

# Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*)

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## Abstract

This study was to assess effects of the pretreatment in all-plant based diets with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). Pretreatment trials were conducted using phytase at graded doses to determine the optimal dose of phytase. Available phosphorus (P) levels increased significantly with the increased doses of phytase and the dose of 1000 U kg<sup>-1</sup> was most efficient. Based on the pretreatment trials, plant based diets for Nile tilapia were formulated by pretreating with phytase at 1000 U kg<sup>-1</sup>. Experimental diets were supplemented with graded levels of mono calcium phosphate (MCP) at 25, 18.75, 12.5, 6.25 and 0 g kg<sup>-1</sup> diet. In addition, there were three controls: one phytase control, one inorganic P control and one pretreatment control. The results showed that diets pretreated with phytase gave better growth performance, feed conversion ratio and protein efficiency ratio of Nile tilapia compared with the phytase control diet and pretreatment control diet ( $P < 0.05$ ). There were no significant differences in growth performance of Nile tilapia between the inorganic control diet and phytase pretreated diets supplemented with MCP at 25, 18.75 and 12.5 g kg<sup>-1</sup> ( $P > 0.05$ ), which resulted in significantly better performance than those at 6.25 and 0 g kg<sup>-1</sup> ( $P < 0.05$ ). Dietary interaction effects of phytase were observed for phosphorus retention efficiency and phosphorus load. Apparent digestibility coefficient of P (ADC<sub>P</sub>) was improved significantly by phytase pretreatment ( $P < 0.05$ ). No significant difference was detected on ADC of crude protein among all experimental diets ( $P > 0.05$ ).

**KEY WORDS:** digestibility, growth, microbial phytase, Nile tilapia *Oreochromis niloticus*, phosphorous, phytate

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## Introduction

Currently, there are increasing interests in the complete replacement of fish meal by various grain products, such as soybean meal, wheat flour and corn gluten meal (Cain & Garling 1995). One of the major problems limiting the use of grain products in fish feed is the presence of phytate (inositol hexaphosphate). Phytate is the primary form of phosphorus (P) in grains, accounting for approximately two-thirds of the total P bound as phytate P (Lall 1991). Phytate is an indigestible P form, which is practically not available for fish such as tilapias [NRC (National Research Council) 1993] because of lack of an intestinal phytase for efficient hydrolysis of phytate during digestion (Jackson *et al.* 1996). Consequently, the available P in all-plant diets may not meet the requirements of fish, and inorganic P is usually supplemented to the diets. On the other hand, undigested phytate P excreted via faeces and contributes to environmental pollution, causing eutrophication in receiving water bodies.

Exogenous phytase has been used successfully to hydrolyse phytate and increase nutrient digestibility (Cao *et al.* 2007). Specifically for hydrolysis of phytate, phytase is most promising in producing cost-effective fish feed formula that can reduce the need to supplement of inorganic P in feeds and also lower

phytate P levels in fish excretion (Simons *et al.* 1990; Liener 1994). Phytase is not heat stable and should be applied by avoiding excess heat during extrusion and other steps in diet manufacture (Vielma *et al.* 2002). It is deactivated at temperatures above 65 °C, while the temperature during feed processing often exceeds 65 °C (Cao *et al.* 2007). Phytase can be utilized in fish feeds by pretreating feedstuffs (Cain & Garling 1995), or spraying onto pellets (Vielma *et al.* 2004) to avoid deactivation.

Studies on the role of supplemental phytase for nutrient utilization or growth of fish focus on commonly cultured species, such as rainbow trout (*Oncorhynchus mykiss*) (Rodehutschord & Pfeffer 1995; Forster *et al.* 1999), common carp (*Cyprinus carpio* L.) (Schäfer *et al.* 1995), channel catfish (*Ictalurus punctatus*) (Jackson *et al.* 1996; Li & Robinson 1997) and Nile tilapia (*Oreochromis niloticus*) (Furuya *et al.* 2001; Liebert & Portz 2005). Biological factors such as the rearing temperature of fish may influence the function of phytase applied onto a diet. Thus, pretreatment seems to be more promising than top-spraying as the dephytinization process happens *in vitro* of fishes. As a result of the increasing price and decreasing resources, inorganic P supplementation in fish diets could be replaced partially or fully by phytase (Cao *et al.* 2007).

However, there have been inconsistent results from studies evaluating the effects of phytase pretreatment on feed quality and utilization. Cain & Garling (1995) reported that pretreatment of soybean meal with phytase enhanced growth performance and P utilization of juvenile rainbow trout. Similarly, dephytinization of soy protein concentrate increased protein and P utilization in Atlantic salmon in seawater (Storebakken *et al.* 1998), whereas a decrease in rapeseed protein quality by dephytinization was noted by Teskeredzic *et al.* (1995) in rainbow trout. Results related to weight gain, apparent digestibility of crude protein and amino acids after phytase addition also showed great inconsistency (Sajjadi & Carter 2004). Some studies found that addition of phytase had positive effects on weight gain of rainbow trout (Rodehutschord & Pfeffer 1995; Vielma *et al.* 1998) and channel catfish (Jackson *et al.* 1996; Li & Robinson 1997), while other studies detected no effects for the same species (Lanari *et al.* 1998; Yan & Reigh 2002). Many studies have demonstrated that addition of phytase to fish feeds can improve the utilization of phytate P and decrease the total P load to the environment for several species (Vielma *et al.* 1998; Liebert & Portz 2005).

In Nile tilapia, phytase supplementation between 500 and 1500 U kg<sup>-1</sup> improved P availability, crude protein digestibility and bone mineralization (Furuya *et al.* 2001). Liebert & Portz (2005) reported that phytase supplementation of 750 U kg<sup>-1</sup> in Nile tilapia diets resulted in the same performance as inorganic P addition (NaH<sub>2</sub>PO<sub>4</sub>; 15 g kg<sup>-1</sup> diet).

The available P requirement of Nile tilapia is between 5 [NRC (National Research Council) 1993] and 9 g kg<sup>-1</sup> (Watanabe *et al.* 1980; Beveridge & McAndrew 2000), and tilapia juveniles usually need higher P levels than adults. When all-plant diets are used, total P content from plant ingredients is usually less than that in fishmeal-based diets. Thus, P content in all-plant diets may not meet the P requirement of Nile tilapia juveniles and partial supplementation of inorganic P may be needed. Whether inorganic P supplementation could be partially or completely replaced in Nile tilapia feed needs further investigation. Besides, the approximate amount of phytate P converted to available P in plant ingredients after phytase pretreatment is also uncertain.

The purposes of this study was to determine the optimal dose of phytase for pretreatment of plant ingredients and the amount of available P released from phytate P, to investigate the effects of the phytase pretreatment and replacement levels of inorganic P on growth performance as well as apparent digestibility of crude protein and P of Nile tilapia juveniles fed all-plant diets.

## Materials and methods

### Pretreatment trials

Two pretreatment trials were conducted using a completely randomized design at the Asian Institute of Technology (AIT), Thailand, from 5 January to 5 February 2006. In the first trial, soybean meal was treated with microbial phytase (Habio® Phytase-5000 FTU g<sup>-1</sup>, Sichuan Habio Bioengineering Co. Ltd, Chengdu, Sichuan, China) at 0 (control), 500, 750, 1000 and 1500 U kg<sup>-1</sup>. The second trial was conducted to treat mixed plant meal consisting of soybean meal (644 gKg<sup>-1</sup>), wheat meal (119 gKg<sup>-1</sup>) and corn gluten meal (237 gKg<sup>-1</sup>) with phytase at 0 (control), 500, 750, 1000, 1500 and 3000 U kg<sup>-1</sup>. Each treatment had three replicates. The pretreatment method described by Cain & Garling (1995) was used and adapted in the present study. The procedures of the method were to: (i) prepare 0.2 M citrate buffer (dissolve 15.38 g citric acid and 37.29 g sodium citrate into 1-L distilled water) at room temperature; (ii) dissolve 2 g phytase (5000 U g<sup>-1</sup>) into 1-L citrate buffer using a magnetic stirrer for 30 min; (iii) add meal to the buffer at 1 : 1 (w : v), and place the mixture in aluminum trays and rapidly heat with constant stirring to 50–55 °C (cover the mixture with aluminum foil and incubate at 55 °C for 6 h); (iv) dry the mixture in a forced-air drying oven at 60 °C for 24 h to reduce the moisture content to approximately 100 gKg<sup>-1</sup>. In the control, the same method was used but without adding phytase (Riche *et al.* 2001).

### Experimental diets

Experimental design and basal diet formulations were based on the assumption that effects of phytase pretreatment can be most accurately measured using P utilization as the indicator of phytic acid hydrolysis (Vielma *et al.* 1998). Therefore, an all-plant basal diet was formulated to contain low levels of available P (Table 1).

Five treatment diets were supplemented with mono calcium phosphate (MCP) at 25 g kg<sup>-1</sup> (100% MCP supplement, diet 3), 18.75 g kg<sup>-1</sup> (75% MCP supplement, diet 4), 12.5 g kg<sup>-1</sup> (50% MCP supplement, diet 5), 6.25 g kg<sup>-1</sup> (25% MCP supplement, diet 6) and 0 g kg<sup>-1</sup> (0% MCP supplement, diet 7) respectively, by cassava replacement for different experimental treatments (Table 2). In addition, there were three controls: one phytase control (diet 1, basal diet) with neither phytase pretreatment nor inorganic P supplement; one inorganic P control (diet 2) without phytase pretreatment but with 25 g kg<sup>-1</sup> supplement of MCP by replacing the same amount of cassava starch in the basal diet;

**Table 1** Composition of the basal diet for Nile tilapia juveniles

Ingredients	As-fed basis (g kg <sup>-1</sup> )
Soybean meal (oil-extracted)	536.5
Wheat	100.0
Corn gluten meal	200.0
Cassava	103.5
Mineral premix <sup>1</sup>	10.0
Fish oil	35.0
Soy lecithin	10.0
Vitamin premix <sup>2</sup>	5.0
Total	1000.0
Proximate analysis	Dry matter basis (g kg <sup>-1</sup> )
Moisture	109.1
Crude protein	363.8
Crude lipid	57.1
Fibre	28.2
Ash	51.0
Nitrogen-free extract <sup>3</sup>	499.9
Calcium	2.0
Total P	4.5
Phytate P	3.0
Available P <sup>4</sup>	1.5

P, phosphorus.

<sup>1</sup> Mineral premix (per kg of diet): MnSO<sub>4</sub>, 54 mg; ferric citrate, 142 mg; CuSO<sub>4</sub>, 10 mg; ZnCO<sub>3</sub>, 29 mg; NaCl, 3.3 mg; KI, 0.9 mg; K<sub>2</sub>SO<sub>4</sub>, 90 mg; CoCl, 0.21 mg; MgO, 10 mg.

<sup>2</sup> Vitamin premix Roche 2118 (Hoffman-La Roche, Inc., Nutley, NJ, USA) (per kg of diet): Vitamin A, 12 000 UI; Vitamin D<sub>3</sub>, 5000 UI; Vitamin E, 30 mg; Vitamin K<sub>3</sub>, 3 mg; Vitamin B<sub>1</sub>, 2.2 mg; Vitamin B<sub>2</sub>, 8 mg; Vitamin B<sub>6</sub>, 5 mg; Vitamin B<sub>12</sub>, 11 mg; Folic acid, 1.5 mg; Biotin, 150 mg; Pantothenic acid: 25 mg; inositol, 65 mg.

<sup>3</sup> Calculated as: NFE = [1000 - (moisture + ash + crude protein + lipid% + fibre)] g kg<sup>-1</sup>.

<sup>4</sup> Calculated as: available P = total P - phytate P (Sajjadi & Carter 2004).

and one pretreatment control (diet 8) with neither phytase pretreatment nor inorganic P supplement but with phytase added directly to feed ingredients. Diet 8 was prepared at a phytase dose of 1000 U kg<sup>-1</sup> by dissolving phytase in distilled water before pelleting.

For diet preparation, dry ingredients were mixed in a Hobart-type mixer (Anoma Taiwan Machinery Co., Taiwan). Fish oil in the diets was included in the mixer prior to extrusion. Diets were pelleted (3 mm in diameter) with a laboratory extrusion system at 80 °C. Pelleted diets were air dried, crumbled into 1–2 mm in diameter with a grinder, and stored in sealed plastic bags at 4 °C until use.

### Growth trial

A growth trial was conducted in 24 in-door recirculating circular cement tanks (1 m in both depth and diameter) at AIT from 20 March to 21 May 2006, to assess effects of experimental diets pretreated with phytase on the growth performance of Nile tilapia. Water flow rate was fixed at 1 L min<sup>-1</sup> to maintain a water depth of 0.7 m in all experimental tanks. Eight experimental diets were assigned randomly to experimental tanks with three replicates each. Sex-reversed all-male Nile tilapia fingerlings of 0.7–0.8 g in size were stocked at 30 fish tank<sup>-1</sup>, and fed with the experimental diets to apparent satiation twice daily at 09:00–10:00 and 16:00–17:00 h. All experimental fish were purchased from the hatchery of AIT. Samples of diets and six fish per tank were collected at the beginning for analyses of whole body composition. Uneaten pellets and faeces in all tanks were removed by siphon everyday. Tank water samples, taken weekly at 09:00 h, were analysed for total alkalinity, total ammonia nitrogen, total Kjeldahl nitrogen and total P, using standard methods [APHA (American Public Health Association), American Water Works Association and Water Pollution Control Federation 1985]. Temperature, dissolved oxygen and pH (at 20 cm below the water surface) were measured *in situ* according to the same schedule. Water quality parameters in the experimental tanks were maintained within acceptable levels for tilapia (Plumb 1999) by using the physical and biological filters in the recycling system. Feed intake was calculated by subtracting the number of uneaten from supplied pellets. Feed intake was monitored in this way everyday throughout the experiment. At the end of the growth trial, all fish were deprived of food for 1 day before counting and weighing. Six fish per tank were collected for analyses of whole body composition. Then feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER) were calculated.

**Table 2** Experimental diets for Nile tilapia juveniles

	Phytase control (diet 1)	Inorganic-P control (diet 2)	100% MCP supplement (diet 3)	75% MCP supplement (diet 4)	50% MCP supplement (diet 5)	25% MCP supplement (diet 6)	0% MCP supplement (diet 7)	Pretreatment control (diet 8)
<b>Ingredients (g kg<sup>-1</sup>, as-fed basis)</b>								
Soybean meal	536.5	536.5	536.5	536.5	536.5	536.5	536.5	536.5
Corn gluten meal	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Wheat	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cassava	103.5	78.5	78.5	84.75	91.0	97.25	103.5	103.5
Fish oil	35	35	35	35	35	35	35	35
Soy lecithin	10	10	10	10	10	10	10	10
Mineral premix <sup>1</sup>	10	10	10	10	10	10	10	10
Vitamin premix <sup>2</sup>	5	5	5	5	5	5	5	5
Ca (H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	0	25.0	25.0	18.75	12.5	6.25	0	0
Total	1000	1000	1000	1000	1000	1000	1000	1000
Phytase <sup>3</sup> (U kg <sup>-1</sup> )	0	0	1000 <sup>4</sup>	1000 <sup>4</sup>	1000 <sup>4</sup>	1000 <sup>4</sup>	1000 <sup>4</sup>	1000 <sup>5</sup>
<b>Proximate composition (g kg<sup>-1</sup>, dry matter basis)</b>								
Dry matter	890.9	893.3	891.2	892.7	892.1	891.5	890.1	891.9
Crude protein	363.8	363.1	364.2	364.7	363.9	363.5	364.3	363.0
Crude fat	57.1	57.9	56.8	57.5	56.9	57.7	57.0	56.6
Ash	51.0	73.2	75.9	69.7	63.4	57.2	51.6	52.2
Calcium	2.0	6.2	6.1	5.2	4.1	3.1	1.9	2.0
Total-P	4.5	11.1	11.1	9.6	7.8	6.2	4.5	4.4
Phytate-P	3.0	3.3	0.9	0.8	0.6	1.0	0.7	2.0
Available-P	1.5	7.8	10.2	8.8	7.2	5.2	3.8	2.4

MCP, mono calcium phosphate; P, phosphorus.

<sup>1</sup> Mineral premix (per kg of diet): the same as Table 1 stated.

<sup>2</sup> Vitamin premix Roche 2118 (Hoffman-La Roche, Inc., Nutley, NJ, USA) (per kg of diet): the same as Table 1 stated.

<sup>3</sup> Phytase: provided by Sichuan Habio Bioengineering Co. Ltd., Chengdu, Sichuan, China. Enzyme activity: 5000 U g<sup>-1</sup>

<sup>4</sup> Plant ingredients were pretreated with phytase at 1000 U kg<sup>-1</sup> before mixed with other feed ingredients.

<sup>5</sup> Phytase mixed directly with other ingredients without pretreatment before pelleted

Nutrient retention efficiency of phosphorus was calculated as:

$$\text{Phosphorus retention \%} = \frac{100 \times (\text{FBW} \times \text{Nf} - \text{IBW} \times \text{Ni})}{(\text{feed intake} \times \text{N diet})}$$

where FBW is the final body weight and IBW is the initial body weight of fish, N is the concentration of nutrient (P) in the fish at the start (Ni) and end (Nf) of the experiment (Storebakken *et al.* 1998).

Phosphorus load was calculated as (Vielma *et al.* 2002):

$$\text{P load (g P kg}^{-1}\text{)} = \frac{(\text{P fed, g}) - (\text{P deposited, g})}{(\text{Weight gain, kg})}$$

### Digestibility trial

A digestibility trial was conducted after the termination of the growth trial in twenty-four 80-L fibreglass tanks. Ten sex-reversed all-male Nile tilapia fingerlings of approximately 25 g in size were stocked in each fibreglass tank with an air stone. Experimental diets used in the growth trial were the same as those used in the digestibility trial with three replicates for each diet. The digestibility trial was conducted from 25 May to 10

June 2006 for 15 days. Fish were fed commercial feed during a 3-day acclimation period, after which the experimental diets were fed twice daily (09:00–10:00 and 16:00–17:00 h) at a rate of 3% body weight per day for 5 days for dietary adaptation. Starting from day 6, faeces were collected twice a day for 10 days and pooled by each treatment tank. One hour after feeding, water was completely changed in all tanks to remove uneaten feed, and faeces were collected using complete membrane from the tank bottom 3 h after each feeding according to the method described by Lim *et al.* (2004). Daily collected faeces were air-dried at 55 °C. Samples of faeces were kept in a desiccator for analyses of crude protein and total P using standard methods [AOAC (Association of Official Analytical Chemists) 2000]. Apparent digestibility coefficients (ADC) of crude protein and P for eight diets were determined by the hydrolysis resistant organic matter (HROM) method (Buddington 1980). ADC was calculated according to:

$$\text{ADC \%} = 100 - (100) \left[ \frac{\% \text{HROM in feed}}{\% \text{HROM in faeces}} \times \frac{\% \text{Nutrient in faeces}}{\% \text{Nutrient in feed}} \right]$$

### Chemical analysis

The chemical analyses of diets, fish whole-body and faeces were performed according to standard methods [AOAC (Association of Official Analytical Chemists) 2000]: dry matter (dried at 105 °C), crude protein (Kjeldahl method), crude lipid (ether extract according to soxhlet method) and ash (combusted at 550 °C for 6 h). The measurement of crude fibre (CF) involves extraction of a ground feed sample with diethyl ether followed by sequential boiling in dilute acid and dilute base. The residue is then burned in a 500–600 °C muffle furnace. CF % is calculated from the difference in weight of the sample before and after burning [AOAC (Association of Official Analytical Chemists) 2000]. Calcium was analysed using atomic absorption phase spectrophotometer (Spectra-55, Varian) after acid digestion. Phosphorus content in fish whole body was determined in three replicates using the vanadium–molybdate method described by AOAC (Association of Official Analytical Chemists) (2000). Absorbance of the vanado-molybdo phosphorus complex was measured at 430 nm using a UV–VIS spectrophotometer (Specord S100, Carl Zeiss, CA, USA). Phytate P in feed ingredients was analysed by anion-exchange method (Latta & Eskin 1980; Harland & Oberleas 1986) using a 100–200 mesh AG1-X4 chloride anion exchange column (Bio-Rad Laboratory, Hercules, California, USA). Phytate is extracted from feed ingredients using diluted HCl. Extraction is then placed on an ion-exchange column. Phytate is eluted with 0.7 M NaCl solution and wet-digested with mixture of concentrated HNO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub> to release phytate P, which is measured colorimetrically at 640 nm using a UV–VIS spectrophotometer (Specord S100, Carl Zeiss) (Harland & Oberleas 1986). Available P was calculated by deducting phytate P from total P (Sajjadi & Carter 2004). Phytase efficacy was calculated as follows:

$$\text{Efficacy} = 100 \times \frac{\text{initial phytate P} - \text{final phytate P}}{\text{initial phytate P}}$$

### Statistical analysis

Data were analysed statistically using ANOVA and regression (Steele & Torrie 1980) with SPSS (version 11.5) statistical software package (SPSS Inc., Chicago, IL, USA). ANOVA for percentage data was performed on arcsine transformed data, while the percentage data were presented in the original scale. Treatment means were compared using Least Significant Difference and differences among treatments were considered significant at an alpha level of 0.05. Mean values were given with  $\pm$  SD.

## Results

### Pretreatment of plant ingredients

After the pretreatment, phytate P contents of both soybean meal and mixed plant meal decreased, while the available P contents increased for all levels of phytase doses including the controls (Tables 3 & 4).

Final available P and efficacy of converting phytate P to available P increased significantly with increasing doses of phytase for both soybean meal (Fig. 1) and mixed plant meal (Fig. 2). Based on two figures, curve equations for soybean meal and mixed plant meal can be written as:  $Y = 11.1907 X^{0.2841}$  ( $R^2 = 0.9078$ ,  $n = 15$ ,  $P < 0.05$ );  $Y = 29.7048 X^{0.14567}$  ( $R^2 = 0.9158$ ,  $n = 18$ ,  $P < 0.05$ ), where  $Y$  is the efficacy of phytase indicating how much phytate P in the plant materials can be converted to available P;  $X$  is the dose of phytase used for pretreatment. According to the inflexion calculation of curves (Lanari *et al.* 1998; Jiang *et al.* 2006), the dose of 1000 U kg<sup>-1</sup> was determined as the most cost effective dose.

### Growth performance

Pretreatment of plant ingredients with phytase and P supplementation both had significantly positive effects on growth performance of Nile tilapia. Survivals were all higher than 90%, ranging from 90.0% to 96.7% among treatments (Table 5). Growth performance in terms of final mean weight, weight gain and daily weight gain was best in the treatments supplemented with 12.5, 18.75 and 25 g kg<sup>-1</sup> MCP and the inorganic P control, intermediate in the treatments supplemented with MCP at 6.25 and 0 g kg<sup>-1</sup>, and poorest in the pretreatment control and the phytase control ( $P < 0.05$ ). SGR showed similar patterns of differences among treatments to other growth performance parameters, with the highest SGR in the treatment with MCP supplement at 12.5 g kg<sup>-1</sup>. Fish fed diets untreated with phytase showed poorer FCR, while FCR ranging from 1.10 to 1.85 was best in the treatments with MCP supplement at 12.5 and 25 g kg<sup>-1</sup> as well as the inorganic P control ( $P < 0.05$ ), which were not significantly different from the treatment with MCP supplement at 18.75 g kg<sup>-1</sup> ( $P > 0.05$ ), but were significantly better than all other treatments ( $P < 0.05$ ). PER ranged from 1.49 in the phytase control to 2.66 in the diet supplemented with MCP at 12.5 g kg<sup>-1</sup>.

The addition of phytase and inorganic P to the basal diet significantly increased the contents of ash, P and crude protein in the whole body of Nile tilapia (Table 6), while dry

Phytase (U kg <sup>-1</sup> )	Total P (g kg <sup>-1</sup> )	Phytate P (g kg <sup>-1</sup> )		Available P <sup>2</sup> (g kg <sup>-1</sup> )	
		Initial	Final	Initial	Final
0	6.40 ± 0.03	4.30 ± 0.02	3.56 ± 0.005 <sup>a</sup>	2.10 ± 0.05	2.82 ± 0.014 <sup>a</sup>
500	6.40 ± 0.03	4.30 ± 0.02	1.44 ± 0.005 <sup>b</sup>	2.10 ± 0.05	4.96 ± 0.012 <sup>b</sup>
750	6.40 ± 0.03	4.30 ± 0.02	1.27 ± 0.012 <sup>c</sup>	2.10 ± 0.05	5.13 ± 0.008 <sup>cd</sup>
1000	6.40 ± 0.03	4.30 ± 0.02	0.78 ± 0.005 <sup>d</sup>	2.10 ± 0.05	5.62 ± 0.011 <sup>de</sup>
1500	6.40 ± 0.03	4.30 ± 0.02	0.47 ± 0.005 <sup>e</sup>	2.10 ± 0.05	5.93 ± 0.017 <sup>e</sup>

<sup>1</sup> Values are means ± SD ( $n = 3$ ). Values in the same column with different superscript letters are significantly different ( $P < 0.05$ ).

<sup>2</sup> Calculated as: available phosphorus (P) = total P – phytate P (Sajjadi & Carter 2004).

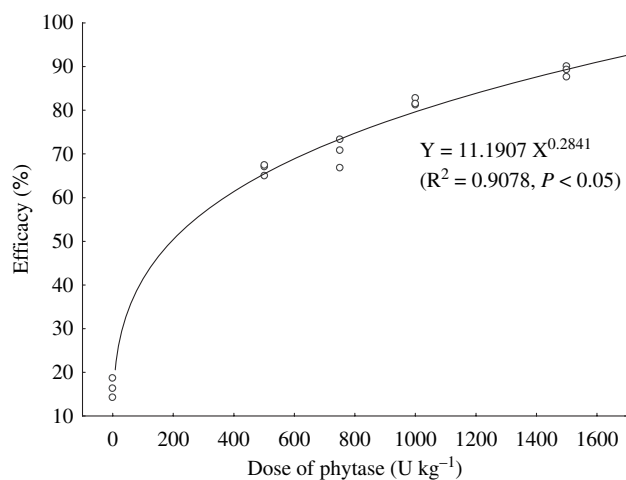
**Table 3** Changes of phytate P and available P after the pretreatment of soybean meal using phytase at different doses<sup>1</sup>

Phytase (U kg <sup>-1</sup> )	Total P (g kg <sup>-1</sup> )	Phytate P (g kg <sup>-1</sup> )		Available P <sup>2</sup> (g kg <sup>-1</sup> )	
		Initial	Final	Initial	Final
0	4.50 ± 0.002	3.00 ± 0.004	2.26 ± 0.002 <sup>a</sup>	1.50 ± 0.006	2.24 ± 0.002 <sup>a</sup>
500	4.50 ± 0.002	3.00 ± 0.004	0.73 ± 0.007 <sup>b</sup>	1.50 ± 0.006	3.77 ± 0.007 <sup>b</sup>
750	4.50 ± 0.002	3.00 ± 0.004	0.63 ± 0.009 <sup>b</sup>	1.50 ± 0.006	3.87 ± 0.009 <sup>b</sup>
1000	4.50 ± 0.002	3.00 ± 0.004	0.49 ± 0.010 <sup>c</sup>	1.50 ± 0.006	4.01 ± 0.010 <sup>c</sup>
1500	4.50 ± 0.002	3.00 ± 0.004	0.36 ± 0.005 <sup>d</sup>	1.50 ± 0.006	4.14 ± 0.005 <sup>d</sup>
3000	4.50 ± 0.002	3.00 ± 0.004	0.14 ± 0.003 <sup>e</sup>	1.50 ± 0.006	4.36 ± 0.003 <sup>e</sup>

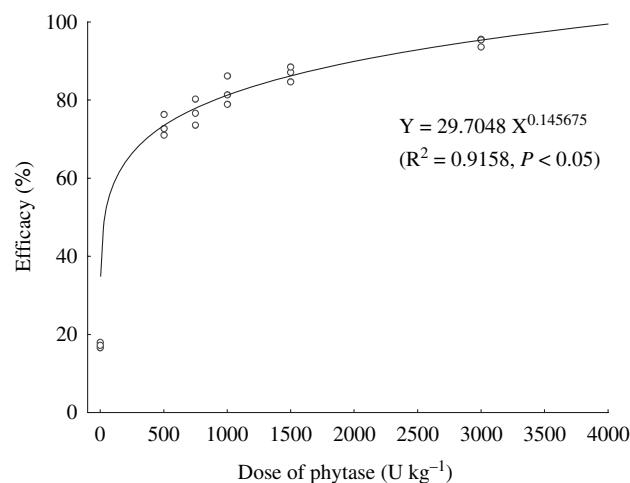
<sup>1</sup> Values are mean ± SD ( $n = 3$ ). Values in the same column with different superscript letters are significantly different ( $P < 0.05$ ).

<sup>2</sup> Calculated as: available phosphorus (P) = total P – phytate P (Sajjadi & Carter 2004).

**Table 4** Changes of phytate P and available P after the pretreatment of the mixed plant meal using phytase at different doses<sup>1</sup>



**Figure 1** Efficacy of converting phytate phosphorus (P) to available P by the pretreatment of soybean meal using phytase at different doses.



**Figure 2** Efficacy of converting phytate phosphorus (P) to available P by the pretreatment of the mixed plant meal using phytase at different doses.

matter and lipid contents decreased significantly ( $P < 0.05$ ). Compared with the phytase control and pretreatment control, significantly higher levels of P contents in tilapia carcass ranging from 1.68 to 1.83 g kg<sup>-1</sup> were found in the inorganic P control (diet 2), 25 g kg<sup>-1</sup> MCP supplement (diet 3), 18.75 g kg<sup>-1</sup> MCP supplement (diet 4), 12.5 g kg<sup>-1</sup> MCP supplement (diet 5), 6.25 g kg<sup>-1</sup> MCP supplement (diet 6)

and 0 g kg<sup>-1</sup> MCP supplement (diet 7) ( $P < 0.05$ ). There were no significant differences in P contents for whole body of Nile tilapia between the inorganic P control (diet 2), 25 g kg<sup>-1</sup> MCP supplement (diet 3), 18.75 g kg<sup>-1</sup> MCP supplement (diet 4) and 12.5 g kg<sup>-1</sup> MCP supplement (diet 5) ( $P > 0.05$ ). P deficiency of Nile tilapia in the phytase control (diet 1), 0 g kg<sup>-1</sup> MCP supplement (diet 7) and pretreatment

**Table 5** Growth performance of Nile tilapia juveniles fed the experimental diets for 60 days

Parameter	Unit	Experimental diets							
		Phytase control (diet 1)	Inorganic P control (diet 2)	100% MCP supplement (diet 3)	75% MCP supplement (diet 4)	50% MCP supplement (diet 5)	25% MCP supplement (diet 6)	0% MCP supplement (diet 7)	Pretreatment control (diet 8)
Initial mean weight	g	0.74 ± 0.09	0.74 ± 0.09	0.73 ± 0.00	0.74 ± 0.09	0.70 ± 0.04	0.77 ± 0.05	0.80 ± 0.00	0.74 ± 0.09
Final mean weight	g	19.1 ± 0.60 <sup>e</sup>	34.6 ± 0.76 <sup>a</sup>	34.9 ± 1.23 <sup>a</sup>	34.6 ± 0.56 <sup>a</sup>	35.8 ± 1.21 <sup>a</sup>	30.8 ± 0.74 <sup>b</sup>	26.8 ± 0.45 <sup>c</sup>	23.0 ± 1.07 <sup>d</sup>
Survival	%	95.0 ± 2.3 <sup>a</sup>	96.6 ± 0.0 <sup>a</sup>	91.6 ± 2.3 <sup>c</sup>	93.3 ± 4.2 <sup>b</sup>	91.6 ± 2.3 <sup>c</sup>	93.3 ± 4.2 <sup>b</sup>	90.0 ± 0.0 <sup>c</sup>	96.6 ± 0.0 <sup>a</sup>
FCR <sup>1</sup>	% day <sup>-1</sup>	1.85 ± 0.03 <sup>e</sup>	1.12 ± 0.07 <sup>a</sup>	1.14 ± 0.00 <sup>a</sup>	1.16 ± 0.01 <sup>ab</sup>	1.10 ± 0.06 <sup>a</sup>	1.24 ± 0.04 <sup>b</sup>	1.35 ± 0.02 <sup>c</sup>	1.64 ± 0.04 <sup>d</sup>
SGR <sup>2</sup>		5.45 ± 0.16 <sup>e</sup>	6.43 ± 0.25 <sup>ab</sup>	6.44 ± 0.06 <sup>ab</sup>	6.43 ± 0.18 <sup>ab</sup>	6.56 ± 0.06 <sup>a</sup>	6.16 ± 0.14 <sup>bc</sup>	5.85 ± 0.03 <sup>cd</sup>	5.75 ± 0.14 <sup>de</sup>
PER <sup>3</sup>		1.49 ± 0.02 <sup>e</sup>	2.59 ± 0.04 <sup>a</sup>	2.54 ± 0.08 <sup>a</sup>	2.61 ± 0.04 <sup>a</sup>	2.66 ± 0.08 <sup>a</sup>	2.36 ± 0.06 <sup>b</sup>	2.16 ± 0.04 <sup>c</sup>	1.68 ± 0.04 <sup>d</sup>

MCP, mono calcium phosphate; P, phosphorus.

<sup>1</sup> Feed conversion ratio (FCR) = dry feed weight given (g)/wet fish weight gained (g).

<sup>2</sup> Specific growth rate (SGR) (% day<sup>-1</sup>) = 100 × [(ln final mean weight – ln initial mean weight)/days of the experiment].

<sup>3</sup> Protein efficiency ratio PER = fish weight gained (g)/protein given (g).

Values are mean ± SD (*n* = 3), values with different superscript letters in the same row are significantly different (*P* < 0.05).

**Table 6** Whole-body chemical composition (g kg<sup>-1</sup>, dry matter basis) of Nile tilapia juveniles fed the experimental diets for 60 days

Parameter	Experimental diets							
	Phytase control (diet 1)	Inorganic P control (diet 2)	100% MCP supplement (diet 3)	75% MCP supplement (diet 4)	50% MCP supplement (diet 5)	25% MCP supplement (diet 6)	0% MCP supplement (diet 7)	Pretreatment control (diet 8)
Dry matter	259.3 ± 0.4 <sup>a</sup>	231.0 ± 0.3 <sup>e</sup>	230.5 ± 0.1 <sup>e</sup>	230.9 ± 0.6 <sup>e</sup>	231.3 ± 0.4 <sup>e</sup>	232.9 ± 1.0 <sup>d</sup>	237.8 ± 1.1 <sup>c</sup>	253.6 ± 0.4 <sup>b</sup>
Ash	88.0 ± 0.1 <sup>d</sup>	106.1 ± 0.3 <sup>a</sup>	106.1 ± 1.0 <sup>a</sup>	105.5 ± 0.7 <sup>a</sup>	105.3 ± 0.4 <sup>ab</sup>	104.2 ± 0.6 <sup>b</sup>	96.5 ± 0.7 <sup>c</sup>	89.0 ± 0.1 <sup>d</sup>
Lipid	188.4 ± 0.1 <sup>a</sup>	133.8 ± 0.3 <sup>cd</sup>	132.9 ± 0.7 <sup>d</sup>	134.0 ± 0.6 <sup>cd</sup>	133.1 ± 0.4 <sup>d</sup>	134.8 ± 2.4 <sup>cd</sup>	135.9 ± 1.1 <sup>c</sup>	175.3 ± 0.9 <sup>b</sup>
Crude protein	507.6 ± 6.5 <sup>c</sup>	553.4 ± 4.0 <sup>a</sup>	549.7 ± 3.9 <sup>a</sup>	548.1 ± 2.7 <sup>a</sup>	550.0 ± 3.4 <sup>a</sup>	531.4 ± 6.0 <sup>b</sup>	527.2 ± 6.3 <sup>b</sup>	513.7 ± 9.7 <sup>c</sup>
Phosphorous	13.3 ± 0.5 <sup>e</sup>	18.3 ± 0.4 <sup>a</sup>	18.6 ± 0.5 <sup>a</sup>	17.9 ± 0.4 <sup>a</sup>	18.1 ± 0.5 <sup>a</sup>	17.4 ± 0.2 <sup>b</sup>	16.8 ± 0.3 <sup>c</sup>	14.3 ± 0.3 <sup>d</sup>

MCP, mono calcium phosphate; P, phosphorus.

Values are means ± SD (*n* = 3); values with different superscript letters in the same rows are significantly different (*P* < 0.05).

control (diet 8) was observed by abnormal head, curved vertebra and loose scale during the trial period. The lowest levels of ash, P and crude protein contents and highest contents of dry matter and lipid in the whole body of Nile tilapia were all found in the phytase control (diet 1).

### Phosphorus retention efficiency and load

Dietary interaction effects of phytase were observed for phosphorus retention efficiency and phosphorus load (Table 7). P retention efficiency was significantly higher for diet 5, 6 and 7, intermediate for 2, 3 and 4 and significantly lower for 1 and 8 (*P* < 0.05). P load was significantly higher for diet 1 and 8, intermediate for 2, 3 and 4 and significantly lower for 5, 6 and 7 (*P* < 0.05).

### Apparent digestibility

Apparent digestibility coefficient of phosphorus (ADC<sub>P</sub>) were enhanced by the phytase pretreatment, however, there

were no significant differences in apparent digestibility coefficient of crude protein (ADC<sub>CP</sub>) among all treatments. ADC<sub>CP</sub> of the eight experimental diets ranged from 83.10% to 85.27% did not significantly differ each other (*P* > 0.05, Table 8). However, phytase pretreatment significantly increased ADC<sub>P</sub> of the experimental diets (*P* < 0.05). ADC<sub>P</sub> values in the diets supplemented with 12.5 g kg<sup>-1</sup> or less MCP (diets 5, 6 and 7) were significantly higher than those in all other diets including the diets supplemented with more than 12.5 g kg<sup>-1</sup> MCP as well as the three control diets (*P* < 0.05).

### Discussion

Pretreatment of plant ingredients using microbial phytase significantly increased available P content in feed, indicating that the indigestible phytate P was successfully converted to available P by phytase in the present study. The relationships between the amount of available P released from phytate P and the doses of phytase administered can be used to predict

Diet	Treatment	P retention (%)	P load (g kg <sup>-1</sup> )
Phytase control (diet 1)	None	28.56 ± 1.17 <sup>c</sup>	6.39 ± 0.17 <sup>a</sup>
Inorganic P control (diet 2)	+ 25 g MCP kg <sup>-1</sup>	49.67 ± 1.11 <sup>b</sup>	4.64 ± 0.55 <sup>b</sup>
100% MCP supplement (diet 3)	Phytase + 25 g MCP kg <sup>-1</sup>	51.75 ± 1.02 <sup>b</sup>	4.32 ± 0.05 <sup>b</sup>
75% MCP supplement (diet 4)	Phytase + 18.75 g MCP kg <sup>-1</sup>	52.48 ± 2.33 <sup>b</sup>	4.06 ± 0.34 <sup>b</sup>
50% MCP supplement (diet 5)	Phytase + 12.5 g MCP kg	59.84 ± 1.34 <sup>a</sup>	3.49 ± 0.02 <sup>c</sup>
25% MCP supplement (diet 6)	Phytase + 6.25 g MCP kg	59.69 ± 2.01 <sup>a</sup>	3.73 ± 0.05 <sup>c</sup>
0% MCP supplement (diet 7)	Phytase	57.38 ± 1.07 <sup>a</sup>	3.30 ± 0.02 <sup>c</sup>
Pretreatment control (diet 8)	Phytase added directly	32.59 ± 1.14 <sup>c</sup>	6.11 ± 0.19 <sup>a</sup>

MCP, mono calcium phosphate; P, phosphorus.

Data are means ± SD (*n* = 3); values in the same column with different superscript letters are significantly different (*P* < 0.05).

**Table 7** P retention efficiency and P load of Nile tilapia fed test diets for 60 days

Diet	Treatment	ADC <sub>CP</sub> (%)	ADC <sub>P</sub> (%)
Phytase control (diet 1)	None	84.40 ± 1.47	28.23 ± 1.15 <sup>f</sup>
Inorganic P control (diet 2)	+25 g MCP kg <sup>-1</sup>	84.37 ± 0.83	52.37 ± 2.31 <sup>c</sup>
100% MCP supplement (diet 3)	Phytase + 25 g MCP kg <sup>-1</sup>	84.43 ± 0.56	47.10 ± 0.65 <sup>d</sup>
75% MCP supplement (diet 4)	Phytase + 18.75 g MCP kg <sup>-1</sup>	84.57 ± 0.61	55.33 ± 1.68 <sup>b</sup>
50% MCP supplement (diet 5)	Phytase + 12.5 g MCP kg	85.27 ± 0.65	69.03 ± 2.00 <sup>a</sup>
25% MCP supplement (diet 6)	Phytase + 6.25 g MCP kg	83.63 ± 0.47	69.47 ± 0.38 <sup>a</sup>
0% MCP supplement (diet 7)	Phytase	83.10 ± 0.91	67.83 ± 1.84 <sup>a</sup>
Pretreatment control (diet 8)	Phytase added directly	83.40 ± 1.17	36.03 ± 0.97 <sup>e</sup>

MCP, mono calcium phosphate; P, phosphorus.

Values are means ± SD (*n* = 3); values in the same column with different superscript letters are significantly different (*P* < 0.05).

**Table 8** Apparent digestibility coefficients of crude protein (ADC<sub>CP</sub>) and P (ADC<sub>P</sub>) for the experimental diets fed to Nile tilapia juveniles

the amount of available P to be released and the amount of inorganic P that may need to be supplemented at different dosages of phytase. In the present study, the release rates of available P from phytate P in soybean meal were about 66% and 82% at phytase doses of 500 and 1000 U kg<sup>-1</sup> respectively, which are similar to those achieved by Yu & Wang (2000). Other studies have shown that soybean meal treated by phytase at 500 U kg<sup>-1</sup> released about 20% of phytate P (Schäfer *et al.* 1995), which increased to about 40% when the dose of phytase increased to 1000 U kg<sup>-1</sup> (Cain & Garling 1995). In the present study, the release rates of available P from phytate P were 16.73% for soybean meal and 24.67% for the mixed plant ingredients when no phytase was added in the controls, due probably to the effect of citrate acid on hydrolysis of phytate in plant ingredients during the incubation. Sugiura *et al.* (2001) found that citric acid could chelate minerals and prevent their precipitation, indicating that citric acid can convert phytate P to available P to some extent. Thus, the pretreatment method might be an effective alternative for fish to maximize phytase activity and overcome temperature limitation (Cain & Garling 1995; Yoo *et al.* 2005).

The growth performance and FCR of Nile tilapia after the 60-day growth trial was improved significantly when the pretreated diets with phytase at 1000 U kg<sup>-1</sup> were used,

compared with the phytase control diet in the present study. Higher PER of fish fed the diets containing phytase-treated than untreated meal indicates that the phytase or heating of the meal during the phytase treatment process improved the nutritional quality of the mixed plant meal. Liebert & Portz (2005) reported the optimal growth of Nile tilapia was achieved by phytase supplementation at 750–1250 U kg<sup>-1</sup> in plant-based diets. Growth improvement was also observed in rainbow trout fed phytase-supplemented diets (Rodehuts-cord & Pfeffer 1995; Papatryphon *et al.* 1999), and salmon and rainbow trout fed the diets with phytase pretreated ingredients (Cain & Garling 1995; Vielma *et al.* 2002). However, the inclusion of phytase did not improve the growth of rainbow trout fed diets containing canola protein concentrate (Forster *et al.* 1999) and juvenile Korean rockfish (*Sebastes schlegeli*) fed diets containing soybean meal (Yoo *et al.* 2005). The available P contents in the phytase control diet, the diets without MCP supplement and the pretreatment control diet were lower than the minimum P requirement of Nile tilapia (5 g kg<sup>-1</sup>), and caused poor fish growth. The available P content in the diet supplemented with 6.25 g kg<sup>-1</sup> MCP was 5.6 g kg<sup>-1</sup>, which was slightly higher than the minimum requirement, but still resulting in poor growth of Nile tilapia than the inorganic P control in the present study, probably because that juvenile fish have



higher P requirements than large fish [NRC (National Research Council) 1993; Cain & Garling 1995]. Diets supplemented with more than 12.5 g kg<sup>-1</sup> MCP and the inorganic P control diet that contained around 9 g kg<sup>-1</sup> of available P gave tilapia better growth than other diets in the present study, in agreement with that reported by Watanabe *et al.* (1980). Vielma *et al.* (2002) found better growth and feed utilization efficiency by pretreatment could be attributed partially to more efficient phytic acid hydrolysis in comparison to top-spraying phytase. Forster *et al.* (1999) reported that phytase top-sprayed onto soy protein-based diets at doses of 0, 500, 1500 or 4500 U kg<sup>-1</sup> diet did not have effect on growth or feed utilization efficiency in rainbow trout. Similarly, 2000 U phytase kg<sup>-1</sup> mixed in the feed prior to pelleting did not enhance performance of sea bass (*Dicentrarchus labrax*) despite a clear increase in P digestibility (Oliva-Teles *et al.* 1998). Further studies should be conducted to compare the economic benefits between the pretreatment and top-spraying with phytase at industrial level.

Ash, crude protein and P contents in whole Nile tilapia fed the phytase pretreated diets (diet 3, 4, 5, 6 and 7) were significantly higher than those fed diets 1 and 8, indicating that phytase pretreatment led to improvement in carcass quality and P retention. Among all the diets, diets 2, 3, 4 and 5 performed best in terms of the growth, nutrient utilization and inorganic P replacement level in the present study. Compared with diets 2, 3 and 4, diet 5 supplement the least inorganic P, only 50% of the inorganic control (diet 2) and diet 3. And the cost of diet 5 is less than the other three diets. So diet 5 supplemented with phytase at 1000 U kg<sup>-1</sup> and 12.5 g kg<sup>-1</sup> MCP is the best, indicating that phytase at 1000 U kg<sup>-1</sup> is comparable with 12.5 g MCP kg<sup>-1</sup> diet. Schäfer *et al.* (1995) suggested that diets mainly based on plant proteins such as soybean meal have high contents of phytic acid, and the supplementation of phytase at levels of either 500 or 1000 U kg<sup>-1</sup> can replace 1.9 g P from MCP. Phytase also increased deposition of P in the whole fish body, indicating that P load to the environment was reduced. Therefore, using phytase in plant-based diets can reduce the need for inorganic P supplementation in diets, leading to the reductions of P discharged to the environment from fish farms. Besides, lipid and dry matter contents in the whole body increased when Nile tilapia fed the low available P diets (diets 1 and 8), which agreed with the results in carp (Onishi *et al.* 1981) and rainbow trout (Rodehutschord 1996). The enhanced lipid and dry matter contents were probably caused by the inhibition of the  $\beta$ -oxidation of fatty acids resulting from a deficiency of P (Schäfer *et al.* 1995).

Phosphorus retention efficiency was significantly higher in fish fed phytase-supplemented diets compared with phytase control in the present study, indicating that phytase could improve phosphorus utilization. The lower retention values in diets 2, 3 and 4 were because of their exceeding the phosphorus requirement, demonstrating improved phosphorus utilization at dietary phosphorus near to requirement. When dietary phosphorus concentration increases above the requirements, the retention efficacy decreases. Factors like FCR, ingredient digestibility and dietary phosphorus content affect phosphorus load (Vielma *et al.* 2002). Using phytase in plant based diets in comparison with phytase control reduced phosphorus load. Hence, the inclusion of phytase in the diets is effective for aquaculture waste management. In the present study, phosphorus utilization was higher in the fish fed either the phytase or MCP-phytase diets. But in combination of phytase and MCP diets, there was no further advantage when the P level is above P requirement of tilapia. Because of the positive effect of phytase, the P retention in juvenile Nile tilapia was improved while the total P load to the environment was reduced. Similar results have also been reported in salmon (Cain & Garling 1995) and rainbow trout (Lanari *et al.* 1998; Sugiura *et al.* 2001) fed soybean meal based diets.

The pretreatment of mixed plant ingredients using phytase at 1000 U kg<sup>-1</sup> diet significantly improved ADCp in the present study, compared with the phytase control diet and pretreatment control diet. The ADCp was reported to be the highest when dietary P level reached the P requirement of cultured fish, then declined with increasing dietary P levels (Riche & Brown 1996). In the present study, the dietary P level in the diet with MCP supplement at 25 g kg<sup>-1</sup> (diet 3) was the highest among all diets, and even higher than the P requirement of juvenile Nile tilapia because of the release of available P from phytate P. However, the ADCp in this diet was significantly lower than the diets with less MCP supplements (diets 4, 5, 6 and 7) as well as the P control ( $P < 0.05$ ), but significantly higher than that in the phytase control. The results with respect to digestibility of diets with MCP supplement and phytase pretreatment may also probably because of the interaction between MCP and phytase. Sugiura *et al.* (2001) also observed that the ADCp reached 93% in rainbow trout fed with a phytase pretreated diet containing 4.21 g kg<sup>-1</sup> of total P. However, when the total P level increased to 14.7 g kg<sup>-1</sup>, the ADCp in diet containing phytase of 1000 U kg<sup>-1</sup> was only 62%. No significant difference was observed in the ADCcp in the present study, and the result was consistent to those reported by other studies (Riche *et al.* 2001; Sajjadi & Carter 2004; Yoo *et al.* 2005). However, Furuya *et al.* (2001) observed that phytase at

500–1500 U kg<sup>-1</sup> improved P availability and crude protein digestibility in Nile tilapia. More research should focus on the apparent digestibility of crude protein and profiles of amino acids in tilapia fed phytase pretreated plant-based diets.

In conclusion, the results of the present study have indicated that the optimal dose of phytase for the pretreatment of plant ingredients is 1000 U kg<sup>-1</sup>, which can efficiently convert phytate P to available P and replace 12.5 g kg<sup>-1</sup> of the inorganic P supplementation to the diets for Nile tilapia juveniles without affecting their growth performance, apparent digestibility coefficients of both crude protein and P. The addition of phytase in the diets of Nile tilapia juveniles can reduce the use of MCP, enhance the utilization of P in plant ingredients, and thus minimize the P discharge to the environment.

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