

Treatments	ADC_p	ADC_{CP}
C	39.42 ± 0.48[a]	71.94 ± 0.26[a]
T_1	52.08 ± 0.62[b]	73.90 ± 0.90[a]
T_2	57.63 ± 0.60[c]	77.68 ± 0.75[b]
T_3	65.39 ± 1.21[d]	82.13 ± 0.79[c]
T_4	57.56 ± 0.45[c]	78.60 ± 0.43[b]

CP = crude protein. Means in the same column with same superscript are not different significantly ($P > 0.05$, Duncan's new multiple range test). Data are means of four replicates ± SE.

[1] Natuphos 5000 G produced from *Aspergillus niger* (BASF India Limited).

a level of 750 U/kg diet, after which it decreased. This indicates that 750 U/kg diet was optimum for enhancing the bioavailability of nutrients for *L. rohita* fingerlings. However, the optimum dosage of phytase supplementation differs in the different studies and among fish species. The positive effect of microbial phytase on the growth performance of the fingerlings in the present study is consistent with the results obtained by various authors (Forster et al. 1999; Cheng and Hardy 2002; Vielma et al. 2002; Zongjia et al. 2003; Debnath et al. 2005a; Liebert and Portz 2005; Yoo et al. 2005). However, some authors (Vielma et al. 2000; Masumoto et al. 2001; Yan and Reigh 2002; Sajjadi and Carter 2004; Yoo et al. 2005) have reported no effect of dietary phytase on weight gain of various fish species fed plant-based diets. This discrepancy in their results may be associated with differences in their diet composition and also to different rearing conditions.

The effect of phytic acid on growth depends primarily on its amount in the diet and the presence or the absence of a distinct stomach. Inclusion of 1% phytic acid or more in the diet of agastric *L. rohita* significantly decreased the growth performance (Alvi 1994). Similar effects are evident on the growth performance of *C. mrigala* fry (Usmani and Jafri 2002). Moreover, common carp fed purified diets containing 0.5 or 1% phytic acid also had a significant reduction in growth (Hossain and Jauncey 1991), whereas in catfish, *Ictalurus punctatus* (gastric species), growth was reduced only when diets containing 2.2% or more phytic acid were fed (Satoh et al. 1989), but diets containing up to 1.5% phytic acid had no effect (Gatlin and Phillips 1989; Satoh et al. 1989). In comparison with low (0.16%) or medium (0.65%) levels of phytate, juvenile chinook salmon, *Oncorhynchus tshawytscha*, showed reduced weight gain when fed 2.6% phytate (Richardson et al. 1985). On the contrary, the growth performance increased when microbial phytase was incorporated in the plant-based diets. An increase in weight gain has been reported in channel catfish fed phytase-supplemented diets containing only plant protein sources (Jackson et al. 1996). Weight gain was increased by 23.52. Similar performance of rainbow trout (Vielma et al. 1998, 2002) and stripped bass (Papatryphon et al. 1999) was also reported and attributed solely to improved use of P from the phytate. But in the present study, improvement in growth performance was as a result of improved use of phytate-P as well as phytate-bound protein. Vielma et al. (2004), in rainbow trout; Debnath et al. (2005a), in *Pangasius pangasius*; and

TABLE 4. *Bone ash, P, and Ca contents of* Labeo rohita *fingerlings fed experimental diets containing various levels of microbial phytase*[1] *for 60 d.*

Treatments	Bone ash (%)	Bone Ca (%)	Bone P (%)
C	40.27 ± 0.92[a]	20.70 ± 0.17[a]	10.09 ± 0.05[a]
T_1	42.92 ± 0.23[b]	22.34 ± 0.55[b]	10.90 ± 0.18[b]
T_2	45.66 ± 0.47[c]	23.04 ± 0.11[b]	11.97 ± 0.03[c]
T_3	46.23 ± 0.32[c]	25.17 ± 0.50[c]	11.88 ± 0.07[c]
T_4	42.76 ± 0.42[b]	23.25 ± 0.29[b]	10.87 ± 0.42[b]

Means in the same column with same superscript are not different significantly ($P > 0.05$, Duncan's new multiple range test). Data are means of four replicates ± SE.

[1] Natuphos 5000 G produced from *Aspergillus niger* (BASF India Limited).

Liebert and Portz (2005), in Nile Tilapia, also observed improvement in growth performance when fed phytase-supplemented diet and attributed this to increased bioavailability of protein and minerals. In our present study, no mortality was recorded during the feeding trial.

Apparent P digestibility is one of the most sensitive criteria for assessing the influence of phytase on P utilization in fish (Sajjadi and Carter 2004). In the present study, digestibility of P was increased with phytase supplementation, confirming the established properties of phytate with respect to dietary nutrient availabilities. The P digestibility increased from 39.42 to 65.39%, but the relationship was not linear. The P digestibility was highest in the group fed T_3 diet containing 750 U phytase/kg diet; then there was no further advantage when the level of phytase was increased to 1000 U/kg diet. The increase in the phosphorus digestibility is in accordance with other studies carried out in common carp (Schäfer et al. 1995), salmonids (Forster et al. 1999; Sugiura et al. 2001; Sajjadi and Carter 2004), channel catfish (Jackson et al. 1996), sea bass (Oliva-Teles et al. 1998), Japanese flounder (Masumoto et al. 2001), striped bass (Papatryphon and Soares 2001), rainbow trout (Sugiura et al. 2001; Cheng and Hardy 2002; Zongjia et al. 2003; Vielma et al. 2004), Nile tilapia (Liebert and Portz 2005), *P. pangasius* (Debnath et al. 2005b), and Korean rockfish (Yoo et al. 2005).

There are conflicting and inconsistent reports as to the efficacy of phytase in improving protein digestibility. Phytase has been reported to increase (Sugiura et al. 2001; Vielma et al. 2004; Debnath et al. 2005a; Liebert and Portz 2005) and not affect (Papatryphon and Soares 2001; Yan and Reigh 2002) or decrease (Hossain and Jauncey 1991; Teskeredzic et al. 1995) protein digestibility. However, in this study, there was an improvement in protein digestibility with phytase supplementation as high as 750 U/kg diet. However, fish fed T_0 diet did not vary significantly from those fed T_1 diet, indicating that 250 U phytase/kg diet was not sufficient enough to release the phytate-bound protein.

Bone ash and bone P are sensitive indicators of the P status in fish (Vielma and Lall 1998). This is because the P requirement for maximum bone mineralization is greater than the requirement for maximum body weight gain. Insufficient P intake leads to the mobilization of P from the bone and transfer to soft and metabolic processes (Baeverfjord et al. 1998). Bone ash increased in fish fed phytase-supplemented diets, indicating that the mineral bioavailability was significantly increased by dietary manipulation. This observation is in accordance with studies conducted with rainbow trout (Vielma et al. 2002), channel catfish (Yan and Reigh 2002), striped bass (Papatryphon et al. 1999), *P. pangasius* (Debnath et al. 2005b), Atlantic salmon (Sajjadi and Carter 2004), and Nile tilapia (Miranda et al. 2000; Liebert and Portz 2005).

As expected, the effect of dietary phytase on bone P and Ca contents was also significant, and this indicated that phytase increased the bioavailability of P and Ca by the breakdown of the bonds between them and phytate. These results agrees with reports by other authors who had observed positive effects of dietary phytase on bone mineralization in *L. rohita* (Baruah et al. 2005), common carp (Schäfer et al. 1995), and other fish species (Storebakken et al. 1998; Papatryphon et al. 1999; Yan and Reigh 2002; Debnath et al. 2005b; Liebert and Portz 2005). In this study, phytase supplementation at a level of 250 U/kg diet was sufficient to significantly improve retention of dietary P and Ca by *L. rohita* fed a plant protein diet, but 750 U/kg diet of phytase appeared necessary for maximum retention of Ca and P.

In conclusion, the present study demonstrated that dietary microbial phytase supplementation at 750 U/kg diet with 35% CP level improved growth performance, P and CP digestibility, and bone mineralization in *L. rohita* fingerlings. Increased use of naturally occurring minerals in plant ingredients reduces the need for mineral supplements in formulated diets and results in decreased elimination of minerals in feces. Thus, supplementation of phytase in carp feeds can be expected to provide both economic and environmental benefits through decreased expenditures on supplemental minerals and mineral outputs to the aquatic ecosystem. However,

diet preparation with different feed ingredients and rearing conditions seem to have an important influence on the effect of phytase in carp feeds. Further studies are required to investigate the rates near 750 U/kg under different dietary and rearing conditions.

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