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Optimum dietary levels of phytase from *Bacillus megaterium* on the utilization of phosphorus in the diets of Nile tilapia, *Oreochromis niloticus*

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Abstract. A feeding trial was conducted to determine the effects of dietary *B. megaterium* phytase at 250, 500, 1,000 and 1,500 Phytase Units (FTU) kg^{-1} diet and diets containing P_i 1.5% mono-sodium phosphate and 1.0% calcium carbonate (positive control). Fish fed diets containing 250 FTU kg^{-1} phytase and above exhibited comparable results on weight gain and specific growth rate (SGR) with those of fish fed the diets containing P. Fish fed the P_i diet exhibited lower feed conversion efficiency (FCE), protein efficiency ratio (PER) and protein retention than did fish fed diets supplemented with 500 FTU and 1,000 FTU kg^{-1} phytase. No significant differences in body crude protein, ash, P, Ca and Mg among fish fed the dietary treatments. Fish fed the diet supplemented with 1,000 FTU kg^{-1} phytase exhibited significantly the highest body crude lipid. Phosphorus concentration in scales, bone and vertebrae of fish fed the P_i diets displayed lower concentration than did fish fed 500 FTU kg^{-1} and 1,000 FTU kg^{-1}, respectively. Fecal P was significantly lower in fish fed diets containing various levels of phytase than in those fed the inorganic phosphorus P*i* diet. Findings of the present study revealed that addition of 500–1,000 FTUkg^{-1} of *B. megaterium* kg^{-1} to tilapia diet improved bioavailability of phytate in Nile tilapia diets thereby it could potentially lessen the P content in pond effluent from 27% to 39%.
Key Words: tilapia, phytase, phosphorus, enzyme, *Bacillus megaterium*.

Introduction. Eutrophication of freshwater has been a key problem in recent years, partly because of the impact of phosphorus (P) that is being discharged from aquaculture systems into the environment (EPA 1973). Phosphorus is a critical pollutant in the aquatic environment; excessive excretion of phosphorus in fresh water can stimulate growth of algae and phytoplankton, consequently reducing dissolved oxygen thus, causing water pollution (Boyd 1990; Sugiura et al 1999; Caipang et al 2011; Baruah et al 2004). Excessive discharge of phosphorus, nitrogen, macro- and trace minerals affect water quality (Hardy & Cheng 2002). Accordingly, reducing the amount of these nutrients discharged into the aquatic environment is of great importance in intensive aquaculture operations.

Interest in phytase enzyme as an additive in feeds has been developed as a means to reduced P in effluents from aquaculture production systems. About two – thirds of the P in plant products is present in the form of phytic acid or phytate (Lall 1991), which is nutritionally unavailable to fish and other monogastric animals that lack intestinal phytase (Pointillart et al 1987). The addition of microbial phytase to the diet has been reported to improve the utilization of phytate P in rainbow trout *Oncorhynchus mykiss* (Cain & Garling 1995; Rodehutscord & Pfeffer 1995), common carp *Cyprinus carpio* (Schafer et al 1995) and the Asian sea bass *Lates calcarifer* (Ganzon-Naret 2013). Studies conducted using three phytases from *Bacillus megaterium*, *B. licheniformis* and *B. pumilus* did not enhance growth performance but mineralization of bones and scales were improved (Dechavez & Serrano 2012). The most remarkable finding was the marked improvement of P retention and lessening of fecal P and P load in all the *Bacillus* phytase-supplemented diets indicating that ameliorating water quality was the main benefit of incorporating these bacterial phytases into the diet of the tilapia *Oreochromis mossambicus* (Dechavez & Serrano 2012).

The increased utilization of P in the diets should decrease the amount of P in waste products. It was reported that incorporating 500 units of microbial phytase kg^{-1} diet results in maximum weight gain and maximum bone P deposition in channel catfish (Jackson et al 1996). The use of bacterial phytase has been tested in tilapia by incorporating in the diets that showed improved phosphorus utilization and absorption (Furuya et al 2001; Liebert & Portz 2005; Dechavez et al 2011; Dechavez & Serrano 2012). Hence, this study aimed to evaluate the efficacy of bacterial phytase at various levels incorporated in plant-based diet; to determine the effects of bacterial supplementation on growth, feed utilization and body composition of tilapia and to evaluate the optimum bacterial supplementation on improving P bioavailability in plant - based diets fed to Nile tilapia, *O. niloticus*.

Material and Method. The experiment was done from June to October 2008 at the hatchery facility of the Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas in Iloilo, Philippines.

The basal diet was formulated to contain 35% protein, about 3,600 kcal digestible energy kg^{-1} and not less than 0.9% available P. The bacterial phytase from *B. megaterium* was added to plant-based diets at 250, 500, 1,000 and 1,500 phytase units (FTU) kg^{-1} diet. Prior to formulation, all feed ingredients were analyzed for proximate composition as well as P, Ca and Mg content. Pure isolates of *B. megaterium* were sub-cultured and the phytase was assayed and prepared as described by Dechavez et al (2011). *Bacillus* phytase in solution was sprayed just before feeding at 250, 500, 1,000 and 1,500 FTU kg^{-1} diet. One unit of enzyme activity (FTU) was defined as the amount of enzyme hydrolyzing 1 μmol of P_i min^{-1} under the assay conditions. The P_i diet (control diet) was supplemented with 1.5% mono-sodium phosphates (NaH_2PO_4) and 1.0% calcium carbonate ($CaCO_3$) (Table 1). The diets were stored at 4^oC until use. Each dietary treatment was assigned randomly in triplicate to fifteen concrete tanks. At random, twenty five (25) Nile tilapia fingerlings (6.0 g average body weight (ABW)) were stocked in 500 L capacity circular concrete tanks. The tanks were supplied with fresh tap water and continuous aeration. The feeding trials were conducted following a two-weeks acclimatization to the diet and experimental conditions. Fish were fed twice daily at 5% body weight for 10 weeks. Fish were weighed at the start and every 14 days until termination of the experiment on the 10th week. Growth and feed efficiency were evaluated in terms of weight gain, feed conversion efficiency, specific growth rate, protein efficiency ratio, protein retention and mineral content (%) of nutrient intake. At the end of the experimental period, 10 fish were weighed; scale and vertebral samples were collected in every treatment. Bone samples were immersed in ethyl alcohol for one week, oven dried and ground for mineral analysis. Scale, bone and vertebra and fish samples were separately pooled for each treatment and pulverized with mortar and pestle for analysis of Phosphorus (P), Calcium (Ca) and Magnesium (Mg) contents.

Chemical analyses of ingredients, diets and fish were done at SEAFDEC Fish Nutrition Laboratory. Chromium analysis was determined using acid digestion method (Furukawa & Tsukahara 1966). Metabolizable energy (ME) was estimated using the following physiological fuel values: 4.0 kcal g^{-1} protein and nitrogen-free extract and 8.0 kcal g^{-1} lipids according to Lorico-Querijero & Chiu (1989) for tilapia.

Calcium and magnesium in fish whole body, scale, vertebral bone and in formulated feeds were analyzed using Flame Atomic Absorption Spectrometry (Spectra 55B AAS) after wet ashing and acid digestion. Phosphorus content in the fish whole body, scale, vertebral bone, feces and concentration in feed ingredients was done using the ammonium-molybdate method (AOAC 1990; Lovell 1975; Pearson 1976). The optical density of the solution due to ammonium-molybdophosphorus complexes was measured at 430 nm (Sunny UV-7802PC Ultraviolet – Visible spectrophotometer).

Differences among treatments were analyzed using one way analysis of variance (ANOVA) and Tukey's test was used to determine the significant differences at 5% level of significance. All statistical calculations were performed using a Statistical Package for

Social Sciences (SPSS) version 16.0 Windows software. Data were tested for homogeneity of variance and normality of data prior to ANOVA.

Table 1

Composition of dietary plant-based test diets fed to Nile tilapia (*O. niloticus*) fingerlings for 10 weeks (g kg^{-1} dry matter)

Ingredient (g kg^{-1} as fed basis)	*Weight (g) (experimental diet)*	*Feed with 1.5% mono-sodium phosphate and 1% calcium carbonate (P_i diet)*
Fish meal	150.00	150.00
Soybean meal	272.20	272.20
Rice bran	250.00	250.00
Cassava leaf meal	50.00	50.00
Wheat flour	232.80	232.80
Soybean oil	10	10
Vitamins mix	25	25
Mineral mix	20	20
Proximate Composition (%)		
Moisture	4.34	4.34
Crude Protein	34.08	34.08
Crude Fat	2.63	2.63
Crude fiber	3.14	3.14
Ash	10.73	10.73
Nitrogen Free Extract	49.42	49.42
Phosphorus	0.91	1.02
Calcium	1.82	2.03
Magnesium	0.73	0.96

Results and Discussion. Growth curve of Nile tilapia fed diets with different levels of bacterial phytase showed that the P_i diet (50.7 g) resulted in the highest growth followed by 500 FTU kg^{-1} (50.1g), 1,500 FTU kg^{-1} (49.9 g), 250 FTU kg^{-1} (48.7 g) and 1,000 FTU kg^{-1} (48.6 g) level in decreasing order although differences were not significant. Plant–based diet supplemented with bacterial phytase resulted in comparable growth rates with that of diet supplemented with P_i.

Survival rates ranged from 95 to 100%; feed conversion efficiency, protein efficiency ratio, protein retention and condition factor (K) were also not significantly affected by the different levels of bacterial phytase and P_i supplementation.

Diet supplemented with 1,000 FTU kg^{-1} (35.30%) resulted in significantly highest crude lipid. Crude protein, protein retention, ash, P, Ca and Mg were not significantly affected by various levels of *B. megaterium* phytase and P_i supplementation. Although not significant, the highest Mg and Ca content were observed in fish fed the diet containing 1,000 FTU kg^{-1} phytase (0.21% and 4.72%, respectively), while the highest P content was observed in fish fed a diet 250 FTU kg^{-1} phytase.

Minerals such as P, Ca and Mg in scales, bone and vertebrae of Nile tilapia were not significantly affected by the experimental diets. Fish fed the P_i diet displayed the highest final Ca and Mg content in scales and bone and vertebrae while fish fed diets with 500 FTU kg^{-1} and 1,000 FTU kg^{-1} phytase exhibited the highest P content in scale, bone and vertebra, respectively. On the other hand, the fecal P concentrations were lower in fish fed diets containing bacterial phytase than did those fed the P_i. The P_i diet resulted in significantly the highest fecal P. The fecal P of fish fed diets supplemented with 1,000 FTU kg^{-1} and 500 FTU kg^{-1} bacterial phytases were reduced by 39 % and 27% respectively, compared to those fed the P_i diet (Table 2).

Table 2

Final average concentration (% dry matter) of crude ash, P, Ca and Mg in scales and bone and vertebrae of Nile tilapia fingerlings fed with dietary levels of *B. megaterium* phytase or P_i diets for 10 weeks

Dietary level	*Scales (%)*				*Bone & vertebrae (%)*				*P*
	Ash	*P*	*Ca*	*Mg*	*Ash*	*P*	*Ca*	*Mg*	*Feces*
250 FTU kg^{-1}	31.18 ± 0.5	3.78 ± 0.4	12.10 ± 0.8	0.32 ± 0.1	49.39 ± 2.0	4.47 ± 0.01	18.98 ± 0.04	0.60 ± 0.01	1.29 ± 0.1^{b}
500 FTU kg^{-1}	35.45 ± 0.9	3.93 ± 0.5	13.54 ± 1.0	0.36 ± 0.03	49.34 ± 1.0	4.82 ± 0.01	19.19 ± 0.9	0.61 ± 0.01	1.11 0.03^{ab}
1,000 FTU kg^{-1}	36.64 ± 0.1	3.63 ± 0.4	11.61 ± 1.0	0.35 ± 0.03	47.12 ± 1.3	5.19 ± 0.2	17.89 ± 0.5	0.58 ± 0.02	0.93 ± 0.1^{a}
1,500 FTU kg^{-1}	34.63 ± 0.04	3.53 ± 0.4	13.47 ± 0.7	0.37 ± 0.1	47.80 ± 1.3	4.58 ± 0.2	18.42 ± 1.0	0.60 ± 0.04	1.19 ± 0.1^{b}
P_i diet	37.12 ± 0.6	3.81 ± 0.3	14.43 ± 0.6	0.38 ± 0.02	51.30 ± 1.6	4.96 ± 0.4	19.58 ± 1.1	0.63 ± 0.03	1.52 ± 0.03^{c}

Values are means of triplicate groups, values in the same column not sharing a common superscript are significantly different ($p < 0.05$).

Microbial phytase is commercially available for use in animal diets especially in poultry and swine diets but are not available in some tropical countries including the Philippines. In the present study, crude phytase from *Bacillus* spp. (*B. megaterium*) was used as a feed additive for Nile tilapia. Crude phytase extract is more practical than using purified enzyme because the former has a much lower cost of production. The phytase extract was sprayed onto the surface of the feed pellets after the manufacturing process was completed. Apparently, it was an effective manner of application considering that no significant differences were observed in feed consumption, weight gain, specific growth rate, survival rate, feed conversion efficiency, protein efficiency ratio, protein retention, crude ash, P, Ca and Mg content of scales, bone and vertebrae of fish fed the various levels of bacterial phytase.

The present study demonstrated that reduction of P wastes could be achieved by incorporating phytase in diets. Phosphorus content in the feces demonstrated a significant reduction when fish fed with 1,000 FTU kg^{1}. The higher the incorporation of phytase the lower is the releases of P in the surrounding water. Nile tilapia fed with plant-based diet supplemented by 500-1000 FTU kg^{-1} phytase exhibited the highest feed conversion efficiency, protein efficiency ratio and protein retention than those fed a diet supplemented by P_i.

Scale P appears to be easily mobilized for utilization in the body when needed and therefore closely reflects dietary P status (Hughes & Soares 1998). Similar observations were made in the present study, where the highest P content was present in bone and vertebrae, scales and final body composition. The results agreed with the recommendation levels of 500 to 1,000 units phytase kg^{-1} diet reported for optimum performance of other animals (Simons et al 1990; Cromwell et al 1993; Cain & Garling 1995; Schafer et al 1995). One would expect that minimizing the amount of P going into the pond water would lower the P concentration, thereby potentially reducing phytoplankton growth (Robinson et al 2002).

Conclusions. The concentrations of ash, P, Ca and Mg in the scales, bone and vertebrae were not significantly different among treatments. Higher level of incorporation of phytase resulted in a remarkably higher absorption of minerals than did the incorporation of P_i. Higher level of P is retained in the bone and fecal P is reduced by about 33% less in fish fed supplemental phytase. Results of the present study showed that there was a significant reduction of 27% and 39% P in feces using the plant-based diet supplemented with 500 FTU kg^{-1} and 1,000 FTU kg^{-1} respectively, over that of the P_i diet. Crude extracts of bacterial phytase as described in the present study could replace the imported commercial phytase and its effect could be comparable to that of P_i supplementation.

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