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Effect of Dietary Phytase on Growth, Enzyme Activities and Phosphorus Load of Nile Tilapia (*Oreochromis niloticus*)

L.C. Nwanna
Department of Fisheries and Wildlife, Federal University of Technology, PMB 704 Akure, Nigeria

Abstract: This study assessed the effect of phytase on the growth and enzyme activities of Nile tilapia (*Oroechromis niloticus*) and on environmental Phosphorus (P) loadings. A basal diet of 30% crude protein, 2.0 g kg^{-1} phytate P and total P of 5.6 g kg^{-1} was supplemented with phytase (Ronozyme P (5000) CT) at 0, 2000, 4000, 6000 and 8000 units per kg diet. The diets were tested on Nile tilapia (14.0±0.60 g) for 70 days. Results indicated that all fish fed diets with phytase had higher Mean Weight Gain (MWG), Specific Growth Rate (SGR), mineral contents and better feed conversion ratio than the fish fed diet without phytase. The MWG (11.6 g) and SGR (0.86%) of the fish fed diet with 8000 units phytase kg^{-1} diet were higher ($p<0.05$) than those of the fish fed diets 1 (8.30 g; 0.67%), 2 (8.70 g; 0.69%), 3 (8.90 g; 0.70% and 4 (9.60 g; 0.74%). Analyses of the gut of the fish after the feeding indicated increased amylase, lipase and protease activities in the fish fed diets with phytase in comparison with the fish fed diet without phytase. These activities increased with increase in the level of the phytase. Magnesium (Mg) contents of the fish fed diets with 6000 (72.4 mg g^{-1} DM) and 8000 (71.6 mg g^{-1} DM) units phytase kg^{-1} diet were statistically the same. These contents were however higher ($p<0.05$) than those of the fish fed diets 1 (45.2 mg g^{-1} DM), 2 (59.6 mg g^{-1} DM) and 3 (68.5 mg g^{-1} DM). Ca and Zn deposition were the same in all the fish, while Mn contents of the fish fed diets with phytase, diet 2 (8.49 ug g^{-1} DM), diet 3 (8.29 ug g^{-1} DM), diet 4 (8.80 ug g^{-1} DM) and diet 5 (9.35 ug g^{-1} DM) were higher ($p<0.05$) than that of the fish fed diet 1 (5.50 ug g^{-1} DM) without phytase. The Phosphorus (P) load decreased linearly with increase in the level of phytase, such that the P released by fish fed diets 2 (19.5 mg g^{-1} DM), 3 (19.1 mg g^{-1} DM), 4 (18.9 mg g^{-1} DM) and 5 (16.8 mg g^{-1} DM) was significantly lower than 24.0 mg g^{-1} (DM) P released by fish fed diet 1 without phytase.

Key words: Phytase, *Nile tilapia*, enzyme activities, P loadings, MWG, phosphorus

INTRODUCTION

According to Cheng and Hardy (2002) about 2/3 of Phosphorus (P) in plants feed stuffs are in the form of phytate, which is not digestible by fishes; because they lack intestinal phytase (Libert and Portz, 2005). Phytate reduces the bioavailability of dietary components including multivalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} (Anderson, 1985) resulting in increasing cost of feed production through supplemental minerals(Satoh *et al*., 1991). Phytate complexes with proteins, lipids and starch (Cosgrove, 1966) thereby reducing their availability in feeds. The reduced solubility of proteins as a result of such complexing can adversely affect certain functional properties of proteins, which are dependent on their hydration and solubility (Cheryam, 1980). Phytate protein interactions can also influence the digestion and absorption of protein and amino acids (Ravindran *et al*., 1995). Phytate also inhibits the activities of a number of digestive enzymes such as pepsin, α-amylase and trypsin (Ravindran *et al*., 1995). Selle *et al*. (2000) observed that phytate may increase secretion of digestive enzymes into the gut, thus increasing the endogenous loss of amino acids. Richardson *et al*. (1985) associated structural abnormalities observed in the epithelial layer of the pyloric caecal region of fish intestine with either a toxic effect of phytic acid or as a result of reduced magnesium bioavailability by phytate. Therefore, hydrolysis of phytate is necessary in fish diets. Phytase has been used for dephosphorylation of phytate in fish feeds (Ketola, 1994; Li and Robinson, 1997) because microbial phytase is well suited to feed application as the pH optimum is low and compatible with gastric conditions where phytate is most soluble (Campbell and Bedford, 1992). Besides, phytase can liberate bound minerals in plant feedstuffs, thus sparing a certain portion of dietary P and minerals. Therefore, this study was conducted to investigate the effect of different levels of phytase on the growth, mineral deposition and enzyme activities of Nile tilapia; and on environmental P loadings.

MATERIALS AND METHODS

Diet preparation: Five isoproteic diets of 30% protein were formulated to contain different levels of phytase (Ronozyme P (5000) CT). Diet 1 is a control diet and contained no phytase. Diet 2 contained 2000 units of phytase per kg diet. Diet 3 contained 4000 units of phytase per kg diet. Diet 4 contained 6000 units of phytase per kg diet and Diet 5 contained 8000 units of phytase per kg diet. All the ingredients used (Table 1) were purchased from a local market and milled into powder form before use. The respective quantities of the soybean meal and phytase in each diet were firstly mixed together before mixing with other ingredients. The ingredients were thoroughly mixed in Hobart A-200 (Troy Ohio USA) pelleting machine until homogenous masses were obtained. The homogenised masses were extruded through 0.8mm die and pelleted into noddle-like strands which were mechanically broken into sizes, oven dried at 48°C for 48h, packed in cellophane bags and stored at –20°C prior to use.

Analysis of dietary phytate: About 8 g of finely ground sample of the 5 diets was soaked in 200 mL of 2% HCl for 3 h and then filtered using Whatman No 1 filter paper. Fifty millilitres of the filtrate was pipetted into 400 mL beaker and 10 mL of 0.3% ammonium thiocyanate solution was added as an indicator. One hundred seven millilitres of distilled water was added to give pH 4.5. The solution was then titrated with standard ferric chloride solution containing 0.00195g Fe mL^{-1} until a brownish yellow colour persisted for 5 min. The Fe equivalent was multiplied by 1.19 to get phytate-phosphorus. This was converted to phytate by multiplying the value of phytate–phosphorus by 3.55 (Young and Greaves, 1940). For analysis of total P, about 2.0g of the diets were ashed for 48 h at 480°C. After the ash had cooled to room temperature, 6 mL of 6 N HCl was added and the mixture was brought to boiling point. After cooling to room temperature, another 2.5 mL of 6 N HCl was added and the mixture was warmed to dissolve all the solutes. The solution was then cooled and diluted to 25 mL with distilled deionized water. P contents were determined using the vanadomolybophosphoric acid colorimetric method.

Feeding trial: A total of 320 juveniles (14.0±0.60g) of Nile tilapia, *Oreochromis niloticus* were obtained from the Federal University of Technology Akure Teaching and Research Fish Farm. The fish were acclimated for 2 weeks in glass tanks in the laboratory. After acclimation, 12 healthy and strong fish were randomly stocked into flow-through glass tanks with flow rate of 1 L min^{-1}. The fish were fed the 5 diets to satiation twice daily. Each treatment was replicated thrice. All fish in each tank were weighed bi-weekly. The feeding lasted for 70 days. The water quality parameters of the culture tanks were measured at 0900 h using standard methods. Water temperature and dissolved oxygen were measured daily using a combined digital YSI DO meter (YSI model 57); pH was monitored weekly using an electronic pH meter (Metler Toledo 320 model).

Table 1: Gross composition of experimental diets (30% Crude Protein, CP)

	Diets/Treatments				
Ingredients (g kg^{-1} DM)	1	2	3	4	5
Menhaden fish meal (65% CP)	100	100	100	100	100
Soybean meal (45% CP)	446	446	446	446	446
Wheat (18% CP)	191.1	191.1	191.1	191.1	191.1
Maize	182.9	182.9	182.9	182.9	182.9
Vitamin-mineral premix	20	20	20	20	20
Carboxymethyl cellulose	10	10	10	10	10
Vegetable oil	50	50	50	50	50
Phytate P (g kg^{-1})	2.00	2.00	2.00	2.00	2.00
Total P (g kg^{-1})	5.60	5.60	5.60	5.60	5.60
Phytase (g kg^{-1})	0	0.4	0.8	1.2	1.6

Collection of faecal sample: Faecal samples were collected from each of the tanks four weeks before the end of the feeding trials. After feeding the fish for the second time in the day, the uneaten feeds were siphoned out, after which detachable glass tubes were connected to the flow-systems; and after 8 h the faeces were collected from the settling column of the tubes. The collected samples were dried at 105°C for 5h, pooled for each treatment and stored at -20°C before use.

Proximate analysis: The five diets, fish samples (whole body) before and after the experiments were prepared and analyzed for their proximate composition according to the methods of AOAC (1990).

Mineral analyses: Three replicates of the fish carcass (whole body) and faeces were analysed for minerals according to the methods of AOAC (1990). About 2.0 g of the samples were ashed for 48 h at 480°C. After the ash had cooled to room temperature, 6 mL of 6 N HCl was added and the mixture was brought to boiling point. After cooling to room temperature, another 2.5 mL of 6 N HCl was added and the mixture was warmed to dissolve all the solutes. The solution was then cooled and diluted to 25 mL with distilled deionized water. Then the minerals (Mg, Ca, Zn, Mn) were measured in Atomic Absorption Spectrophotometer (AAS). P contents were determined using the vanadomolybophosphoric acid colorimetric method.

Determination of digestive enzyme activities: Digestive enzyme activities in the fish were assayed as follows: At the end of the experiment, 3 fish each per treatment were sacrificed. The dead fish were dissected and their gut contents removed. The respective gut contents were homogenised in 10 mL of 0.2M phosphate buffer (pH 7) solution. The homogenate was centrifuged at 2,500 rev g^{-1} for 30 min at 37°C in an "MSE" minor centrifuge. The clear supernatant was obtained as crude enzyme extract. The supernatant was stored at –20°C before further analyses. The enzyme activities (amylase, lipase and protease) were assayed in duplicate. The amylase activity was measured based on the method of Rauscher *et al.* (1986) using DNSA (Dinitrosalicytic acid) as substrate. Total protein was measured using the Bio Rad test kit based on the method of Bradford (1976) using Bovine Serum Albumin (BSA) as a standard.

The turbidimetric method described by Neumann *et al.* (1984) was used for measuring lipase activity. The results were expressed as units per mg total protein. The absorbance was measured in a spectrophotometer (SPECTRONIC model 21D; Rochester, NY,USA) at 540nm.

Statistical analysis: Data resulting from the experiment were subjected to one way Analysis of Variance (ANOVA) test using the SPSS (Statistical Package for Social Science 1998 version). Individual differences (p=0.05) among treatment means were separated using Duncan`s multiple range test (Duncan, 1955).

RESULTS

Proximate contents of the diets (Table 2) are closely related. This is an indication that differences in growth and other parameters may not be attributed to dietary composition. The culture water temperature ranged between 27 and 30°C, dissolved oxygen between 6.0 and 8.2 mg L^{-1} while pH varied from 8.0-8.9. These values are good for culturing tropical fishes and did not pose any stress factors to the fish during the experiment. The growth and nutrient utilization data (Table 3) indicated no significant differences in the mean weight gain of the fish fed diets without phytase (control diet) and those fed diets that contained 2000 units of phytase kg^{-1} diet (diet 2) , 4000 units of phytase kg^{-1} diet (diet 3) and 6000 units of phytase kg^{-1} diet (diet 4). However, the fish fed diets that contained 8000 units of phytase kg^{-1} diet (diet 5) had significantly higher mean weight gain than the fish fed diets 1-4. Table 3 also showed no significant differences in the daily feed intake and feed conversion ratio of the fish fed the different diets. The Specific Growth Rate (SGR) was similar (p>0.05) in the fish fed diets 1-4. However, the fish fed diet 5 had the highest significant SGR. The results of the fish carcass composition (Table 4) showed that the values of protein, lipid, Nitrogen Free Extract (NFE), fibre and ash were similar indicating good dietary acceptance and digestibility. The results of the mineral composition of the fish and faecal P load after the experiment are presented in Table 5. The table indicated that Mg and Mn deposited in all the fish fed diets that contained phytase

Table 2: Proximate composition of experimental diets

	Treatments				
Parameters	1	2	3	4	5
Protein	31.6±1.2	31.1±1.0	29.7±0.3	30±0.6	30.2±1.1
Lipid	17.8±3.2	19.3±2.3	18.2±0.8	19.3±1.4	19.2±0.7
NFE	31.0±2.3	30.0±2.1	31.2±0.2	31.8±1.8	30.9±4.2
Fibre	4.28±3.0	3.94±2.1	3.48±0.3	3.23±3.6	2.94±1.3
Ash	6.01±0.3	6.05±2.6	6.43±0.5	6.6±0.7	6.60±2.3

Table 3: Growth performance of Nile tilapia fed phytase diets for 70 days

	Treatments				
Parameters	1	2	3	4	5
Initial mean weight (g)	14.0±0.6	14.0±0.1	14.1±0.6	14.1±0.2	14.1±0.0
Final mean weight (g)	22.3±0.6	22.7±1.2	23.0±0.1	23.7±1.6	25.7±2.1
Mean weight gain (g)	8.30±0.6^{a}	8.70±1.1^{a}	8.90±0.8^{ab}	9.60±1.4^{ab}	11.6±1.9^{c}
SGR (%)[1]	0.67±0.1^{a}	0.69±0.1^{a}	0.70±0.2^{a}	0.74±0.1^{a}	0.86±0.2^{b}
FCR [2]	1.69±3.2^{a}	1.49±2.1^{a}	1.31±1.1^{a}	1.31±5.8^{a}	1.20±1.0^{a}

1. Specific growth rate = 10^{2} (Log_{e} final weight -Log_{e} initial weight)/culture period (days), 2. Food conversion ratio = Dry weight of feed fed (g)/ fish weight gain

Table 4: Carcass composition of *O. niloticus* fed phytase diets for 70 days

	Treatments				
Parameters	1	2	3	4	5
Protein	61.3±2.9	60.3±3.1	61.2±2.6	60.4±1.8	60.0±2.0
Lipid	12.9±2.3	13.8±2.6	13.8±1.3	12.6±1.4	13.0±2.2
NFE	2.95±2.1	2.03±2.3	2.20±1.4	2.84±1.2	2.20±2.7
Fibre	1.81±1.2	1.76±0.6	1.77±1.3	1.79±2.4	1.68±2.2
Ash	21.1±3.2	22.1±2.3	21.0±2.2	22.4±4.2	23.1±4.3

Table 5: Mineral contents and P load of *O. niloticus* fed phytase diets for 70 days

	Treatments				
Parameters	1	2	3	4	5
Mg (mg g^{-1})	45.2±0.1^{a}	59.6±4.0^{b}	68.5±5.8^{c}	72.4±0.1^{c}	71.6±0.6^{c}
Ca (mg g^{-1})	22.0±0.1^{a}	32.4±3.8^{a}	27.1±9.4^{a}	39.0±18.8^{a}	29.0±0.9^{a}
Zn (µg g^{-1})	13.7±0.1^{a}	16.3±2.5^{a}	14.4±1.3^{a}	19.5±5.2^{a}	19.0±4.3^{a}
Mn (µg g^{-1})	5.50±0.6^{a}	8.49±1.7^{b}	8.29±0.2^{b}	8.80±0.6^{b}	9.35±0.1^{b}
P load (mg g^{-1})	24.0±0.6^{a}	19.5±0.2^{b}	19.1±0.1^{b}	18.9±0.6^{b}	16.8±1.0^{c}

Table 6: Amylase, lipase and protease activities (mg L^{-1}) of *O. niloticus* fed phytase diets for 70 days

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
Amylase	0.12±0.01^{c}	0.17±0.01^{b}	0.17±0.02^{b}	0.19±0.02^{ab}	0.22±0.02^{a}
Lipase	0.61±0.02^{c}	0.67±0.01^{b}	0.68±0.04^{b}	0.70±0.01^{ab}	0.73±0.01^{a}
Protease	1.58±0.01^{d}	1.75±0.01^{c}	1.96±0.02^{b}	2.04±0.02^{a}	2.11±0.01^{a}

were significantly higher than that deposited in fish fed diet without phytase. Ca and Zn contents of all the fish were the same ($p>0.05$). The table also showed that all the fish fed diets with phytase discharged less P than the fish fed diet without phytase. Also the P discharged decreased with increase in phytase levels. Table 6 showed the enzyme activities of the fish fed the various diets. The table indicated that amylase, lipase and protease activities were stimulated, apparently in response to phytase present in the diets. This enhancement also increased with increase in the levels of phytase. The presence of phytase in the diets significantly mproved the enzyme activities of the fish, which resulted in corresponding increase in the performance of the fish (Table 3).

DISCUSSION

The experimental tanks, water parameters, temperature, pH and dissolved oxygen were within the acceptable ranges recommended by Boyd (1990) for good warm water fish culture. The results of the growth and nutrient utilization indices indicated that the effect of the phytase improved with increase in the level of inclusion. Yan *et al.* (2002) reported that dephosphorylation of phytate in the stomach of channel catfish *Ictalurus punctatus* increased with the level of phytase supplementation. This further explains that more nutrients were made availabe in phytase treated diets, which the fish converted well into flesh. Shäfer *et al.* (1995) reported increase in the weight gain and better feed conversion by common carp fed phytase treated soybean meal based diets. This observation is in consonant with the findings from the present study that phytase treatment of soybean meal based diets increased weight gain and promoted better feed conversion in Nile tilapia, *Oreochromis niloticus.* Cain and Garling (1995) also reported that weight gain and feed conversion ratio of salmonid fed phytase treated soybean meal were equal or greater than in the same fish fed diet without phytase, while Hughes and Soares (1998) reported improved feed conversion by striped bass (*Morone saxatilis)* fed phytase treated diets.

The mineral deposition in the fish after the experiment increased with increase in the enzyme levels. Supplementation of 2000, 4000 and 6000 units of phytase kg^{-1} diet increased Mg and Mn deposition significantly relative to that deposited in fish fed diet without phytase. Similarly addition of 2000, 4000 and 6000 units of phytase kg^{-1} diet marginally improved Ca and Zn deposition. The significant differences in the Mg and Mn obtained from the present study are in line with work of Cheng and Hardy (2002) who reported significant differences in Mg and Mn deposition in rainbow trout as a result of phytase treatment of the plant proteins. Storebakken *et al.* (1998) similarly reported higher Ca, Mg and Zn deposition in Atlantic salmon fed phytase treated soy-protein concentrate, than in the same fish fed diet without phytase. The report of Sugiura *et al.* (2001) that phytase supplementaion increased the apparent absorption of protein, Ca, Mg and Zn in low-ash diets containing soybean meal further supports the findings from the present study.

Phosphorus loadings reduced with increase in the phytase levels. This means that more P was absorbed by the fish. Cain and Garling (1995) recorded that pretreatment of soybean meal with phytase increased P availability in soybean meal by hydrolyzing phytin P to an available inorganic form; which improved feed digestibility, feed conversion and growth rate in salmonid fish. The reduction in the P loadings is an indication that phytase can regulate the level of P discharged into the environment and consequently control pollution in fish culture systems. Sugiura *et al.* (2001) reported that excretion of Phosphorus (P) in the faeces of fish fed a low-ash diet containing phytase-treated soybean meal was reduced by between 95 and 98% compared with P excretion by fish that consumed commercial diet without phytase.

Digestive enzymes are physiological parameters functionally linked to fish performances (Blier *et al.*, 1997). In the present study, supplementation of phytase in the diets increased the amylase, lipase and protease activities in the gut of the fish which enhanced the hydrolysis of the diets and nutrient liberation, digestibility, growth and mineralization in the fish.

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