A THESIS

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Utilization of Microbial Phytase in Diets for Juvenile Olive Flounder (*Paralichthys olivaceus*)

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GRADUATE SCHOOL

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I. INTRODUCTION

Replacement of fish meal by plant protein sources is of great interest (Fontainhas-Fermandes et al., 1999; Mabahinzireki et al., 2001) and become increasingly important for the development of low-cost fish feed (Baruah et al., 2004) because of high cost and limited availability of fish meal in many countries (Naylor et al., 2000). Suitable alternative feed ingredients, such as soybean meal and cottonseed meal are promising sources of protein for aqua-feeds in the future. The major obstacle in using these plant protein sources is the presence of anti-nutritional factors, such as phytic acid, gossypol, saponins, and trypsin inhibitors (NRC, 1993; Masumoto et al., 2001). Phytic acid (myo-inositol hexakisphosphate) is the major phosphorus storage compound in plant seeds and composing of 80% of total phosphorus that can not be digested and absorbed by monogastric animals, including fish (Baruah et al., 2004). The discharge of unutilized phytate phosphorus into water can stimulate the growth of algae and phytoplankton, thus reducing dissolved oxygen and causing water pollution around fish farms (Sugiura et al., 1999). Furthermore, phytic acid can chelate other divalent and trivalent cations, such as iron (Fe), zinc (Zn), magnesium (Mg), copper (Cu) and calcium (Ca), resulting in decreased bioavailability of these minerals (Wise, 1983). It also can react directly with charged groups of protein mediated by mineral cations, thus adversely influence protein digestion and bioavailability (Barbara et al., 1999; Urbano et al., 2000; Chen and Li, 2003).

Phosphorus is an essential mineral required by fish for important physiological functions (NRC, 1993). Recently, many processes have been reported

to liberate free phosphorus from phytic acid (Urbano et al., 2000; Hotz and Gibson, 2001), but the better results were obtained by the use of enzymatic hydrolysis (Silva et al., 2005). Phytase has been reported not only to increase phosphate utilization efficiency from phytate in feeds but also to decrease phosphorus pollution into the water (Broz et al., 1994; Yanke et al., 1998). Hughes and Soares (1998) showed that phytase supplementation to a diet containing high level of phytate improved the absorption and utilization of phosphorus in striped bass. Dietary phytase also improved the nutritive value of canola protein concentrate and decreased phosphorus output in rainbow trout (Forster et al., 1999). Baruah et al. (2004) reported that microbial phytase supplementation in diets increased bioavailability of nitrogen and phosphorus and reduced feed costs.

Olive flounder (*P. olivaceus*) is one of the most important culture species and accounts for higher than 98% of the flatfish production in Korea. Recently, many studies were conducted to investigate the use of plant protein sources for fish meal replacement in diets for olive flounder (Kikuchi et al., 1994; Kikuchi, 1999; Masumoto et al., 2001; Saitoh et al., 2003; Pham et al., 2005). The deficiency of phosphorus has been reported to limit the inclusion of plant proteins in fish feeds. Masumoto et al. (2001) observed that the dietary supplementation of phytase improved the bioavailability of phosphorus in soybean meal or soybean protein concentrate based diets for olive flounder. Addition, there is few information on the effects of phytase on digestibility of phosphorus and protein in diets containing cottonseed and soybean meal for olive flounder. Therefore, this study was conducted to investigate the effects of supplementation of phytase on growth performance, feed

utilization, protein digestibility and phosphorus availability in juvenile olive flounder fed diets containing cottonseed and soybean meal.

II. MATERIALS AND METHODS

2.1 Experimental diets

Four experimental diets (CS0, CS30, CS30+P, and CS40+P) were formulated to be isonitrogenous (48% crude protein) and isocaloric (18 MJ/kg). The energy value of each diet was estimated on the basis of mammalian physiological fuel value, i.e., 16.7/KJ g protein or carbohydrate and 37.7 KJ/g lipid (Lee and Putman, 1973). The diet formulation and proximate compositions are presented in Table 1, 2. Diet CS0 is fish meal based diet (control diet). In diet CS30, 30% fish meal protein in the control diet was replaced by cottonseed and soybean meal in equal proportion. Based on the results in our previous experiments, diet (CS40) containing 40% cottonseed and soybean meal for fish meal substitute impaired the growth of juvenile olive flounder compared to the control diet. Therefore, diet CS40 was excluded in present study. In diets CS30+P and CS40+P, 30% and 40% fish meal was replaced by cottonseed and soybean meal with phytase supplementation of 1000 FTU/kg diet. Microbial phytase was used in the experimental diets as described by Cheng and Hardy (2003) and Yoo et al. (2005). The phytase activities measured in diets CS30+P and CS40+P were 1065 and 1097 FTU/kg diet, respectively (Table 2). All dry ingredients were thoroughly mixed with distilled water and fish oil. Pellets were extruded through the meat chopper machine (SMC-12, Korea) in 3.0 mm diameter size, dried by fan at room temperature, crushed into desirable particle sizes (0.4 – 2.0 mm), and stored at –20 ^{o}C until use.

Table 1. Formulation of the experimental diets (% dry matter)

	Diets			
Ingredients	**CS0**	**CS30**	**CS30+P**	**CS40+P**
White fish meal	54.00	37.80	37.80	32.40
Soybean meal	0.00	11.80	11.80	15.74
Cottonseed meal[1]	0.00	12.70	12.70	16.94
Corn gluten meal	6.60	7.20	7.20	7.40
Wheat flour	24.00	13.30	13.30	9.72
Mineral mix[2]	0.50	0.50	0.50	0.50
Vitamin mix[3]	0.50	0.50	0.50	0.50
Squid liver oil	12.00	13.00	13.00	13.30
CMC	1.00	1.00	1.00	1.00
Lysine	0.00	0.60	0.60	0.80
Methionine	0.00	0.30	0.30	0.40
Ferrous Sulfate-7H_2O	0.00	0.30	0.30	0.30
Phytase[4]	0.00	0.00	0.01	0.01
Cellulose	0.90	0.50	0.49	0.49
Chromic oxide	0.50	0.50	0.50	0.50

[1] Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA.

[2] Mineral mixture (g/kg of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3] Vitamin mixture (g/kg of mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[4] Phytase (10,000 FTU/g) was purchased from Easy Bio System, Inc., Seoul, Korea.

Table 2. Proximate composition of the experimental diets (% dry matter)

	Diets			
Proximate composition	**CS0**	**CS30**	**CS30+P**	**CS40+P**
Dry matter, %[1]	98.46	98.04	96.09	95.59
Protein, % DM[2]	48.06	48.32	48.58	48.12
Lipid, % DM[3]	17.12	16.98	16.33	16.45
Ash, % DM[4]	8.80	8.15	8.23	8.12
Gross energy, MJ/kg DM	17.87	17.87	17.87	17.85
Phytase activity (FTU/ kg DM)[5]	0.00	0.00	1065.00	1097.40

[1] Dry matter was analyzed according to AOAC (1995).

[2] Protein was analyzed using Kjeltec 2003 Analyzer Unit (Foss Tecator, Sweden).

[3] Lipid was analyzed using the method described by Folch et al. (1957).

[4] Ash was analyzed according to AOAC (1995).

[5] Phytase activity was analyzed according to the method described by Han et al. (1999) and Kim and Lei (2005).

2.2 Fish and feeding trial

Olive flounder juveniles were transported from a private hatchery in Jeju Island to Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. The fish were fed with a commercial diet for 2 weeks to be adapted to the experimental facilities. Three hundreds fish (mean body weight 2.5 g) were randomly distributed into twelve 35 L tanks (25 fish/tank) in a flow through system supplied with sand filtered seawater at a flow rate of 3 L/min. One of the experimental diets was fed 3 groups of fish to apparent satiation (twice per day at 8:00 and 18:00 hours) for 10 weeks. Aeration was also provided to maintain enough dissolved oxygen. The growth of fish was measured every 2 weeks.

2.3. Phytase activity

Phytase activity the experimental was measured according to the method described by Han et al. (1999) and Kim and Lei (2005) (Fig. 1). Dietary samples were finely ground with a grinding machine (Korea) in a cold room (4 ^{o}C) until all materials pass through a 1-mm sieve. Five grams sample (triplicates for each diet) was weighed into a 125 ml flask and 50 ml of 0.2 M citrate buffer (pH 5.5) was added into the flask. The phytase was extracted by constant stirring (magnetic stir bar) at room temperature for 30 min. The mixture was transferred into an enppendorf tube and centrifuge at 4 ^{o}C and 15,000 x g for 20 min with a centrifuge machine (Micro-TR17, Hanil Science Industrial Co., Ltd, Korea). The supernatant fraction was transferred into a clean eppendorf tube. Two aliquots (0.2 ml each) of samples were pipetted into

10 ml test tubes. The test tube was incubated at 37 ^{o}C water bath for 5 min. Then 0.2 ml of 1% (wt/vol) sodium phytate was added in the selected buffer and pH to start the enzymatic hydrolysis of phytate, and incubated for 15 min at 37 ^{o}C. The reaction was terminated by adding 0.4 ml of 15% trichloroacetic acid (room temperature). The mixture was centrifuged at 2,000 x g for 10 min and transferred the supernatant fraction to a new tube. The supernatant (0.2 ml) was mixed with 1.8 ml of nanopure water. Two ml of fresh color reagent was added into each tube and mixed well. The tube was incubated at 50 ^{o}C for 15 min and cooled down at room temperature. The absorbance of each sample solution was measured at 820 nm using a spectrophotometer (Geneysys 10 UV, Rochester, NY, USA) using distilled water as the blank and the series of diluted potassium phosphate solutions as standards. Phytase activity was calculated per gram of feed. One unit of phytase is defined as the amount of enzyme required to release 1 μmol of inorganic P/min from sodium phytate at 37 ^{o}C.

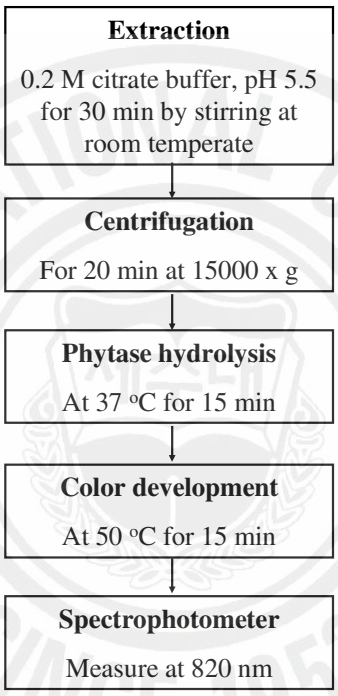

Fig. 1. Flow chart of the direct method for determining phytase activity in the experimental diets.

2.4 Whole body composition

At the end of feeding trial, all fish were weighed and counted for the calculation of feed intake, feed conversion ratio, protein efficiency ratio, and specific growth rate. Three fish from each tank (9 fish per diet) were sampled and stored at –20 ^{o}C for whole body proximate analysis. Analyses of crude protein, moisture and ash were performed by the standard procedures (AOAC, 1995). Lipids were determined according to the method described by Folch et al. (1957).

2.5 Feces collection and apparent digestibility test

The indirect method described by Cho and Kaushik (1990) was used to calculate the apparent digestibility coefficient of protein and phosphorus with chromic oxide (0.5% in diets) as the inert indicator. Feces were collected with a modified fecal collection system for olive flounder (Yamamoto et al., 1998). After ten weeks of feeding trial, fish of each treatment (three groups) were transferred to four 150 L fecal collection tanks. To collect the feces, all the fish were fed their respective diets containing 0.5% chromic oxide to satiation in the evening at 19:00 hour and the feces were collected in the next morning and afternoon at 7:00 and 14:00 hours, respectively. The collected feces were immediately frozen at -20 ^{o}C until analysis. Dietary and fecal protein was analyzed using Kjeltec 2003 Analyzer Unit (Foss Tecator AB, Sweden). Chromic oxide in feces and diets was determined according to the method described by Furukawa and Tsukahara (1966). Total phosphorus in diets and feces were measured using an inductively coupled plasma (ICP) emission spectrophotometer as described by Leske and Coon (1999).

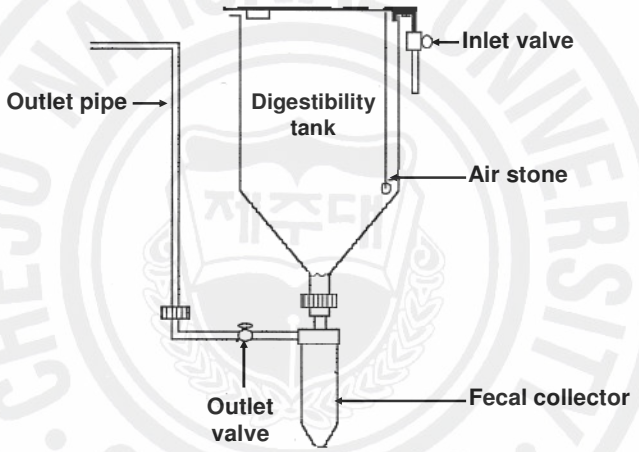

Fig. 2. Design of a digestive tank.

2.6 Serum cholesterol

At the end of feeding trial, 3 fish per tank (9 fish per diet) were randomly selected and anaesthetized in tricaine methane sulfonate (MS-222) solution (100 mg/l). Blood were taken from caudal veins with non-heparinised syringes, kept at room temperature for 2 h and centrifuged at 5,000 rpm for 10 min at 4 ^{o}C using a micro-centrifuge (Micro-TR17, Hanil Science Industrial Co., Ltd, Korea). The serum cholesterol was determined using automatic Photometer CH100 Plus (Calenzano, Firenze, Italy).

2.7 Antioxidant capacity assay

Antioxidant capacity of experimental diets and fish livers was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as described by Brand-Williams (1995) with some modifications. Two g of diets (2 replicates per diet) were homogenized in 20 ml aqueous methanol (80%) and kept at room temperature for 10 min. The homogenates were centrifuged at 5,000 rpm (4 ^{o}C) for 10 min and filtered through a 0.45 μm syringe filter (Whatman Inc., Clifton, NJ) prior to the assay. Whole livers of 3 bled fish per tank (9 fish per diet) were homogenized in the aqueous methanol (80%) at a ratio of 1:4 (whole livers: aqueous methanol) for 60 sec using a homogenizer (X-120, Germany). The homogenate was centrifuged at 5,000 rpm (at 4 ^{o}C) for 10 min. The supernatant was filtered through a 0.45 μm syringe filter. One hundred μl of filtered extract was pipetted into a 1.5 ml cuvette, then 900 μl of DPPH methanolic solution (100 μM) was added to obtain a

final volume of 1 ml. The absorbance of the mixture was measured at 517 nm with 1 min intervals for 10 min by a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The antioxidant capacity of the extract against the DPPH radicals was calculated as percent inhibition. Percent inhibition = $[(A_0 - A_s)/A_0] \times 100$, where A_0, A_s are the absorbance of sample at 0 and s min, respectively.

2.8 Total polyphenol compounds

Total polyphenol compounds in the experimental diets were measured by a colorimetric method described by Skerget et al. (2005). Briefly, 1 g of diets was extracted with 250 ml methanol for 2 h at 40 ^{o}C. The solution was cooled and filtered through a 0.45 μm syringe filter (Whatman Inc., Clifton, NJ). To 0.5 ml filtered extract, 2.5 ml of Folin-Ciocalteu reagent (0.2 N, Sigma) was added and kept for 5 min at room temperature, then 2 ml of Na_2CO_3 solution (75g/l) was added. The mixture was incubated for 5 min at 50 ^{o}C and cooled. The absorbance was measured at 760 nm using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The results were expressed in gram of gallic acid per kilogram of dry diet.

2.9 Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple tests. Data were presented as means ± standard deviations (SD). The percentage data were

arcsine transformed before the ANOVA analysis. Differences were considered significantly at $P<0.05$.

III. RESULTS

3.1 Growth performance

Growth performance and feed utilization of juvenile olive flounder (initial body weight 2.5 g) fed the diets containing cottonseed and soybean meal (CS) with or without phytase supplementation for 10 weeks are presented in Table 3. Weight gain, specific growth rate, and protein efficiency ratio of fish fed the diets CS30 and CS30+P were not significantly different compared to those of fish fed the control diet. Feed conversion ratio and feed intake did not differ among fish groups fed all the experimental diets. Survival of all fish groups was over 90% and was not significantly different.

Table 3. Growth performance and feed utilization of juvenile olive flounder fed the experimental diets for 10 weeks[*]

Diets	**CS0**	**CS30**	**CS30+P**	**CS40+P**
Initial body weight, g	2.47 ± 0.15	2.47 ± 0.04	2.50 ± 0.11	2.49 ± 0.02
Final body weight, g	15.1 ± 0.59[a]	14.96 ± 1.07[ab]	15.30 ± 0.31[a]	14.08 ± 0.57[b]
Specific growth rate[1]	1.09 ± 0.02[a]	1.06 ± 0.05[ab]	1.06 ± 0.03[ab]	1.02 ± 0.03[b]
Protein efficiency ratio[2]	1.67 ± 0.01[a]	1.56 ± 0.04[ab]	1.59 ± 0.04[ab]	1.47 ± 0.17[b]
Feed conversion ratio[3]	1.24 ± 0.01	1.30 ± 0.07	1.26 ± 0.01	1.32 ± 0.08
Feed intake (g/g BW)[4]	1.94 ± 0.13	2.36 ± 0.13	1.95 ± 0.05	2.25 ± 0.35
Survival (%)	100.0 ± 0.00	91.70 ± 7.80	98.30 ± 1.30	93.30 ± 8.89

[*] Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

[1] Specific growth rate (%) = [(log final body weight - log initial body weight)/days] x100

[2] Protein efficiency ratio = wet weight gain/total protein given.

[3] Feed conversion ratio = dry feed fed/wet weight gain.

[4] Feed intake (g/g body weight) = dry feed consumed (g)/body weight (g).

3.2 Whole body composition

For whole body composition (Table 4), there were no significant differences among fish groups fed the experimental diets, except for ash content. Ash in whole body of juvenile olive flounder was gradually increased with the increasing of dietary cottonseed and soybean meal inclusion.

Table 4. Whole body composition of juvenile olive flounder fed the experimental diets for 10 weeks*

Diets	**CS0**	**CS30**	**CS30+P**	**CS40+P**
Moisture content, %	74.6 ± 0.5	75.1 ± 0.9	74.6 ± 0.5	74.6 ± 0.3
Protein, % DM	65.2 ± 4.0	64.1 ± 3.2	64.3 ± 3.0	66.1 ± 2.2
Lipid, % DM	23.5 ± 2.2	20.9 ± 1.4	23.2 ± 0.9	21.9 ± 1.7
Ash, % DM	9.8 ± 0.7^{a}	10.9 ± 1.3^{ab}	11.8 ± 0.9^{bc}	12.7 ± 0.6^{c}

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

3.3 Apparent digestibility coefficient

The apparent digestibility coefficient (ADC) of protein in the diet CS40+P was significantly higher than that of other experimental diets (Fig. 3). No differences in ADC of protein were found between the diets CS0, CS30, and CS30+P, even though diets CS30 and CS30+P resulted in numerically increased values. Phosphorus availability was gradually increased ($P<0.05$) with the increment of dietary CS inclusion and/or phytase supplementation (Fig. 4).

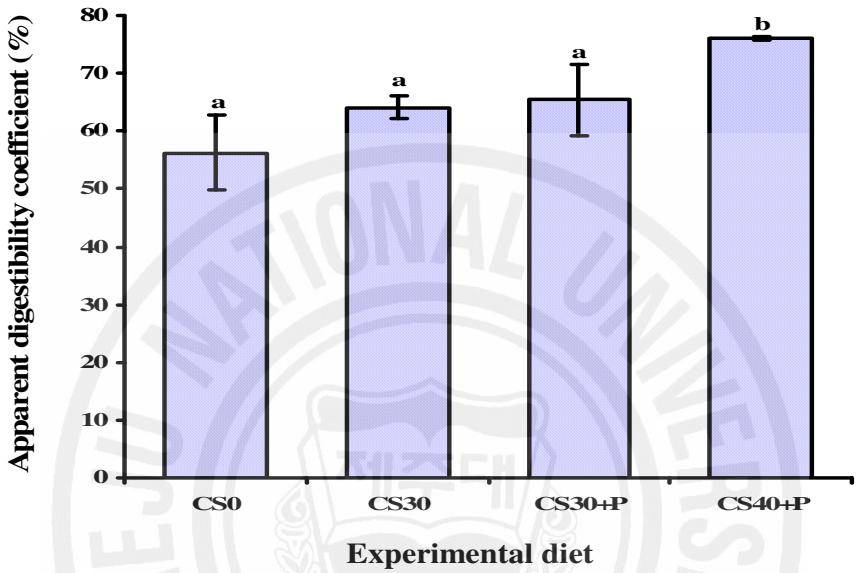

Fig. 3. Apparent digestibility coefficient of protein in juvenile olive flounder fed the diets containing cottonseed and soybean meal for 10 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

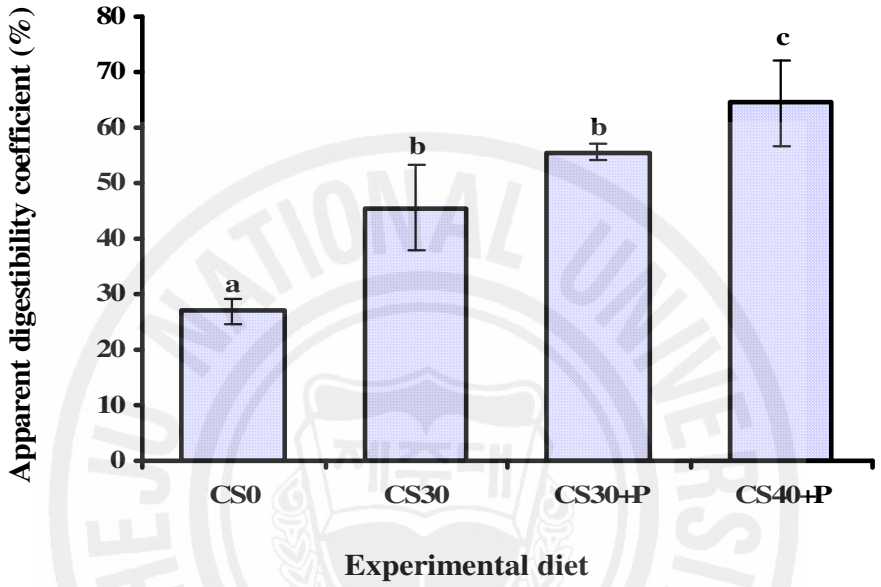

Fig. 4. Apparent digestibility coefficient of phosphorus in juvenile olive flounder fed the diets containing cottonseed and soybean meal for 10 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

3.4 Serum cholesterol

Serum cholesterol of fish fed the diets CS30 and CS40+P was significantly lower than that of fish fed the control diet. Cholesterol content in serum of fish fed the diet CS30+P with phytase supplementation was comparable to that of fish fed the control diet (Fig. 5).

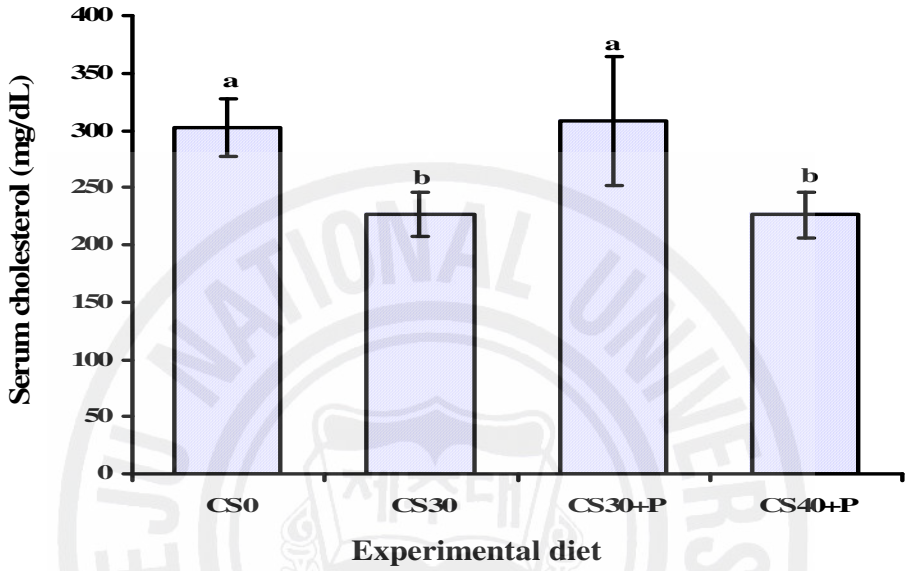

Fig. 5. Cholesterol in serum of juvenile olive flounder fed the experimental diets for 10 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

3.5 Antioxidant activity

Antioxidant activity in the experimental diets was significantly increased with the increment of dietary cottonseed and soybean meal (Fig. 6). The DPPH radical scavenging capacity of liver tended to increase with the increment of dietary cottonseed and soybean meal (Fig. 7). Total polyphenol content was significantly increased in CS40+P diet compared to that in the other diets (Fig. 8).

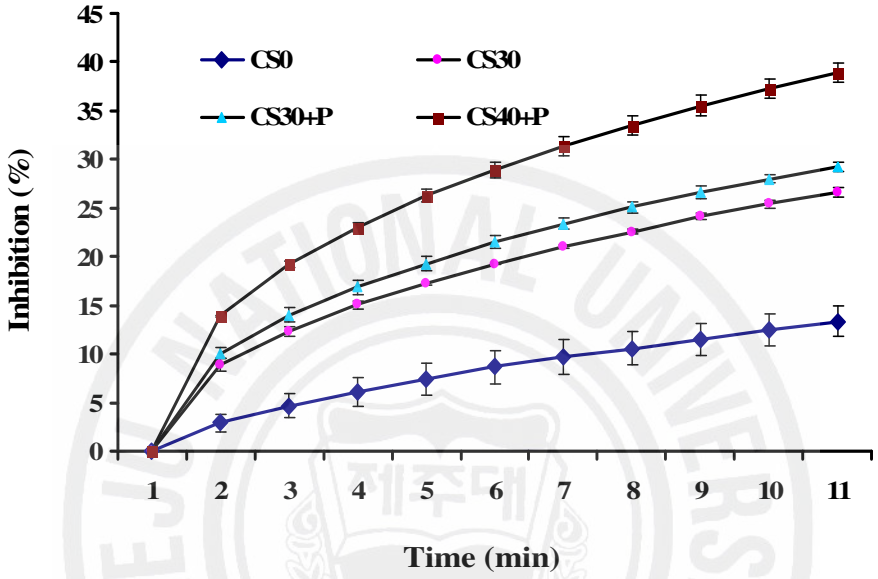

Fig. 6. DPPH radical scavenging activity (%) in the experimental diets containing different proportion of cottonseed and soybean meal for 10 weeks. Absorbance was measured at 517 nm for 10 min at interval of 1 min.

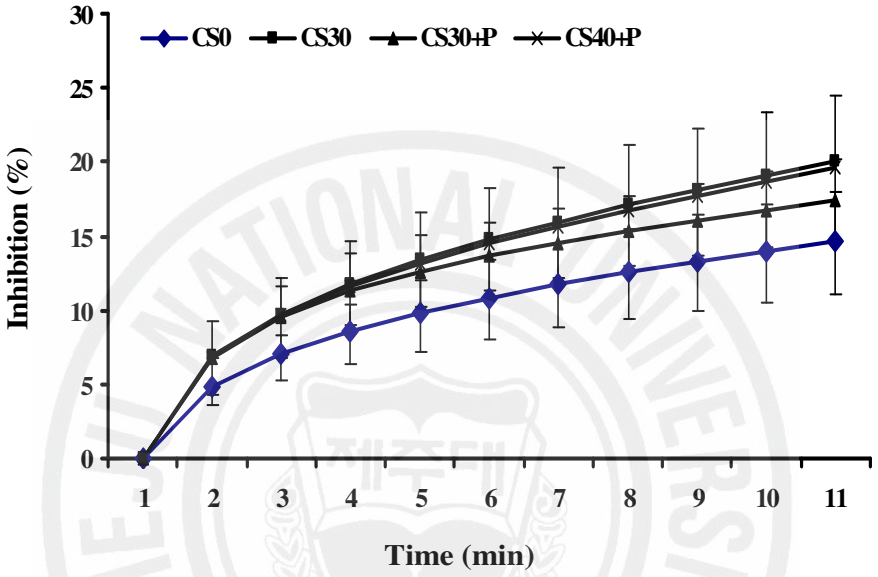

Fig. 7. DPPH radical scavenging activity (%) in liver of juvenile olive flounder fed the experimental diets for 10 weeks. Absorbance was measured at 517 nm for 10 min at interval of 1 min.

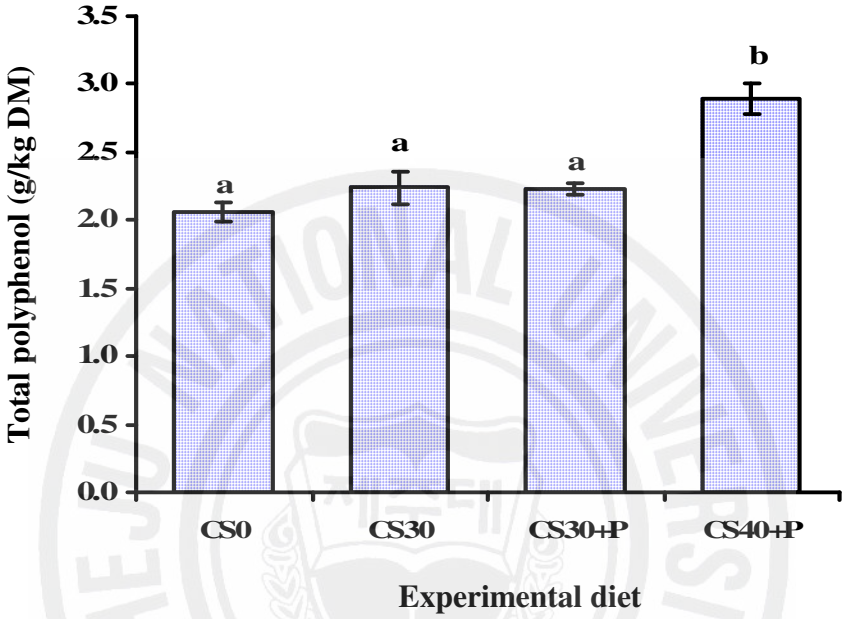

Fig. 8. Total polyphenol compounds in the experimental diets containing cottonseed and soybean meal with or without phytase supplementation. Values are mean of two replicates per diet. Bars with different letters are significantly different ($P<0.05$).

III. DISCUSSION

The use of exogenous phytase can increase the availability of phosphorus in fish feed containing larger proportion of plant proteins and consequently reduce the effluent phosphorus in aquaculture (Lanari et al., 1998; Vielma et al., 2002; Cheng and Hardy, 2003; Sajjadi and Carter, 2004). In the present study, the level of supplemented phytase in the experimental diets was based on a study conducted by Yoo et al. (2005). At the end of 10 week feeding trial, the supplementation of phytase significantly increased apparent digestibility coefficient of phosphorus and protein of olive flounder fed the diets containing 40% cottonseed and soybean meal as fish meal substitute compared to the control diet. This is in agreement with many previous studies which resulted in positive effects of phytase inclusion in many fish species, such as rainbow trout (Sugiura et al., 2001), Atlantic salmon (Sajjadi and Carter, 2004), Korean rockfish (Yoo et al., 2005), striped bass (Paratryphon and Soares Jr, 2001), African catfish (Van Weerd et al., 1999), channel catfish (Li et al., 2004), olive flounder (Masumoto et al., 2001), and Nile tilapia (Portz and Liebert, 2004). Yoo et al. (2005) concluded that supplementation of microbial phytase significantly improved the apparent digestibility coefficient of phosphorus in rockfish diets replacing 30% and 40% fish meal with soybean meal regardless of the level and method of phytase supplementation. A higher absorption of phosphorus was observed in juvenile olive flounder fed a diet containing soybean protein with phytase compared to fish fed a fish meal-based diet (Masumoto et al., 2001). However, no significant differences in phosphorus digestibility, retention, conversion and the phosphorus budget were found between fish fed diets containing soybean

meal with 1000 FTU phytase/kg by pretreatment or simple supplementation (Van Weerd et al., 1999; Yoo et al., 2005). The efficacy of nutrient digestibility in fish feeds depends on the type of ingredients used (Cheng and Hardy, 2002; 2003), processing techniques (Nwanna et al., 2005), and chemical composition of diets, particularly high ash content (Sugiura et al., 2001). A significant improvement in protein digestibility of fish diets containing plant protein sources have been demonstrated in several studies (Portz and Liebert, 2004; Debnath et al., 2005). The results of the present study indicate that supplementation 1000 FTU of phytase/kg in juvenile olive flounder diet containing 40% cottonseed and soybean meal can improve the apparent digestibility coefficient of protein and phosphorus (Fig. 3, 4). However, the positive effects of dietary phytase on protein and phosphorus digestibility of did not clearly demonstrate.

The dietary crude protein (48% DM) and energy content (18 MJ/kg DM) in the present study were formulated based on the requirement of juvenile olive flounder reported by Kim et al. (2002). In the diets containing cottonseed and soybean meal, limiting amino acids, such as lysine and methionine were supplemented to meet their requirements of fish. In the present study, up to 30% replacement of fish meal protein by cottonseed and soybean meal with phytase supplementation did not affect the growth of fish (Table 3). In our previous study (Pham et al., 2005), growth performance of juvenile olive flounder (initial body weight 0.74 g) were not affected by dietary cottonseed and soybean meal up to 30% fish meal replacement. However, higher incorporation of dietary cottonseed and soybean meal (diet CS40+P) resulted in impairment in growth performances that were also demonstrated in the previous study (Pham et al., 2005). The lower growth

performance of fish fed diet CS40+P might be due to the presence of other anti-nutrient factors in cottonseed and soybean meal, particularly gossypol. Gossypol, a yellow pigment found in the gland of cottonseed, has been demonstrated to be toxic for many fish species (Dorsa et al., 1982; Dabrowski et al., 2000; Lee et al., 2002; Garcia-Abiado et al., 2004). Moreover, phytase has no influence on growth performance and whole body composition of fish in the present study as previously proved by Li et al. (2004) and Yoo et al. (2005). The results in the present study suggest that the total gossypol contents in diet CS40+P (18.8% dietary cottonseed meal inclusion, Table 1) might be higher than the tolerant level of gossypol in juvenile olive flounder and thereby resulted in the depression of fish growth.

The increment in whole body ash content of fish fed phytase diets could be related to the excess of inorganic phosphorus and/or other minerals released from the experimental diets by phytase and their retention in the fish body. Sajjadi and Carter (2004) observed that there was an interaction between phytase and inorganic phosphorus on bone ash, bone phosphorus and whole body phosphorus, and concluded that supplementation of phytase or inorganic phosphorus or both resulted in higher whole body ash (Table 4).

Cholesterol lowering effect of soybean and other plant proteins has been intensively investigated on vertebrates including fish (Golgberg et al., 1982; Kaushik et al., 1995; Ali et al., 2004; Chisholm et al., 2005; Dias et al., 2005). Goldberg et al. (1982) demonstrated that decreased cholesterol level in hypercholesterolemia patients was contributed by soybean protein in diets. Anderson and Wolf (1995) suggested that cholesterolemia is affected by various non-protein components of soy, such as trypsin inhibitors, saponins, phytoestrogens, fiber, phytosterols, phytic acid

and minerals. Ali et al. (2004) also observed that soy isoflavones lowered plasma cholesterol in rats. It is evident that there are various factors including nutrition, non-nutrition, endocrines involved in the modulation of cholesterol synthesis and metabolism in vertebrate animals, except for teleosts. Cholesterol metabolism has not well understood in fishes (Este'vez et al., 1996). Kaushik et al. (1995) reported that plasma cholesterol levels were reduced in rainbow trout fed soybean protein in comparison to the fish fed fish meal based diet. Similar result was also observed by Dias et al. (2005) who reported that level of plasma cholesterol was lower in European sea bass fed soybean diets than those of fish fed fish meal based diet. In the present study, interestingly, serum cholesterol was significantly reduced the fish fed diet CS30 and CS40+P containing cottonseed and soybean meal (Fig. 5). Recent studies have shown that the cottonseed products have an ability to reduce the serum cholesterol in animals (Nwoha and Aire, 1995; Edwards and Radcliffe, 1995; Radcliffe at el., 2001). The mechanism for the gossypol in cottonseed products on cholesterol has not been clearly determined, but studies have revealed a significant interaction between gossypol and lipid metabolism. The current results imply that dietary inclusion of both cottonseed and soybean meal could affect cholesterol metabolism in fish. Further study is needed on this issue.

An increased antioxidant activity in diets containing cottonseed and soybean meal (Fig. 6) seemed to be attributed to higher levels of polyphenols (Fig. 8) in the present study. This is supported by the fact that DPPH radical scavenging activities are closely related with polyphenol contents (Skerget et al., 2005). However, the increased antioxidant capacity was not clearly demonstrated in the liver of fish fed the diets, even though the values of the DPPH radical scavenging activities were

numerically increased in the fish groups fed the diets containing cottonseed and soybean meal (Fig. 7).

In conclusion, these results suggest that supplementation of phytase in diets containing cottonseed and soybean meal could improve the apparent digestibility of phosphorus in juvenile olive flounder. Growth performances might not be affected by dietary cottonseed and soybean meal up to 30% fish meal protein replacement in juvenile olive flounder. Also, dietary replacement of plant protein sources, such as cottonseed and soybean meal could reduce the serum cholesterol in the fish. Further research needs to study on the effect of phytase on nutrients digestibility of dietary containing plant protein for other fish species.

V. REFERENCES

Ali, A.A., Velasquez, M.T., Hansen, C.T., Mohamed, A.I., Bhathena, S.J., 2004. Effects of soybean isoflavones, probiotics, and their interactions on lipid metabolism and endocrine system in an animal model of obesity and diabetes. J. Nutr. Biochem. 15, 583-590.

Anderson, R.L., Wolf, W.J., 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. J. Nutr. 125, 581S-588S.

AOAC, 1995. Official methods of analysis, 16th edition, Arlington VA. Association of Official Analytical Chemists.

Barbara, F., Harland, PH.D., Gurleen Narula, M.S., 1999. Food phytate and its hydrolysis products. Nutr. Res. 19, 947-961.

Baruah, K., Sahu, N.P., Pal, A.K., Debnath, D., 2004. Dietary phytase: An ideal approach for a cost effective and low-polluting aquafeed. NAGA, World Fish Center Quarterly 27, 15-19.

Brand-William, W., Cuvelier, M.E., Berset C., 1995. Use of a free radical method to evaluate antioxidant activity. Food Sci. Technol. 28, 25-30.

Broz, J., Oldale, P., Perrin-voltz, A.H., Tychen, G., Schulze, J., Simoes Nunes, C., 1994. Effects of supplemental phytase on performance and phosphorus utilization in broiler chickens fed a low phosphorus diet without addition of inorganic phosphates. Br. Poult. Sci. 35, 273–280.

Chen, Q.C., Li, B.W., 2003. Separation of phytic acid and other related inositol phosphates by high-performance ion chromatography and its applications. J. Chromatogr. A, 1018, 41-52.

Cheng, Z.J., Hardy, R.W., 2002. Effect of microbial phytase on apparent digestibility of barley, canola meal and wheat middlings, measured *in vivo* using rainbow trout (*Oncorhynchus mykiss*). Aquacult. Nutr. 8, 271-277.

Cheng, Z.J., Hardy, R.W., 2003. Effects of extrusion and expelling processing, and microbial phytase supplementation on apparent digestibility coefficients of nutrients in full-fat soybeans for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 218, 501-514.

Chisholm, A., Auley, K.Mc. Mann, J., Williams, S., Skeaff, M., 2005. Cholesterol lowering effects of nuts compared with canola oil enriched cereal of similar fat composition. Nutr. Metabol. Cardiovas. Dis. 15, 284-292.

Cho, C.Y., Kaushik, S.J., 1990. Nutrition energetics in fish: energy and protein utilization in rainbow trout (*Salmo gairdneri*). World Rev. Nut. Diet. 6, 132.

Dabrowski, K., Rinchard, J., Lee, K.-J., Blom, J.H., Cierezko, A., Ottobre, J., 2000. Effects of diets containing gossypol on reproductive capacity of rainbow trout (*Oncorhynchus mykiss*). Biol. Reprod. 62, 227-234.

Debnath, D., Pal, K.P., Sahu, N.P., Jain, K.K., Yengkokpam, S., 2005. Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. Aquacult. Res. 36, 180-187.

Dias, J., Alvarel, M.J., Corraze, G., Diez, A., Bautista, J.M., Kaushik, S.J., 2005. Dietary protein source affects lipid metabolism in the European seabass (*Dicentra labrax*). Comp. Biochem. Physiol. A 142, 19-31.

Dorsa, W.J., Robinette, R.H., Robinson, H.E., Poe, E.W., 1982. Effects of dietary cottonseed meal and gossypol on growth of young channel catfish. T. Am. Fish. Soc. 111, 651-655.

Edward, M.S., Radcliffe, J.D., 1995. A comparison of the effect of cottonseed oil and corn oil on lipid status in the rat. Biochem. Arch. 11, 103-109.

Este'vez, A., Delgado, A., Hortelano, P., Alejandre, M., 1996. Characterization of mevelonate metabolism in the sea bass, *Dicentrarchus labrax* L. liver. Fish Physiol. Biochem. 15, 205-211.

Folch, J., Lee, M., Sloane -Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Fontainhas-Fernandes, A., Gomes, E., Reis-Henriques, M.A., Coimbra, J., 1999. Replacement of fish meal by plant protein in the diet of Nile tilapia: digestibility and growth performance. Aquacult. Int. 7, 57-67.

Forster, I., Higgs, D.A., Dosanjh, B.S., Rowshandeli, M., Parr, J., 1999. Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11 ^{o}C fresh water. Aquaculure 179, 109-125.

Furukawa, H., Tsukahara, H., 1996. On the acid digestion method for the determination if chromic oxide as an index substance in the study of digestibility of fish fed. Bull. Jpn. Soc. Sci. Fish. 32, 502-508.

Garcia-Abiado, M.A., Mbahinzireki, G., Rinchard, J., Lee, K.-J., Dabrowski, K., 2004. Effects of diets containing gossypol on blood parameters and spleen structure in tilapia, *Oreochromis sp.*, reared in re-circulating system. J. Fish Dis. 27, 359-368.

Goldberg, A.P., Lim, A., Kolar, J.B., Grundhauser, J.J., Steinke, F.H., Schonfeld, G., 1982. Soybean protein independently lowers plasma cholesterol levels in primary hypercholesterolemia. Atherosclerosis 43, 355-368.

Han, Y., Wilson, D. B., Lei, X. G., 1999. Expression of an *Aspergillus niger* phytase gene (phyA) in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 65, 1915-1918.

Hotz, C., Gibson, R.S., 2001. Assessment of home-based processing methods to reduce the phytate content and phytate/zinc molar ratio of white maize (*Zea mays*). J. Agric. Food Chem. 49, 692-698.

Hughes, K.P., Soares, J.H. Jr., 1998. Efficacy of phytase on phosphorus utilization in practical diets fed to striped bass, *Morone saxatilis*. Aquacult. Nutr. 4, 133-140.

Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B., Laroche, M., 1995. Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout *Oncorhynchus mykiss*. Aquaculture 133, 257-274.

Kikuchi, K., 1999. Use of defatted soybean meal as a substitute for fish meal in diets of Japanese flounder (*Paralihcthys olivaceus*). Aquaculture 179, 3-11.

Kikuchi, K., Furuta, T., Honda, H., 1994. Utilization of soybean meal as a protein source in the diet of juvenile Japanese flounder, *Paralichthys olivaceus*. Suisanzoshoku 42, 601-604.

Kim, K.W., Wang, X.J., Bai, S.C., 2002. Optimum dietary protein level for maximum growth of juvenile olive flounder *Paralichthys olivaceus* (Temminck et Schlegel). Aquacult. Res. 33, 673-679.

Kim, T. W., Lei X. G., 2005. An improved method for rapid determination of phytase activity in animal feed. Journal of Animal Science 83, 1062-1067.

Lanari, D., D'Agaro, D., Turri, C., 1998. Use of nonlinear regression to evaluate the effects of phytase enzyme treatment of plant protein diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 161, 345-356.

Lee, D.J., Putman, G.B., 1973. The response of rainbow trout to varying protein/energy ratios in a test diet. J. Nutr. 103, 916-922.

Lee, K.-J., Dabrowski, K., Blom, J.H., Bai, S.C., Stromberg, P.C., 2002. A mixture of cottonseed meal and soybean meal and animal byproduct mixture as a fish meal substitute: growth and gossypol enantiomer in juvenile rainbow trout (*Oncorhynchus mykiss*). J. Anim. Physiol. Anim. Nutr. 86, 201-213.

Leske, K.L., Coon, C.C., 1999. A bioassay to determine the effect of phytase on phytate phosphorus hydrolysis and total phosphorus retention of feed ingredients as determined with broilers and laying hens. Poultry Sci. 78, 1151-1157

Li, M.H., Manning, B.B., Robinson, E.H., 2004. Summary of phytase studies for channel catfish. Res. Report 23, 1-5.

Masumoto, T., Tamura, B., Shimeno, S., 2001. Effects of phytase on bioavailability of phosphorus in soybean meal-based diets for Japanese flounder, *Paralichthys olivaceus*. Fisheries Sci. 67, 1075-1080.

Mbahinzireki, G.B., Dabrowski, K., Lee, K.-J., El-Saidy, D. Wisner, E.R., 2001. Growth, feed utilization and body composition of tilapia (*Oreochromis sp.*) fed with cottonseed meal-based diets in a re-circulating system. Aquacult. Nutr. 7, 189-200.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of Aquaculture on world fish supplies (review article). Nature 405, 1017-1024.

NRC (National Research Council), 1993. Nutritional Requirements of Fish. National Academic Press, Washington, DC. pp. 114.

Nwanna, L.C., Fagbenro, O.A., Adeyo, A.O., 2005. Effects of different treatments of dietary soybean meal and phytase on the growth and mineral deposition in African catfish *Clarias gariepinus*. J. Anim. Veteri. Advan. 4, 980-987.

Nwoha, P.U., Aire, T.A., 1995. The effects of gossypol and chloroquine interaction on serum electrolytes of protein-malnourished rats. Contraception 52, 255-259.

Paratryphon, E., Soares Jr, J.H., 2001. The effect of phytase on apparent digestibility of four practical plant feed stuffs fed to striped bass, *Morone saxatilis*. Aquacult. Nutr. 7, 161-167.

Pham, M.A., Lee, K.-J., Lim, S.-J., Lee, B.-J., Kim, S.-S., Park, Y.-J., Lee, S.M., 2005. Fish meal replacement by cottonseed and soybean meal in diets for

juvenile olive flounder, *Paralichthys oliveceus*. J. Aquacult. Kor. Soc. 18, 215-221.

Portz, L., Liebert, F., 2004. Growth, nutrient utilization and parameters of mineral metabolism in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) fed plant-based diets with graded levels of microbial phytase. J. Anim. Physiol. A. Anim. Nutri. 88, 311-320.

Radcliffe, J.D., King, C.C., Czajka-Narins, D.M., Imrhan, V., 2001. Serum and liver lipids in rats fed diets containing corn oil, cottonseed oil, or a mixture of corn and cottonseed oils. Plant Foods for Human Nutr. 56, 51-60.

Saitoh, S., Koshio, S., Harada, H., Wantanabe, K., Yoshida, T., Teshima, S.-I., Ishikawa, M., 2003. Utilization of extruded soybean meal for Japanese flounder *Paralichthys olivaceus* juveniles. Fisheries Sci. 69, 1075-1077.

Sajjadi, M., Carter, C.G., 2004. 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.

Silva, L.G., Trugo, L.C., Terzi, S.C., Couri, S., 2005. Low phytate lupin flour based biomass obtained by fermentation with mutant of *Aspergillus niger*. Process Biochem. 40, 951-954.

Skerget, M., Kotnik, P., Hadolin, M., Hras, A.R., Simonic, M., Knez, Z., 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and the antioxidant activities. Food Chem. 89, 191-198.

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*) fed soybean meal-based diets. Aquacult. Res. 32, 583-592.

Sugiura, S.H., Raboy, V., Young, K.A., Dong, F.M., Hardy, R.W., 1999. Availability of phosphorus and trace elements in low-phytate varieties of barley and corn for rainbow trout *Oncorhychus mykiss*. Aquaculture 170, 285-296.

Urbano, G., Lopez-Jurado, M., Aranda, C., Vidal, V., Tenorio, E., Porres, J., 2000. The role of phytic acid in legumes: antinutrient or beneficial function. J. Physiol. Biochem. 56, 283-294.

Van Weerd, J.H., Khalaf, KH.A., Aartsen, F.J., Tijssen, P.A.T., 1999. Balance trials with catfish *Clarias gariepinus* fed phytase treated soybean meal-based diets. Aquacult. Nutr. 5, 135-142.

Vielma, J., Rouhonen, K., Peisker, M., 2002. Dephitinization of two soy proteins increases phosphorus and protein utilization by rainbow trout, *Oncorhynchus mykiss*. Aquaculture 204, 145-156.

Wise, A., 1983. Dietary factors determining the biological activities of phytate. Nutr. Abstract Rev. 53, 791-807.

Yamamoto, T., Akimoto, A., Kishi, S., Unuma, T., Akiyama, T., 1998. Apparent and true availabilities of amino acids from several protein sources for fingerlings rainbow trout, common carp, and red sea bream. Fisheries Sci. 64, 448-458.

Yanke, L.J., Bae, H.D., Selinger, L.B., Cheng, K.-J., 1998. Phytase activity of anaerobic ruminal bacteria. Microbiology 144, 1565-1573.

Yoo, G.-Y., Wang, X., Choi, S., Han, K., Kang, J.-C., Bai, S.C., 2005. Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean

rockfish *Sebastes schelegeli* fed diets containing soybean meal. Aquaculture 243, 315-322.

VI. SUMMARY

Phytase have reported to increase the apparent digestibility coefficient of phosphorus and protein in the diets containing plant protein sources. The results in our study also suggest that supplementation of phytase in diets containing cottonseed and soybean meal significantly improves the apparent digestibility phosphorus in juvenile olive flounder. Growth performances might not be affected by dietary cottonseed and soybean meal up to 30% fish meal protein replacement in juvenile olive flounder. Also, dietary supplementation of plant protein sources, such as cottonseed and soybean meal could reduce the serum cholesterol in the fish. In other hand, phytase was reported to be produced by fungi and micro-organisms, particularly *Aspergillus oryrae* which is abundance in fermented stuffs. The application of fermentation techniques using *A. oryzae* to improve the nutritional quality of cottonseed and soybean meal could be interesting topics for further studies.

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