Egyptian Journal of Aquatic Research (2013) 39, 205–213

National Institute of Oceanography and Fisheries

Egyptian Journal of Aquatic Research

http://ees.elsevier.com/ejar
www.sciencedirect.com

FULL LENGTH ARTICLE

Influences of calcium/phosphorus ratio on supplemental microbial phytase efficiency for Nile tilapia (*Oreochromis niloticus*)

M.S. Hassaan [a,*], M.A. Soltan [b], H.M. Agouz [c], A.M. Badr [d]

[a] *Fish Nutrition Research Laboratory, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt*
[b] *Animal Production Department, Faculty of Agriculture, Benha University, Egypt*
[c] *Central Laboratory for Aquaculture Research, Abbassa, Abou-Hammad, Sharkia, Egypt*
[d] *Regional Center for Food and Feed Ministry of Agriculture, Cairo, Egypt*

Available online 19 October 2013

KEYWORDS

Ca/P ratio;
Calcium-propionate;
Phytase;
Oreochromis niloticus

Abstract A 3 × 3 factorial feeding trial was conducted to evaluate the effects of microbial phytase (MP) supplementation fed with Ca/P ratio on growth, digestibility, vertebral mineralization and some blood parameters in Nile tilapia *Oreochromis niloticus*. Three levels of phytase (0, 500 and 1000 U kg^{-1} diet) were combined with three Ca/P ratios (0.3:1, 0.6:1 and 0.9:1), respectively. The Ca/P ratios were achieved by supplementing calcium propionate at (0, 5 and 10 g kg^{-1} diet). After a 84-day feeding trial, tilapia fish fed 500 and 1000 U kg^{-1} diet at Ca/P ratio (0.6:1) had significantly higher growth rate, feed intake (FI) crud protein, vertebrae ash and phosphorus than other groups. Interaction between Ca/P and MP are significantly ($p < 0.001$) affected by all parameters of digestibility and blood parameters. The highest apparent digestibility recorded by fish fed diet supplemented with 1000 U kg^{-1} with 0.6:1 Ca/P ratio. The highest triglyceride was found in fish fed MP 500 or 1000 U kg^{-1} and combined with 0.6:1 Ca/P ratio, while, fish fed MP 500 U kg^{-1} with Ca/P 0.6:1 showed the highest value of cholesterol and serum. No significant differences were found in serum aspartate aminotransferase (AST) and alanine aminotransferase ALT of fish fed experimental diet.

Introduction

As with terrestrial vertebrates, calcium (Ca) is essential for normal growth and physiological function of aquatic species such as muscle function and nerve transmission in aquatic species (Lovell, 1989; NRC, 1993). Calcium is abundant in water and it is generally accepted that fish can absorb Ca from the surrounding water to fulfill part or all of the metabolic Ca requirement (Love, 1980).

* Corresponding author. Tel.: +20 19490092; fax: +20 242185320.
E-mail address: Mohamed_shaban200065@yahoo.com (M.S. Hassaan).

Peer review under responsibility of National Institute of Oceanography and Fisheries.

Production and hosting by Elsevier

1687-4285 © 2013 Production and hosting by Elsevier B.V. on behalf of National Institute of Oceanography and Fisheries.
http://dx.doi.org/10.1016/j.ejar.2013.09.001

Requirement of dietary Ca has only been studied for a few species, and the results vary depending on species and concentration of Ca in the water. Dietary Ca requirement in channel catfish and tilapia reared in Ca-free water has been estimated to be 4.5 and 7 g kg^{-1}, respectively (Robinson et al., 1986, 1987). In addition, (Shiau and Tseng, 2007) reported that for better growth, bone and scale Ca concentration of juvenile tilapia reared in water containing 27.1–33.3 mg Ca L^{-1} needed adequate dietary Ca supplement of 3.5, 4.3 and 4.2 g kg^{-1}, respectively.

Otherwise Ca and the importance of P supplementation in diet have widely been reported in many species including fresh water species such as common carp (Kim et al., 1998), rainbow trout (Ogino et al., 1979). Several studies suggest that the ratio of Ca to other minerals, particularly P should be considered, because an excess of Ca relative to P has been shown to adversely affect the growth and survival of some species such as *Penaeus vannamei* (Davis et al., 1993) and grouper (Ye et al., 2006). In diet, it is necessary to take into account the Ca/P ratio, because it has important consequences for bone development. However, when this ratio increases harmful effects can appear. Also, several aberrations in bone mineral homeostasis and bone metabolism are associated with higher Ca/P ratio (Kumar et al., 2011). The recommended levels of Ca/P ratio for fish are in range of 1:1 to 1:1.7 (Sanchez et al., 2000; Ye et al., 2006).

Phytase is an enzyme chemically known as myoinositol hexaphosphate phosphohydrolase and belongs to Class 3: hydrolases, that may be produced either by microorganisms or may be present in some plant ingredients. It is very specific to hydrolyze the indigestible phytate that is present in plant protein sources. Supplementation of phytase in fish feeds has been generally reported to improve the bioavailability and utilization of plant phosphorus (P) by fish (Cao et al., 2007).

Efficacy of microbial phytase is governed directly or indirectly by numerous interactive factors. These may include dietary substrate levels, fish species, the inclusion rate and source of phytase (Cao et al., 2007; Liebert and Portz, 2005). However, dietary Ca levels and Ca/P ratios are also crucial to phytase efficacy. Angel et al. (2002) reviewed that dietary level of Ca (and Ca/P ratios) is crucial to phytase efficacy in poultry. Additionally, increasing Ca/P ratio depressed *Escherichia coli* derived phytase action in the pig diet and thus significantly depressed weight gain and feed efficiency (Adeola et al., 2006). As the Ca to P ratio increases, microbial phytase activity decreases (Qian et al., 1996; Tamin et al., 2004). Moreover, Cao et al. (2007) recommended Ca/P ratios in fish meal in the range of 1.1–1.4:1 at which phytase executes high efficiency. In case of fish, there has been a lack of information relating to dietary Ca/P ratio affecting the ability of supplementary phytase to affect nutrient

Therefore the objective of the current study was to characterize the influence of phytase, Ca/P ratio and their interactions on growth, nutrient digestibility and some blood parameters of *Oreochromis niloticus* fed a plant protein–based diet.

Material and methods

Experimental design and diets

Diets were formulated to meet all of the known requirements of *O. niloticus*. A 3 × 3 factorial experiment was designed to study the effects of dietary microbial phytase (MP), Ca/P and their interactions on growth performance, nutrient digestibility and blood chemistry. The Ca/P ratios were achieved by supplementing calcium at three different levels of (0.3:1, 0.6:1 and 0.9:1) using calcium propionate from the Pharmaceutical Company Adoia Cairo Egypt. Ca-propionate was supplemented at 0, 0.5, and 1 g kg^{-1} dry diet as a source of Ca/P ratio. The basal diet was formulated using plant-based ingredient to contain approximately 17.89 MJ kg^{-1} diet and 30% crud protein kg^{-1} diet (Table 1). MP (Natuphos 7500 U g^{-1}) derived from *Aspergillus niger* was supplied by the Regional Center for Food and Feed Ministry of Agriculture Cairo, Egypt. Diets were prepared by blending all the ingredients except the vitamins and minerals mixture in a plastic bowl. Calcium propionate and oil was added to the mixed ingredients, chromic oxide (0.5%) was added in all the tested diets to determine the apparent digestibility coefficient and absorption of minerals. Pellets were prepared by using a laboratory pellet mill (2-mm die) in National Institute of Oceanography and Fisheries, Cairo Governorate, Egypt (CPM, California Pellet Mill Co., San Francisco, CA, USA). Required amount of MP was dissolved in 50 mL of distilled water and sprayed over 1 kg of the finished diet as described by Robinson et al. (2002). Similar amount of distilled water (50 mL) was sprayed to the control diet to maintain an equal level of moisture. The diets were kept at 4 °C until use. Test diets were then prepared by spraying graded levels of phytase to plant protein based diet at 0, 500 and 1000 U kg^{-1} diet. One unit of phytase activity (U) is defined as the enzyme activity that liberates 1 μmol of inorganic orthophosphate min^{-1} at pH 5.5 (37 °C) at a substrate concentration (sodium phosphate) of 5.1 μmol L^{-1} (Engelen et al., 1994).

Fish source and management

Fishes were obtained from the Abbassa hatchery, Abbassa village, Abu-Hammad district, Sherkia Governorate, Egypt. Fish were stocked in fiberglass tanks for two weeks before the start the experiment for acclimation where all fish were fed daily on the basal diet containing 30% crude protein at a rate of approximately 3% of their average body weight to be adapted to pelleted feeds according to Hassan et al. (2013). After the acclimatization the experimental fish were distributed randomly into the experimental plastic tanks (150 L for each) representing the nine treatments studied (triplicate for each treatment). A set of 675 fish of *O. niloticus* L. mono-sex males fingerlings average initial weight of (2.67 ± 0.02 g). Twenty-five of fish were randomly stocked into each tank with three replications per treatment. About one-third of water volume in each tank was daily replaced by aerated fresh water after cleaning and removing the accumulated excreta. All tanks were supplied with compressed air for oxygen requirements.

The water temperature and dissolved oxygen were measured every day using a YSI model 58 oxygen meter (Yellow Spring, OH, USA) total ammonia and nitrite were measured twice weekly using a DREL, 2000 spectrophotometer (Hash Company, Loveland, CO, USA). Total alkalinity and chloride were monitored twice weekly using the titration methods; pH meter (pH pen; Fisher Scientific, Cincinnati, OH, USA). During the 84 days feeding trail, the water quality parameters averaged (±SD): water temperature 27.8 ± 0.8 °C: dissolved oxygen, 5.9 ± 0.7 mg L^{-1}: total ammonia, 0.20 ± 0.12 mg L^{-1} nitrite,

Table 1 Composition and proximate analysis of the experimental diets.

Ingredient (%)	Experimental diet Ca/P ratio								
	0.3:1	0.6:1	0.9:1	0.3:1	0.6:1	0.9:1	0.3:1	0.6:1	0.9:1
Fish meal	5	5	5	5	5	5	5	5	5
Soy meal	35	35	35	35	35	35	35	35	35
Corn gluten	5	5	5	5	5	5	5	5	5
Yellow corn	19	19	19	19	19	19	19	19	19
Wheat bran	9.5	9	8.5	9.5	9	8.50	9.5	9	8.5
DDGs	20	20	20	20	20	20	20	20	20
Soya oil	4	4	4	4	4	4	4	4	4
Vit. and Min[a]	2	2	2	2	2	2	2	2	2
Calcium-P[b]	0	0.5	1	0	0.5	1	0	0.5	1
Chromic oxide	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sum	100	100	100	100	100	100	100	100	100
Chemical analysis (determined on dry matter basis)									
Dry matter	90.23	89.73	89.29	90.01	89.69	89.24	89.93	89.48	89.04
Crude protein	30.18	30.13	30.05	30.47	30.48	30.39	30.25	30.18	30.11
Ether extract	7.57	7.56	7.54	7.56	7.55	7.53	7.58	7.56	7.54
NFE[c]	57.99	57.32	57.85	57.62	57.78	57.89	57.22	57.37	57.46
Ash	4.63	4.87	5.09	4.64	4.87	5.08	4.65	4.87	5.05
GE(MJ kg^{-1} diet) [d]	18.05	17.91	17.78	18.02	17.91	17.79	17.99	17.86	17.74
Total P (%)	1.180	1.130	1.139	1.162	1.171	1.161	1.153	1.720	1.620
Total available P (%)	0.381	0.382	0.384	0.380	0.381	0.383	0.381	0.380	0.384

[a] Vitamin and mineral mix (per kg of diet): $MnSO_4$, 40 mg; MgO, 10 mg; K_2SO_4, 40 mg; $ZnCO_3$, 60 mg; KI, 0.4 mg; $CuSO_4$, 12 mg; Ferric citrate, 250 mg; Na_2SeO_3, 0.24 mg; Co, 0.2 mg; retinol, 40000 IU; cholecalciferol, 4000 IU; α-tocopherolacetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mcg; nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg.

[b] Calcium-P = Calcium propionate (MW = 186.22).

[c] NFE nitrogen free extract = 100-(CP + EE + ash).

[d] Calculated using gross calorific values of 23.63, 39.52 and 17.15 KJ g^{-1} for protein, fat and carbohydrate, respectively according to Brett (1973).

0.08 ± 0.03 mg L^{-1}: total alkalinity, 169 ± 42 mg L^{-1}: chlorides, 565 ± 152 mg L^{-1}: pH 8.6 ± 0.3, all tested water quality criteria (temperature, pH value DO) were suitable and within the acceptable limits for rearing Nile tilapia *O. niloticus* fingerlings and agree with (El-Greirsy and El-Gamal, 2012). A photoperiod of 12-h light, 12-h dark (08:00–20:00 h) was used. Fluorescent ceiling lights supplied the illumination. During the 84-days experimental period, all fish were fed the experimental diets during 6 day $week^{-1}$. Fish were hand-fed with the respective diet to apparent satiation two times daily for 84 days. Thirty minutes after the feeding, uneaten feed were removed by siphoning, and then dried and weighted. Feed intake was the difference between them and expressed as the total feed intake in 84 days per fish. At the termination of the trail a sample of five fish randomly sampled from each tank. Fish samples were pooled, ground, stored in polyethylene bags and frozen for until the chemical analysis. Body moisture, crude protein, lipid and ash contents were determined according to AOAC methods (AOAC, 1995).

Growth performance and feed utilization parameters

Growth performance and feed utilization were measured in terms of final body weight (g), weight gain (WG), specific growth rate (SGR, % day^{-1}) feed conversion ratio (FCR), Protein efficiency ratio (PER) and feed intake. Growth response parameters were calculated as follows:

$$\text{Weight gain(WG)} = \text{final body weight(g)} - \text{initial body weight(g)}$$

$$\text{Specific growth rate(SGR)} = 100 \times ((\text{Ln(W2)} - \text{Ln(W1)})/\text{T})$$

where: Ln = the natural log; W1 = initial body weight; W2 = final body weight and T = period of study (84 day).

$$\text{Feed conversion ratio(FCR)} = \text{Feed intake(FI)(g)}/\text{WG(g)}$$

$$\text{Protein efficiency ratio(PER)} = \text{WG(g)}/\text{Protein intake(g)}$$

$$\text{PPV\%} = (\text{protein gain(g)}/\text{protein intake(g)}) \times 100;$$

$$\text{FR\%} = (\text{fat gain(g)}/\text{fat intake(g)}) \times 100.$$

$$\text{ER\%} = (\text{energy gain(kJ)}/\text{energy intake(kJ)}) \times 100.$$

To determine bone ash and phosphorus, previously frozen fish were boiled for about 10 min in water until the flesh and bone were easily separated. Soft tissues were carefully removed from the vertebrae. Isolated vertebrae were rinsed with distilled water and dried in an oven at 105 °C for 24 h. After drying, samples were ground with a mortar and pestle and then defatted with solvent (chloroform: methanol 1:1), dried and

ashed in a muffle furnace (Vielma et al., 1998). The ash was weighed and subsequently analyzed for phosphorus by molybdovanadate method (AOAC, 1995). Phosphorus in the diet, initial and final whole-body, and feces were analyzed by the same method. Calcium in diet was analyzed by Atomic Absorption Spectrophotometer (AAS; Hitachi Z-2300, Tokyo, Japan)

Apparent nutrient digestibility

After two-month feeding of experimental diets, feces were collected from each aquarium once daily every morning prior to feeding for a one-month period. The feces were collected on a filter paper for drying as described by El-Saidy and Gaber (2002). The chemical analyses were conducted according to AOAC (1995). Chromic oxide was determined according to the procedure described by (Furukawa and Tsukahara, 1966). Apparent nutrient digestibility was calculated using equations of Schneider et al. (2004) as follows:

$$\mathrm{ADC}_{dietarynutrient} = 1 - (\mathrm{marker}_{diet}/\mathrm{marker}_{faces}) \times (\mathrm{nutrient}_{faces}/\mathrm{nutrient}_{diet}).$$

Blood samples and hematological parameters analysis

Blood samples were collected at the end of the experiment. The fish were anesthetized with t-amyl alcohol then the blood samples were taken by puncturing the caudal vessels. Serum was obtained by centrifugation of the blood samples at 3,000*g* for 10 min and stored at −20 °C for further analysis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to the method described by Reitman and Frankel (1957). Serum cholesterol and triglycerides were measured using standard Kits (Modern Laboratory Kits)

Statistical analysis

Data were analyzed using a two-way ANOVA with MP levels and Ca/P ratio. Statistical significance was set at the 5% probability level and means were separated using Duncan's new multiple range test. Data were statistically analyzes by using the software SAS, version 6.03 (Statistical Analysis System, 1993). All data are expressed as mean ± SE.

Results

Growth performance

During the whole growth trial, No mortality occurred over the 84 days. At the end of experiment, the highest FBW, WG, SGR, FI and the best FCR were recorded by fish fed 500 or 1000 U kg^{-1} diet with 0.6: 1 Ca/P ratio, while, fish fed diet not containing MP with 0.3:1 Ca/P ratio recorded the lowest parameters of growth performance and feed utilization (Table 2).

Regardless of the effect of Ca/P ratio, results of Table 2 indicated that FBW, WG and SGR of fish fed diet supplemented with 500 or 1000 U kg^{-1}, while PER, PPV, FR and ER recorded the highest values for fish fed diet supplemented with 1000 U kg^{-1} diet and the same diet showed the best FCR.

Concerning the effects of Ca/P ratio, results in Table 2 revealed that fish fed diet with 0.6:1 Ca/P ratio released the highest in FBW, WG, SGR, FI, best FCR, PER and PPV and ER compared with 0.3:1 and 0.9:1 Ca/P in diet and the differences were significant ($p < 0.05$).

Chemical composition in whole body of fish and vertebra

Chemical compositions of whole fish and vertebrae are shown in Table 3. The dietary MP and different Ca/P ratio and their interaction had significantly ($p < 0.05$) effect on the chemical composition values of whole fish and vertebrae.

The highest crude protein, vertebrae ash, and vertebrae phosphorus were found in fish fed diet supplemented with 1000 U kg^{-1} MP and combined with 0.6:1 Ca/P ratio, while, fish fed diet supplemented with 500 U kg^{-1} MP with the high ratio of Ca/P 0.9:1 and diet supplemented with 1000 U kg^{-1} MP with 0.60:1 or 0.90: 1 Ca/P ratio showed the highest value of ash. Also, high level of MP supplemented in diet with a high level of Ca/P ratio showed a higher lipid content than other diets.

Irrespective of Ca/P ratio, diet supplemented with 1000 U kg^{-1} recorded higher crude protein and vertebrae phosphorus and ash than the un-supplemented diet, while, the higher lipid content was found in fish fed diet supplemented with 500 U kg^{-1} MP. With regard to Ca/P ratio, fish fed diet with 0.6:1 Ca/P ratio showed the highest crude protein than other diets. Whereas, the highest value of vertebrae ash was recorded by diet with 0.9:1 Ca/P ratio. But, both ratios of Ca/P (0.6:1 and 0.9:1) showed the highest vertebrae phosphorus.

Apparent digestibility coefficient

Apparent digestibility coefficient for the experimental diets is presented in Table 4. Dietary Ca/P and MP phytase were significantly ($p < 0.001$) affected the digestibility of protein, fat, carbohydrate and energy. Interaction between Ca/P and MP significantly ($p < 0.001$) affected all parameters of digestibility. The highest apparent digestibility was recorded by fish fed on diet supplemented with 1000 U kg^{-1} with 0.6:1 Ca/P ratio. Supplement 1000 U kg^{-1} MP in diet significantly improved protein, lipid, carbohydrate and energy digestibility with respect to Ca/P. Regardless the effect of phytase, protein, fat, carbohydrate and energy the digestibility seemed to increase with increase of dietary 0.6:1 Ca/P ratio.

Blood parameters

Level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), phosphorus, triglyceride, cholesterol and phosphorus in the serum of Nile tilapia is presented in Table 5. The highest triglyceride was found in fish fed diet supplemented with 500 or 1000 U kg^{-1} MP and combined with 0.6:1 Ca/P ratio, while, fish fed diet supplemented with 500 U kg^{-1} MP with the medium ratio of Ca/P 0.6:1 showed the highest value of cholesterol and serum plasma. No significant differences were found in serum AST and ALT of fish fed on experimental diet. With regard to the effect of MP, phosphorus, triglyceride, cholesterol and phosphorus were higher in serum of *O. niloticus* fed diet supplemented with 1000 U kg^{-1} MP compared with other diets. With respect to

Table 2 Growth performance and feed utilization in *O. niloticus* after 84 days of feeding phytase supplemented with different Ca/P ratio.

Items	Growth performance and feed utilization									
	IBW	FBW	WG	SGR	FI	FCR	PER	PPV	FR	ER
Effect of MPU Kg^{-1}										
0	2.61	15.79^{b}	13.18b	2.00^{c}	26.23^{c}	2.03^{a}	1.67^{c}	30.74^{c}	45.62^{c}	32.05^{c}
500	2.64	17.41^{a}	14.77^{a}	2.10^{a}	28.08^{a}	1.91^{b}	1.75^{b}	32.72^{b}	47.14^{b}	33.39^{b}
1000	2.61	17.48^{a}	14.87^{a}	2.11^{a}	26.90^{b}	1.81^{c}	1.84^{a}	34.45^{a}	48.55^{a}	35.16^{a}
Pooled SE	0.012	0.204	0.078	0.004	0.034	0.011	0.061	0.062	0.336	0.113
Effect of Ca:p ratio										
0.3:1	2.62	16.26^{b}	13.63^{c}	2.02^{c}	26.70^{c}	1.97^{a}	1.70^{c}	31.51^{c}	46.44^{b}	32.74^{c}
0.6:1	2.62	17.64^{a}	15.02^{a}	2.11^{a}	27.63^{a}	1.85^{c}	1.81^{a}	33.78^{a}	46.79^{b}	34.40^{a}
0.9:1	2.61	16.78^{b}	14.17^{b}	2.07^{b}	26.88^{b}	1.90^{b}	1.76^{b}	32.61^{b}	48.08^{a}	33.46^{b}
Pooled SE	0.012	0.204	0.078.	0.004	0.034	0.011	0.061	0.062	0.336	0.113
Interactions										
0 × 0.3:1	2.59	14.27^{d}	11.68^{e}	1.90^{e}	25.28^{f}	2.17^{a}	1.54^{e}	28.37^{h}	44.36e	30.52^{g}
0 × 0.6:1	2.62	15.85^{c}	13.23^{d}	2.00^{d}	26.56^{d}	2.01^{b}	1.66^{d}	29.49^{g}	44.25e	31.15^{f}
0 × 0.9:1	2.60	17.24^{b}	14.64^{b}	2.10^{b}	26.85^{c}	1.83^{cd}	1.82^{bc}	34.33^{c}	48.42^{abc}	34.47^{c}
500 × 0.3:1	2.65	17.12^{b}	14.47^{b}	2.07^{c}	28.28^{a}	1.96^{b}	1.70^{d}	31.89^{e}	46.05^{de}	32.53^{e}
500 × 0.6:1	2.62	18.49^{a}	15.85^{a}	2.17^{a}	28.21^{a}	1.78^{e}	1.87^{a}	35.45^{b}	48.73^{ab}	35.47^{b}
500 × 0.9:1	2.64	16.65^{bc}	14.01^{c}	2.05^{c}	27.75^{b}	1.98^{b}	1.68^{d}	30.82^{f}	46.65^{cd}	32.19^{e}
1000 × 0.3:1	2.63	17.39^{b}	14.60^{b}	2.10^{b}	26.55^{d}	1.79^{d}	1.85^{ab}	34.27^{c}	48.90^{ab}	35.18^{b}
1000 × 0.6:1	2.61	18.60^{a}	15.99^{a}	2.18^{a}	28.12^{a}	1.76^{e}	1.90^{a}	36.40^{a}	47.39^{bcd}	36.59^{a}
1000 × 0.9:1	2.60	16.46^{bc}	13.86^{c}	2.05^{c}	26.03^{e}	1.88^{e}	1.77^{c}	32.68^{d}	49.34^{a}	33.72^{d}
Pooled SE	0.021	0.376	0.135	0.008	0.059	0.019	0.015	0.108	0.582	0.196

Values (± SE, $n = 3$). Means within the same column sharing the same superscript are insignificantly different ($p \leqslant 0.05$).

Table 3 Chemical composition in *O. niloticus* after 84 days of feeding phytase supplemented with different Ca/P ratio.

Items	Chemical composition of whole body fish (%)				Vertabra	
	DM	CP	EE	Ash	Ash	P
Effect of MPU Kg^{-1}						
0	36.61	52.90^{b}	21.70^{b}	13.36	41.32c	10.12^{b}
500	36.40	53.56^{a}	23.29^{a}	13.47	45.62^{b}	11.06^{a}
1000	36.48	53.89^{a}	23.72^{b}	13.60	46.54^{a}	11.17^{a}
Pooled SE	0.243	0.156	0.200	0.106	0.095	0.085
Effect of Ca:p ratio						
0.3:1	36.34	53.33^{b}	22.73	12.51^{c}	42.92^{c}	10.45^{b}
0.6:1	36.77	53.93^{a}	22.97	13.68^{b}	45.10^{b}	10.83^{a}
0.9:1	36.31	53.08^{b}	23.02	14.23^{a}	45.47^{a}	11.055^{a}
Pooled SE	0.243	0.156	0.200	0.106	0.095	0.085
Interactions						
0 × 0.3:1	36.30	52.550^{d}	20.46^{a}	12.50^{d}	37.30e	9.46^{e}
0 × 0.6:1	36.77	51.42^{d}	19.17^{bcd}	13.27^{c}	41.80^{d}	10.23^{d}
0 × 0.9:1	36.78	53.71^{bc}	19.73^{ab}	14.30^{a}	44.87^{c}	10.67^{cd}
500 × 0.3:1	36.31	53.90^{b}	19.44^{abc}	12.37^{d}	45.37^{c}	10.90^{bc}
500 × 0.6:1	37.00	54.24^{b}	18.53^{cd}	13.70^{bc}	46.17^{b}	11.33^{ab}
500 × 0.9:1	35.90	52.53^{d}	19.80^{ab}	14.33^{a}	45.33^{c}	10.93^{bc}
1000 × 0.3:1	36.43	35.61^{bc}	19.20^{bcd}	12.67^{d}	46.10^{b}	11.00^{bc}
1000 × 0.6:1	36.53	55.07^{a}	18.10^{d}	14.07^{a}	47.33^{a}	11.60^{a}
1000 × 0.9:1	36.27	53.01^{d}	20.13^{a}	14.06^{a}	46.20^{b}	10.90^{b}
Pooled SE	0.421	0.271	0.341	0.183	0.165	0.148

Values (± SE, $n = 3$). Means within same column sharing the same superscript are insignificantly different ($p \leqslant 0.05$).

the effect of Ca/P ratio triglyceride was higher in fish fed plant protein diet with 0.6:1 Ca/P ratio than other diets. But, serum phosphorus was increased in fish fed plant protein diet with 0.6:1 and 0.9:1 Ca/P ratio While, low Ca/P ratio 0.3:1 in experimental fish diet showed the highest level of cholesterol in *O. niloticus*.

Table 4 Apparent digestibility of DM, CP, lipid, carbohydrate and energy in *O. niloticus* after 84 days of feeding phytase supplemented with different Ca/P ratio.

Items	Apparent digestibility coefficient				
	Dry matter	Crude protein	Lipid	Carbohydrate	Energy
Effect of MPU Kg^{-1}					
0	85.739	85.69^{c}	85.16^{c}	46.37^{c}	77.09^{c}
500	85.768	87.01^{b}	86.95^{b}	47.87^{b}	78.41^{a}
1000	85.561	87.38^{a}	87.30^{a}	48.40^{a}	78.47^{a}
Pooled SE	1.03	0.077	0.114	0.104	0.073
Effect of Ca:p ratio					
0.3:1	86.26	86.54^{b}	85.87^{c}	47.16^{c}	77.42^{c}
0.6:1	85.53	87.52^{a}	87.27^{a}	47.99^{a}	78.76^{a}
0.9:1	85.27	86.03^{c}	86.26^{b}	47.49^{b}	77.79^{b}
Pooled SE					
Interactions					
0 × 0.3:1	85.613	83.83^{d}	83.59^{e}	45.67^{f}	75.50^{f}
0 × 0.6:1	86.103	85.60^{c}	85.78^{d}	46.38^{e}	77.65^{de}
0 × 0.9:1	85.500	87.50^{ab}	86.100^{cd}	47.05^{d}	78.12^{bc}
500 × 0.3:1	86.550	86.00^{c}	86.61^{c}	47.52^{cd}	78.48^{b}
500 × 0.6:1	83.157	86.40^{bc}	87.84^{ab}	48.27^{b}	79.39^{a}
500 × 0.9:1	87.597	85.32^{c}	86.39^{cd}	47.82^{bc}	77.38^{e}
1000 × 0.3:1	86.630	85.75^{c}	87.41^{b}	48.29^{b}	78.29^{b}
1000 × 0.6:1	86.557	87.68^{a}	88.20^{a}	49.31^{a}	79.24^{a}
1000 × 0.9:1	83.497	86.38^{bc}	86.29^{cd}	47.59^{cd}	77.78^{cd}
Pooled SE	1.80	0.364	0.201	0.364	0.130

Values (±SE, $n = 3$). Means within same column sharing the same superscript are insignificantly different ($P \leqslant 0.05$).

Table 5 Serum blood parameters in *O.niloticus* fingerlings after 84 days of feeding phytase supplemented with different Ca/P ratio.

Items	Blood parameters				
	ALT	AST	Triglyceride mg dL^{-1}	Cholesterol mg dL^{-1}	Phosphorus
Effect of MP U Kg^{-1}					
0	19.12	97.11	125.33^{c}	118.22^{c}	15.72c
500	19.29	96.44	133.11^{b}	125.67^{b}	18.17^{b}
1000	19.21	97.09	135.44^{a}	129.33^{a}	18.70^{a}
Pooled SE	0.258	0.519	0.499	0.454	0.136
Effect of Ca:p ratio					
0.3:1	19.42	96.78	130.22^{b}	125.11^{a}	17.06^{b}
0.6:1	19.19	97.03	132.11^{a}	124.56a^{b}	17.84^{a}
0.9:1	19.01	96.83	131.55a^{b}	123.55^{b}	17.69^{a}
Pooled SE	0.258	0.519	0.499	0.454	0.136
Interactions					
0 × 0.3:1	19.5000	97.33	125.00^{d}	121.00^{e}	14.65^{e}
0 × 0.6:1	19.4000	96.30	124.66^{d}	117.66^{f}	15.63^{d}
0 × 0.9:1	18.4667	97.70	126.33^{d}	116.00^{f}	16.86^{c}
500 × 0.3:1	19.5000	96.67	131.00^{c}	125.67^{cd}	18.04^{b}
500 × 0.6:1	19.0333	97.33	136.00^{a}	124.76^{d}	18.53^{b}
500 × 0.9:1	19.3333	95.38	132.33^{b}	126.66^{cd}	17.95^{b}
1000 × 0.3:1	19.2667	96.42	134.67^{ab}	128.67^{b}	18.48^{b}
1000 × 0.6:1	19.1333	97.47	135.68^{a}	131.33^{a}	19.34^{a}
1000 × 0.9:1	19.2333	97.49	136.00^{a}	128.00^{b}	18.27^{b}
Pooled SE	0.448	0.898	0.865	0.787	0.236

Values (±SE, $n = 3$). Means within same column sharing the same superscript are insignificantly different ($p \leqslant 0.05$).

Discussion

The growth performance of *O. niloticus* fingerlings in terms of final fish weight, weight gain and specific growth rate was significantly improved on plant protein based diets with graded levels of phytase supplementation up to certain limits. The findings of the study provide evidence that supplementation with MP at a level of 1000 U kg^{-1} diet was probably enough for reducing the effect of phytic acid and releasing the chelated protein and minerals of plant based diets. Similar results were

obtained by for *O. niloticus* (Goda, 2007; Liebert and Portz, 2007) and *Labeo rohita* fingerlings (Baruah et al., 2007). In the present study, phytase supplementation significantly improved FI and FCR of *O. niloticus* fingerlings fed on plant based protein. Increasing the palatability and conversion rate of diet may be due to enhanced release of nutrients of plant based diets by breaking down the bonds between phytate-protein and phytate-minerals (Vielma et al., 1998). Furthermore, phytate may chelate with amino acids in the stomach of different fish species and reduces the availability of amino acid (Usmani and Jafri, 2002). The findings of present study are comparable with the findings of Wang et al. (2009) and Baruah et al. (2007) reached to the same results in rainbow trout and *L. rohita* respectively, when fed plant based diet supplemented with phytase.

With respective of Ca/P ratio, obtained results indicated that Ca content in diets from organic Ca sources (calcium propionate) either with or without phytase seemed sufficient to support the growth of *O. niloticus*. These results confirmed with Laining et al. (2011) who indicated that, tiger puffer had the ability to utilize Ca from organic sources in the diet along with Ca uptake from seawater to fulfill part of the entire metabolic Ca requirement.

On the other hand, organic acids and their salts can also contribute in nutritional ways, because they are components in several metabolic pathways for energy generation, for instance, for ATP generation in the (citric acid cycle or carboxylic-acids cycle), organic acids and their salts can also contribute in nutritional ways, because they are components in several metabolic pathways for energy generation (da Silva et al., 2012). Pontoppidan et al. (2007) reported that solubility of phytate is an important factor for phytate degradation by phytase, which is influenced by pH and Ca level.

With regard to the interaction between MP and Ca/P ratio, diet supplemented with either 500 or 1000 U kg^{-1} diet in combination with Ca/P ratio of 0.6:1 significantly improved growth performance parameters among the different groups. This indicates that phytase supplementation was more effective when supplemented with 0.6:1 ratio Ca/P than others supplemented with 0.9:1 of Ca/P. High level of Ca in fish feed will chelate with phytate forming an insoluble complex or compete with phytase for the binding site at the myoinositol ring and thus block the site of phytase mediated substrate hydrolysis (Qian et al., 1996) or increase the pH value to inhibit the activity of phytase (Cao et al., 2007). Similarly Laining et al. (2011) indicated that growth rate was significantly higher in tiger puffer *Tahifugu rubripes*, fed diet supplemented with 2000 FTU kg^{-1} diet and (0.5) Ca/P ratio.

In the present study dry matter and ash content of *O. niloticus* fingerlings were not affected by dietary MP but, The EE content was significantly increased due to dietary MP. Similar results were also obtained by Sajjadi and Carter (2004a) by Atlantic salmon, *Salmo salar*; Debnath et al. (2005) in *P. pangasius*.

Increased Ca/P ratio was not affected DM and EE but significant increase seen in ash content of fish fed high levels of Ca/P ratio and this may be due to addition of organic salt (Ca-propionate) which enhance minerals absorption through the intestine. Similar results were observed in the previous findings Sarker et al. (2012) in juvenile yellowtail, *Seriola quinqueradiata*.

Fish that were fed on diet supplemented with 1000 U kg^{-1} combined with 0.6:1 Ca/P ratio showed the highest body content of CP, ash and vertebra ash and P content. This indicates that phytase supplementation positively affected chemical composition of body and vertebra when combined with 0.6 Ca/P ratio but, increased Ca/P reduces the efficiency of phytase and this also suggested that, added Calcium propionate with 0.5% in diet provides an optimum environment for phytase activity by lowering pH. On the contrary, Laining et al. (2011) indicated that, Ca/P ratios were achieved by supplementing calcium carbonate at 0, 6 and 12 g kg^{-1}, tiger puffer (*T. rubripes*) fed on diet at Ca/P ratio (0.5) with phytase insignificantly affect the values of whole body protein, lipid and ash significantly, also recorded the highest value of P in vertebrae. This inconsistency in the outcome of different authors may be attributed to differences in feed ingredients, nutritional quality of plant ingredients, water quality, fish species and size and culture or experimental conditions.

The higher apparent digestibility coefficient (ADC) of crude protein in plant protein based diets supplemented with MP, observed in the this trail, clearly indicated the acceptability of the alternative plant protein based test diets supplemented with MP has increased as reported by) Vielma et al. (2004), Liebert and Portz (2005), Goda (2007), Nwanna et al. (2008), Laining et al. (2011) and Wang et al. (2009). In the present study, protein digestibility by *O. niloticus* fingerlings fed without or low dose of phytase supplementation confirms the above theories mentioned by (Vielma et al., 1998; Usmani and Jafri, 2002). However, Sajjadi and Carter (2004a) and Dalsgaard et al. (2009) found significant effect on protein digestibility. This discrepancy, observed in several studies for nutrient digestibility, can be linked to variation in protein quality of feed ingredients, pH of fish stomach and drying procedures (Wang et al., 2009).

Generally, the impact of phytase supplementation on nutrient digestibility depends on a variety of factors such as concentration and source of phytate in the diet, (Selle et al., 2000), digestibility of protein source, levels of calcium and phosphorus (Sugiura et al., 2001).

Dietary phytase combined with 0.6:1 Ca/P in diet improved the ADC of protein, lipid, carbohydrate and energy. This results confirmed with Laining et al. (2011) who reported that tiger puffer *T. rubripes* fed the diet supplemented with phytase at Ca/P ratio (0.5) together with phytase had significantly higher protein digestibility, also reported that increasing Ca/P with the same level of phytase decreased the protein digestibility. The authors, Pontoppidan et al. (2007) reported that solubility of phytate is an important factor for phytate degradation by phytase, which is influenced by pH and Ca level. They reported that increasing Ca/P ratio reduced the phytase-related increase in P and ash ADC, suggesting a Ca-induced interference with phytase activity.

The present study clearly demonstrates that the addition of MP to a plant protein–based diet fed to *O. niloticus* significantly increases the serum phosphorus, cholesterol and triglycerides but, no significant effect was shown in ALT and AST in fish fed on MP. This result is in agreement with Liu et al. (2013) they reported that, the addition of neutral phytase did not notably affect the hepatic ALT and AST activities but significant increases were observed in the serum cholesterol triglycerides in grass carp *Ctenopharyngodon idellus* and gibel carp *Carassius auratus gibelio*.Increase in serum P in fish fed phytase supplement may indicate the enhancement of minerals absorption particularly P. Enhancement of mineral utilization

in particular P by supplementing dietary phytase to plant based diet containing phytate has been well documented in monogastric animals including fish species (Sajjadi and Carter, 2004b; Vielma et al., 2004). Currently, little information is available on the effect of dietary phytase addition on these blood characteristics and more investigations are needed to confirm these aspects.

Conclusion

Increasing the Ca/P ratio has been previously reported to reduce the effectiveness of phytase in Nile tilapia. To increase phytase efficacy, dietary Ca/P ratio should be kept to a 0.6 ratio for Nile tilapia fed plant protein based diet.

References

Adeola, O., Olukosi, O.A., Jendza, J.A., Dilger, R.N., Bedford, M.R., 2006. Response of growing pigs to *Peniophora lycii*- and *Escherichia coli*-derived phytases or varying ratios of calcium to total phosphorus. Anim. Sci. 82, 637–644.

AOAC (Association of Official Analytical Chemists), 1995. Official Methods of Analysis. AOAC Inc., Washington, DC, USA, p. 1234.

Angel, R., Tamim, N.M., Applegate, T.J., Dhandu, A.S., Ellestad, L.E., 2002. Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. J. Appl. Poult. Res. 11, 471–480.

Baruah, K., Sahu, N.P., Pal, A.K., Jain, K.K., Debnath, D., Mukherjee, S.C., 2007. Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. Aquac. Res. 38, 109–120.

Brett, J.R., 1973. Energy expenditure of Sockeye salmon *Oncorhynchus nerka*, during sustained performance. J. Fish. Res. Board Can. 30, 1799–1809.

Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., Luo, Z., Li, D., 2007. Application of microbial phytase in fish feed. Enzyme Microb. Technol. 40, 497–507.

da Silva, B.C., Vieira, F.D.N., Mourio, J.L.P., Ferreira, G.S., Seiffert, W.Q., 2012. Salts of organic acids selection by multiple characteristics for marine shrimp nutrition. Aquaculture. http://dx.doi.org/10.1016/j.aquaculture.2012.12.017.

Davis, D.A., Lawrence, A.L., Gatlin, D.M., 1993. Response of *Penaeus vannamei* to dietary calcium, phosphorus and calcium: phosphorus ratio. J. World Aquac. Soc. 24, 504–515.

Debnath, D., Pal, A.K., Sahu, N.P., 2005. Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. Aquacult. Res. 362, 180–187.

Dalsgaard, J., Ekmann, K.S., Pedersen, P.B., Verlhac, V., 2009. Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (*Oncorhynchus mykiss*) and on the magnitude and composition of phosphorus waste output. Aquaculture 286, 105–112.

El-Greirsy, Z.A., El-Gamal, A.E., 2012. Monosex production of tilapia, *Oreochromis niloticus* using different doses of 17a-methyltestosterone with respect to the degree of sex stability after one year of treatment. Egypt. J. Aquat. Res. 38, 59–66.

El-Saidy, D.M.S., Gaber, M.M.A., 2002. Complete replacement of fishmeal by soybean with the dietary L-lysine supplementation in Nile tilapia fingerlings. J. World Aquacult. Soc. 33, 297–306.

Engelen, A.J., Van Der Heeft, F.C., Randsdrop, P.H.G., Smith, E.L.C., 1994. Simple and rapid determination of phytase activity. JAOAC 77, 760–764.

Furukawa, H., Tsukahara, H., 1966. On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. Bull. Jpn. Soc. Sci. Fish. 32 (6), 502–508.

Goda, M.A.-S., 2007. Effect of dietary soybean meal and phytase levels on growth, feed utilization and phosphorus discharge for Nile tilapia (*Oreochromis niloticus* L.). J. Fish. Aquat. Sci. 2, 248–263.

Hassan, B., El-Salhia, M., Khalifa, A., Assem, H., Al Basomy, A., El-Sayed, M., 2013. Environmental isotonicity improves cold tolerance of Nile tilapia, *Oreochromis niloticus*, in Egypt. Egypt. J. Aquat. Res. 39, 59–65.

Kim, J.D., Kim, K.S., Song, J.S., Lee, J.Y., Jeong, K.S., 1998. Optimum level of dietary monocalcium phosphate based on growth and phosphorus excretion of mirror carp, *Cyprinus carpio*. Aquaculture 161, 337–344.

Kumar, V., Sinha, A.K., Makkar, H.P.S., De Boeck, G., Becker, K., 2011. Phytate and phytase in fish nutrition animal physiology and animal. Nutrition. http://dx.doi.org/10.1111/j.1439-0396.2011.01169.x.

Laining, A., Ishikawa, M., Kyaw, K., Gao, J., Binh, N.T., Koshio, S., Yamaguchi, S., Yokoyama, S., Koyama, J., 2011. Calcium/phosphorus ratio influnces the efficacy of microbial phytase on growth, minerals digestibility and vertebral mineralization in juvenile tiger puffer, *Takifugu rubrips*. Aquac. Nutr. 17, 267–277.

Liebert, F., Portz, L., 2005. Nutrient utilization of Nile tilapia *Oreochromis niloticus* fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. Aquaculture 248, 11–19.

Liebert, F., Portz, L., 2007. Different sources of microbial phytase in plant based low phosphorus diets for Nile tilapia *Oreochromis niloticus* may provide different effects on phytate degradation. Aquaculture 267, 292–299.

Liu, L., Luo, Y., Liang, X., Wang, W., Wu, W., 2013. Effects of neutral phytase supplementation on biochemical parameters in grass carp, *Ctenopharyngodon idellus*, and Gibel Carp, *Carassius auratus gibelio*, fed different levels of monocalcium phosphate. J. World Aquacult. Soc. 44, 56–65.

Love, R.M., 1980. In: The Chemical Biology of Fishes, vol. 2. Academic Press, New York, NY.

Lovell, R.T., 1989. Nutrition and Feeding of Fish. Van Nostrand Reinhold, New York, p. 260.

National Research Council (NRC), 1993. Nutrient Requirements of Fish. National Academic Press, Washington, DC.

Nwanna, L.C., Oishi, C.A., Filho, M.P., 2008. Use of phytase to improve the digestibility of alternative feed ingredients by *Amazon tambaqui*, Colossoma Macropomum. Sci. Asia 34, 353–360.

Ogino, C., Takeuchi, L., Takeda, H., Watanabe, T., 1979. Availability of dietary phosphorus in carp and rainbow trout. Bull. Jpn. Soc. Sci. Fish. 45, 1527–1532.

Pontoppidan, K., Pettersson, D., Sandberg, A.S., 2007. Peniophora lycii phytase is stabile and degrades phytate and solubilises minerals in vitro during simulation of gastrointestinal digestion in the pig. J. Sci. Food Agric. 87, 2700–2708.

Qian, H., Kornegay, E.T., Conner Jr., D.E., 1996. Adverse effects of wide calcium:phosphorus ratios on supplemental phytase efficacy for weanling pigs fed two dietary phosphorus levels. J. Anim. Sci. 74, 1288–1297.

Reitman, S., Frankel, S., 1957. Colorimetric determination of glutamic oxaloacetic and glutamic pyruvic transaminases. J. Clin. Pathol. 28, 56–59.

Robinson, E.H., Rawles, S.D., Yette, H.E., Greene, L.W., 1986. Dietary calcium requirement of channel catfish, *Ictalurus punctatus*, reared in calcium-free water. Aquaculture 39, 342–364.

Robinson, E.H., Li, M.H., Manning, B.B., 2002. Comparison of microbial phytase and di calcium phosphate for growth and bone mineralization of pond-raised channel catfish, *Ictalurus punctatus*. J. Appl. Aquacult. 12, 81–88.

Robinson, E.H., LaBomascus, D., Brown, P.B.I.I.I., Linton, T.L., 1987. Dietary calcium and phosphorus requirement of *Oreochromis aureus* reared in calcium-free water. Aquaculture 64, 267–276.

Sajjadi, M., Carter, C.G., 2004a. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar* L.). Aquacult. Nutr. 10, 135–142.

Sajjadi, M., Carter, C.G., 2004b. Dietary phytase supplementation and the utilization of phosphorus by Atlantic salmon (*Salmo salar* L.) fed a canola-meal-based diet. Aquaculture 240, 417–431.

Sanchez, C.C., Palacios, M.C.A., Perez, G.M., Ross, L.G., 2000. Phosphorus and calcium requirements in the diet of the American cichlid Cichlasoma urophthalmus (Gunther). Aquac. Nutr. 6, 1–9.

Sarker, M.S.A., Satoh, S., Kamata, K., Haga, Y., Yamamoto, Y., 2012. Supplementation effect(s) of organic acids and/or lipid to plant protein-based diets on juvenile yellowtail, *Seriola quinqueradiata* Temminck et Schlegel 1845, growth and, nitrogen and phosphorus excretion. Aquacult. Res. 43, 538–545.

Schneider, O., Amirkolaie, A.K., Vera-Cartas, J., Eding, E.H., Schrama, J.W., Verreth, J.A.J., 2004. Digestibility, feces recovery, and related carbon, nitrogen and phosphorus balances of five feed ingredients evaluated as fishmeal alternatives in Nile tilapia, *Oreochromis niloticus* L. Aquacult. Res. 35, 1370–1379.

Selle, P.H., Ravindran, V., Caldwell, R.A., Bryden, W.L., 2000. Phytate and phytase: consequences for protein utilization. Nutr. Res. Rev. 13, 255–278.

Shiau, S.-Y., Tseng, H.C., 2007. Dietary calcium requirements of juvenile tilapia, *Oreochromis niloticus* × *O. aureus*, reared in fresh water. Aquac. Nutr. 13, 298–303.

Statistical Analysis system, 1993. SAS/STAT user Guide Release 6.03 Edition. SAS Institute Inc., Cary, North Carolina, USA.

Sugiura, S.H., Gabaudan, J., Dong, F.M., Hardy, R.W., 2001. Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout *Oncorhynchus mykiss* (Walbaum) fed soybean meal-based diets. Aquacult. Res. 32, 583–592.

Tamin, N.M., Angel, R., Christman, M., 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. Poult. Sci. 83, 1358–1367.

Usmani, N., Jafri, A.K., 2002. Influence of dietary phytic acid on the growth, conversion efficiency and carcass composition of *Cirrhinus mrigala* (H) fry. J. World Aquacult. Soc. 33, 199–204.

Vielma, J., Lall, S.P., Koskela, J., 1998. Effects of dietary phytase and cholecalciferol on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 163, 309–323.

Vielma, J., Ruohonen, K., Gabaudan, J., Vogel, K., 2004. Top-spraying soybean mealbased diets with phytase improves protein and mineral digestibility but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult. Res. 35 (10), 955–964.

Wang, F., Yang, Y.H., Han, Z.Z., Dong, H.W., Yang, C.H., Zou, Z.Y., 2009. Effects of phytase pretreatment of soybean meal and phytasesprayed in diets on growth, apparent digestibility coefficient and nutrient excretion of rainbow trout (*Oncorhynchus mykiss* Walbaum). Aqua Int. 17, 143–157.

Ye, C.X., Liu, Y.J., Tian, L.X., Mai, K.S., Du, Z.Y., 2006. Effect of dietary calcium and phosphorus on growth, feed efficiency, mineral content and body composition of juvenile grouper, *Epinephelus coioides*. Aquaculture 255, 263–271.