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The potential use of legume-based diets supplemented with microbial phytase on the growth performance and feed efficiency of sea bass, *Lates calcarifer*

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Abstract. A feeding trial was conducted for 12 weeks to evaluate the potential use of legume-based diets supplemented with dietary microbial phytase on the growth performance and feed efficiency of juvenile sea bass, *Lates calcarifer*. Fifteen sea bass juveniles (mean initial weight of 0.96 g and mean initial total length (TL) of 4.2 cm) were stocked at three replicates into each of the twelve 100 L conical fibreglass tanks containing 90 L sea water in a closed recirculating system with filtered and aerated sea water. Four isonitrogenous, isolipidic and isocaloric experimental diets were formulated. The control diet (C0) contained fish meal, soybean meal, shrimp meal and squid meal as major protein sources. Legume seed meals of pigeon pea (*Cajanus cajan*), yellow mungbeans (*Phaseolus aureus*), and green mungbeans (*Vigna radiata*) were incorporated in the practical diets D1, D2 and D3 respectively at 18-20% replacing an equivalent amount of 6-7g fish meal protein and supplemented with microbial phytase at the level of 300U kg^{-1} diet. Growth rate, feed conversion ratio (FCR), protein efficiency ratio (PER) and apparent net protein utilization (ANPU) of sea bass were significantly ($P < 0.05$) higher in control diet than those given different legume based diets supplemented with phytase. Histological examination of the liver tissues for the different dietary treatments did not manifest any abnormalities. Phytase supplementation also improved bone ash, phosphorus (P) concentration as well as P content in the carcass for fish in legume fed groups. Results from the present study showed that incorporation of dietary microbial phytase in legume based diets slightly improve the growth performance and P availability in sea bass juveniles.
Key Words: sea bass, microbial phytase, feeding, *Cajanus cajan* and feed conversion ratio.

Introduction. Sea bass are important marine foodfish species in Southeast Asia and Australia owing to their high commercial value (Appelbaum & Arockiaraj 2010; Arockiaraj & Appelbaum 2010; Caipang et al 2011b). Recently, sea bass are mostly raised in floating net cages (Tacon & Rausin 1989), and had been cultured in brackishwater ponds for more than 20 years. In the Philippines, these species are raised in polyculture either with tilapia or milkfish. The global annual production of sea bass in 2006 was currently 400,000 MT according to FAO statistics (Pillay & Kutty 2005; Wang et al 2006). In current feed formulation, fish meal constitutes as the major feed component in artificial diets for fish and crustacean because of its high protein content, good amino acid and fatty acid profile (Boonyaratapalin & William 2001; Tacon et al 2004). However, due to the high market demand and uncertain availability for fish meal (Carter & Hauler 2000; Kissil et al 2000) this led to search for alternative protein sources as complete or partial replacement for the fish meal component in diets to sustain fish production. The use of plant ingredients as dietary protein source had been studied for many years by several researchers for the different aquaculture species (Robaina et al 1997; Eusebio & Coloso 2000; Farhangi & Carter 2001). Hence, more information is needed to improve the overall growth performance and feed utilization of sea bass using plant proteins supplemented with phytase in order to reduce phosphorus loading for the environmental protection against degradation and eutrophication.

Legumes specifically pigeon peas, yellow and green mungbeans are common agricultural products in the Philippines. They are known for their relatively high protein content, carbohydrates and lipids (De la Pena et al 1987; FNRI 1980) that might be considered potential protein sources as substitute for fish meal in formulating sea bass diet. However, the use of legume seeds in fish feeds is limited because of the presence of anti-nutritional factor (De Silva & Andersons 1995) known as phytate or phytic acid (myo-inositol-1,2,3,4,5,6-hexakiphosphates). Phytic acid is an organic form of phosphorus that is abundantly present in plant materials such as legumes, cereals, oilseeds and nuts (Sebastian et al 1996; Yi et al 1996; Caipang et al 2011a; Vats & Banerjee 2004). Phytic acid usually accumulates in the aleurone, the outer most cell layer of cereals and legumes. Reddy et al (1989) found that the highest amount of phytate among cereals is found in maize (0.83 – 2.22%) and among legumes in dolique beans (5.92 – 9.15%). About 80% of the total phosphorus (P) content in plants is in the form of phytate-P. This phytate-P cannot be used efficiently by fish as they lack the intestinal phytase enzyme needed for the hydrolysis of phytate during digestion (Jackson et al 1996). Phytic acid has strong chelating potential to form insoluble complexes with minerals such as Mg, Zn and Cu thus decreasing their bioavailability and absorption in the intestinal tract (Davies 1982). Phytic acid reacts with proteins over a wide pH range, forming phytate-protein complexes. Also, under acidic conditions, phytic acid is likely to bind tightly to plant proteins at around pH 4.0-5.0 (Dechavez et al 2011). It was noted that these organically bound P are almost totally excreted in the rearing water and environment that caused eutrophication due to phosphorus loading.

Phytase is an enzyme specific to phytate hydrolysis. This enzyme is present in the digestive tract of many animals, but the amount is normally too small to digest phytate to a significant extent (Sugiura et al 1999). Natuphos was the first commercially available phytase from a genetically modified *A. niger* strain. Phytase activity (FTU or U) is defined as the quantity of enzyme that liberates 1 micromol of inorganic-P per minute from 0.0015 mol/L sodium phytate at pH 5.5, and 37^{o}C (Simons et al 1990). The phytase feed enzyme derived from *Aspergillus* species is a 3-phytase which liberates the phosphorus (P) at position C3 of the myo-inositol hexaphosphate ring (Sandberg et al 1996; Selle & Ravindran 2006). Many studies have demonstrated that addition of phytase to plant-based diets can improve the bioavailability of phosphorus (P) in rainbow trout (Rodehutscord & Pfeffer 1995); carps (Schaefer & Koppe 1995); and tilapias (Furuya et al 2001). Most microbial phytase especially those of fungal origin has an optimum pH and temperature which varies from 2.2 to 8 and from 45 to 77^{o}C respectively. Phytase are histidine acid phosphatases that catalyze the hydrolysis of phytic acid to P_1 and myo-inositol phosphates. This enzyme when incorporated in the fish diets enhanced the growth performance, increased the bioavailability of phosphorus and decreased fecal P effluents in the culture system. Phytase supplementation in plant-based practical diets can enhance the utilization of phytate P and decreases P load to the aquatic environment (Liebert & Portz 2005). According to Vielma et al (1998) phytase supplementation with semi-purified diet with soybean could significantly improved the protein digestibility in rainbow trout. Cao et al (2007) reviewed that phytase dose at a level of 250 to 2000 U kg^{-1} feed is usually considered optimum for many fish species. Phytase enzymes are thermolabile and have optimal range of 45-60^{o}C (Lei & Porres 2003) therefore spraying the processed feed with liquid suspension of phytase could be the best option to solve the high temperature phase of extrusion. The role of phytase supplementation has been well proven and documented in monogastric animals to improve protein and amino acid utilization through breakdown of phytin-protein complexes (Kornegay 1995) and increase P utilization.

Therefore, the objective of the present study is to determine the effect of legume based diets supplemented with microbial phytase on the growth performance, bone mineralization and phosphorus utilization for juvenile sea bass.

Material and Method

Experimental Diets. Four experimental diets were formulated to be isonitrogenous (40%), isolipidic (10%) and isocaloric (360 kcal/g) as shown in Table 1.

Table 1

Ingredients and proximate analyses of legume-based diets used in the feeding experiment (g/100g dry weight)

	Microbial phytase supplementation (U kg^{-1} diet)			
	0	300	300	300
		Diet No.		
Ingredients	C0	D1	D2	D3
Peruvian fish meal	30.0	24.0	24.0	24.0
Soybean meal (defatted)	25.0	25.0	25.0	25.0
Shrimp meal (Acetes)	10.0	10.0	10.0	10.0
Squid meal	5.0	5.0	5.0	5.0
Pigeon pea meal		18.1		
Yellow mungbean meal			19.6	
Green mungbean meal				20.0
Breadflour	13.0	6.8	5.0	5.0
Rice bran	7.0	1.1	1.4	1.0
Cod liver oil	2.5	2.5	2.5	2.5
Soybean oil	2.5	2.5	2.5	2.5
Vitamin mix	2.5	2.5	2.5	2.5
Mineral mix	2.5	2.5	2.5	2.5
Proximate analyses (% dry basis) of the experimental diets				
Crude protein	40.48	40.4	40.2	40.0
Crude lipid	10.30	10.24	10.18	10.09
NFE[1]	29.60	29.83	29.09	29.86
Crude fiber	2.62	2.42	2.31	2.15
Crude Ash	17.00	17.07	18.22	17.89
Phosphorus	1.04	0.98	0.92	0.92
Metabolizable energy* (Kcal/g)	362.24	362.34	358.34	359.30

[1]Nitrogen free extract;
*Metabolizable energy was calculated based on the standard physiological values of 4.5kcal/g protein, 3.3 kcal/g carbohydrate and 8 kcal/g fat (Brett & Groves 1979).

Legume seeds used in the feeding experiment were pigeon pea (*Cajanus cajan*), yellow mungbean (*Phaseolus aureus*) and green mungbean (*Vigna radiata*). The whole mature seeds were oven dried at 60^{o}C for 6h (Millamena et al 2002) in order to remove some of the anti-nutritional factors present and to increase its nutrient utilization. The seeds were finely ground into the meal form, and sieved using a 60 mesh screen. The legume seed meals were added to replace an equivalent amount of 6-7 g fish meal protein in the control diet.

The proximate analysis of various legume seed meals is presented in Table 2 using standard methods (AOAC 1990). The control diet (C0) contained fish meal as the major protein source without inclusion of legume meals and phytase supplemented in the diet. The three legume-based diets, D1, D2 and D3 contained pigeon pea, yellow mungbean and green mungbean respectively at 18.0-20.0% of the diet with microbial phytase supplemented at the level 300 U kg^{-1} diet. The same amount of fish meal was added in D1 to D3 diets, which was 6% less than the level of fish meal in the control (C0) diet. Soybean meal, shrimp meal (*Acetes)* and squid meal were kept constant in all

diets. The level of bread flour was adjusted to maintain the same dietary protein content in the diets. Diets were prepared by blending all the dry ingredients prior to the addition of vitamins and minerals. Soybean oil and cod liver oil as lipid sources were then poured into the mixture at a ratio of 1:1. Breadflour was gelatinized by cooking in 600 mL water and added to the mixture. The semi-moist mixture were then extruded in a meat pelletizer using a 2 mm die and then dried overnight at 60^{o}C. The enzyme phytase was directly sprayed onto the surface of the processed pellets and stored at 4^{o}C until use. One unit of enzyme activity is defined as the amount of phytase that liberates 1 µmol of inorganic P from 0.0051 mol/L of sodium phytate/min. at 37^{o}C and pH 5.5.

Table 2

Proximate analyses of various legume seed meals used in the experimental diets

	Pigeon pea	Yellow mungbean	Green mungbean
Proximate composition (% dry matter)			
Crude protein	23.00	23.90	23.20
Crude fat	2.00	1.00	1.10
Crude fiber	7.20	4.80	4.20
Nitrogen Free Extract	63.60	66.40	68.00
Ash	4.20	3.90	3.50
Phosphorus	0.30	0.30	0.30

Experimental Fish and Feeding Management. Sixty-day old sea bass fingerlings about 2.54 cm in size were purchased from SEAFDEC Finfish Hatchery. They were placed in two oxygenated plastic bags and transported to the University of the Philippines Visayas (UPV) Multispecies Hatchery in Miag-ao, Iloilo. Sea bass were acclimatized for two weeks in a 1000-L circular fiberglass tank and were given commercial pellets followed by weaning them with control diet prior to the experiment.

The feeding experiment was conducted in 12-100 L conical tanks in a closed recirculating system provided with a sand filter and continuous aeration. Tanks were supplied with filtered sea water at a flow rate of 1 L min^{-1} and photoperiod was maintained under the natural light and dark cycle throughout the study. Sea bass fingerlings (mean initial weight = 0.96g/fish and TL=4.2cm) were randomly distributed at a stocking rate of 15 fish per tank and in three replicate tanks per treatment. The fish were maintained for 5 days using the control feed prior to the feeding experiment.

Diets were fed thrice daily at 12% BW throughout the 12-week feeding experiment. Dissolved oxygen, temperature, pH and salinity were monitored daily in the early morning while chemical parameters such as ammonia-N (NH_3-N) and reactive phosphorus (PO_4^{-3}) content were determined and analyzed on a weekly basis. The range values for the water quality parameters of all tanks are presented in Table 3.

Table 3

Range values of water quality parameters in the experimental tanks

Physico-chemical parameter	Range
Dissolved oxygen	6.5-8.0 ppm
Temperature	25-300 ^{o}C
pH	6.3-7.3
Salinity	32-35 ppt
Ammonia (NH_3-N)	0.01-0.02 ppm
Reactive phosphate (PO_4^{-3})	0.25-0.38 ppm

Feed consumption was monitored at each feeding. Uneaten pellets and feces in all tanks were carefully siphoned off before feeding early in the morning. Fish were weighed every 15 days to adjust its feeding ration.

Analytical Procedures and Calculation. Proximate composition of various legume seed meals, and experimental diets were determined according to AOAC (1990) methods. Moisture content was determined by drying the samples at 105^{o}C to a constant weight. Nitrogen (N) content was measured by using Kjeltec auto digestion and distillation apparatus, and CP was calculated by multiplying N x 6.25. Crude lipid was determined using the Soxhlet unit with diethyl ether as a solvent while crude fiber was determined using a Fibertec set up. Ash content was analyzed by incinerating the sample in a muffle furnace at 600^{o}C for 6 h.

Growth performance and feed utilization of sea bass juveniles were evaluated in terms of percentage weight gain (%WG), specific growth rate (SGR % day^{-1}), food conversion ratio (FCR), protein efficiency ratio (PER), and apparent net protein utilization (ANPU) based on the following standard formulae:

$$\% \text{ WG} = \frac{\text{final weight} - \text{initial weight} \times 100}{\text{initial weight}}$$

$$\text{SGR\%} = [\ (\ln \text{final weight} - \ln \text{initial weight})/\text{time (days)} \times 100]$$

$$\text{FCR} = [\ \text{total dry weight of feed (g)}/\text{total wet weight gain (g)}]$$

$$\text{PER} = [\text{wet weight gain (g)}/\text{CP fed (g)}$$

$$\text{ANPU} = 100\ (\text{final tissue protein} - \text{initial tissue protein})/\text{CP fed}$$

Fish carcass composition was determined at the end of the feeding experiment. All fish were weighed and counted and fifteen sea bass were pooled from each treatment group for the subsequent analyses. The bone sample for mineral phosphorus (P) content was prepared following the method as described by Baruah et al (2007) where the whole fish was boiled for 20 minutes. The excess flesh was stripped off from the vertebrae with a toothbrush and finally washed with distilled water. The vertebrae were then dried for 2h at 110^{o}C and extracted with anhydrous ethyl ether for 7h, pulverized, dried and weighed. The dried samples were ashed at 550^{o}C for 6h, pulverized with mortar and pestle for analysis of P. Ash weight was calculated as a percentage of dry, fat-free bone weight. P content was analyzed spectrophotometrically using the molybdovanadate method (AOAC 1990).

Liver samples from the different dietary treatments were carried out for histological examination at the Institute of Marine Fisheries and Oceanology (IMFO) Histology Laboratory of the University of the Philippines Visayas (UPV), Miag-ao, Iloilo. Tissues were fixed in Bouin's solution, dehydrated and embedded in paraffin, sectioned at 5µm, stained with haematoxylin and eosin (H&E), examined and photographed using a photomicroscope (Nikon Optiphot-2) with camera LWS.

Statistical analyses. All the data were analyzed using analysis of variance (ANOVA) for completely randomized design (CRD). Treatment means were compared using the Duncan's Multiple Range Test (DMRT) and differences among treatments were considered significant at an alpha level of 0.05. The statistical analyses were carried out using the SPSS Software Program for Windows, Version 16. Mean values were given with ± standard error (SE).

Results and Discussion

Growth experiment. The average body weight of sea bass (0.95-0.97g) fed experimental diets at the start of the feeding trial did not differ from each other indicating that the groups of fish were homogeneous. The growth performance of sea bass, *Lates calcarifer* fingerlings fed with control diet and legume based diets supplemented with phytase after 12 weeks of feeding are presented in Table 4.

At the end of the feeding period (12 weeks) the group of fish fed control diet had a significantly *(P<0.05)* higher final mean weight of (17.01g) percentage weight gain (1690%) and specific growth rate (1.49%/day) than the groups of legume diets

supplemented with microbial phytase at 300U kg^{-1}. Among the groups of legume-based diets, there was no significant differences (P>0.05) in their final mean weight, percentage weight gain and specific growth rates, although the highest was achieved with fish fed diet containing yellow mungbean (D2 diet) and the lowest was obtained in sea bass fed D3 diet with green mungbean.

Table 4

Growth performance of sea bass, *Lates calcarifer* after 12 weeks of feeding with control diet and phytase[1] supplemented legume based diets

	Treatments			
	C0	D1	D2	D3
Initial mean weight (g)	0.95 ± .003	0.97 ± .016	0.96 ± .012	0.97±.008
Final mean weight (g)	17.01± .017[a]	15.79 ± 0.1[b]	15.72 ± .012[b]	15.75 ± .00[b]
Weight gain (%)	1690.00 ± 11.2[a]	1528.00 ± 2.3[b]	1536.00 ± 3.0[b]	1524.00 ± 4.0[b]
SGR (%/day)	1.49 ± .014[a]	1.44 ± .01[b]	1.45 ± .017[b]	1.44 ± .021[b]
FCR	2.10 ± .02[a]	2.50 ± 0.15[b]	2.47 ± 0.24[b]	2.49 ± .250[b]
PER	1.50 ± .02[a]	1.39 ± .04[b]	1.35 ± .021[b]	1.37 ± .021[b]
ANPU	43.75 ± .05[a]	42.26 ± 1.3[b]	42.19 ± 1.71[b]	42.07 ± 1.14[b]

SGR= Specific growth rate (%/day); FCR = feed conversion ratio; PER = protein efficiency ratio; ANPU = apparent net protein utilization. Survival ranged from 73.33 – 80% in all dietary treatments. Means in the same row with same superscripts are not significantly different (P > 0.05, Duncan's new multiple range test). Data are means of three replicates ± SE. [1]Natuphos 500 G produced from *Aspergillus niger*.

Feed efficiency. The FCR (2.10) was significantly (P<0.05) lower in sea bass fingerlings fed with control than the rest of the experimental groups. On the other hand, the FCR values for sea bass fed legume based diets with phytase for D1, D2 and D3 were 2.50, 2.47 and 2.49 respectively. The highest PER (1.50) was observed with fish fed control diet and the lowest (1.35) was with fish fed D2 diet, however the value obtained in the control diet was significantly (P<0.05) higher than the rest of the experimental groups. Fish fed control diet had a significantly (P<0.05) higher ANPU (43.75) than those fed phytase supplemented legume diets. No differences were observed in ANPU among sea bass fed legume diets supplemented with 300 U kg^{-1} diet.

Proximate Analyses for Carcass Composition. The proximate analyses for the carcass composition of sea bass *Lates calcarifer* fed experimental diets for 12 weeks is presented in Table 5. The highest carcass crude protein was observed in fish fed control diet (CO) and was comparable with the values obtained for D1, D2, and D3 diets. Although not significant (P>0.05) the highest carcass crude lipid, ash and P content were observed in fish legume based diets containing 300 U kg^{-1} phytase in comparison with fish fed control diet (P>0.05).

Table 5

Carcass proximate composition[1] (% of dry matter) of sea bass *Lates calcarifer* fed various experimental diets for 12 weeks

Diet No.	Crude Protein	Crude Lipid	Ash	Phosphorus
C0	52.79[a]	22.69[a]	10.49[a]	1.86[a]
D1	52.46[a]	22.74[a]	10.54[a]	1.93[a]
D2	52.49[a]	22.87[a]	10.51[a]	1.90[a]
D3	52.62[a]	22.76[a]	10.59[a]	1.92[a]

[1]Data are means of two replicates. Values are not significantly different at P >0.05.

Ash and Bone-P analyses. The results for ash and P contents in bones of sea bass juveniles fed different experimental diets are shown in Table 6. Fish fed phytase supplemented diets D1-D3 had higher percentage of ash (42.29-42.59%) and

phosphorus (10.14–10.23%) when compared with that of fish fed CO diet with 42.18% and 10.10% ash and phosphorus content respectively. Furthermore, no significant (P>0.05) differences in the ash and P contents in sea bass were observed after 12 weeks of feeding of experimental diets.

Table 6

Ash and phosphorus (P) contents in sea bass juveniles fed control diet and phytase supplemented legume based diets

Diet No.	Ash (%)	P (%)
C0	42.18 ±1.16[a]	10.10±.038[a]
D1	42.29± 1.14[a]	10.19±1.22[a]
D2	42.59±1.13[a]	10.23± 0.62[a]
D3	42.36 ±1.15[a]	10.14± 0.67[a]

Means (n=3) in the same column with same superscript are not significantly different (P>0.05). Data are means of three replicates ± SEM

Histological Examination of the Liver Tissues. Histological examination of the liver fed control diet and legume based diets containing 300U phytase kg^{-1} diet did not show any abnormalities in the liver tissues of sea bass juveniles (Figure 1). Macroscopically, the livers removed from the different experimental groups of fish were pulpy and somewhat they are reddish in color.

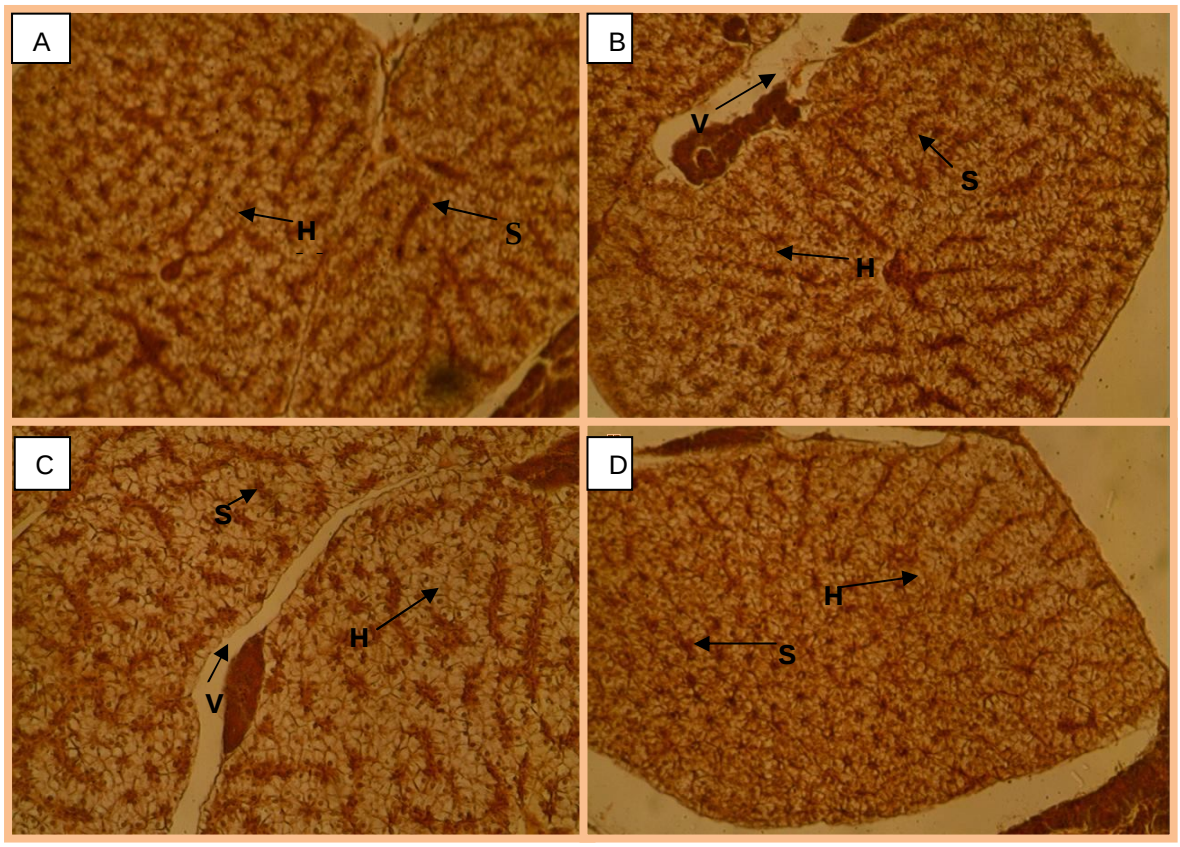

Figure 1. Histological examination of liver tissues of sea bass juvenile fed different experimental diets. Plate A shows the normal appearance of the hepatocytes (H) within the hepatic lobule and normal blood sinusoids fed on CO diet. In Plate B there was a mild vacuolation with regular arrangement of hepatocytes for fish fed D1. The same findings were also noted for liver tissues in plates C and D fed D2 and D3 diets respectively indicating absence of pathological abnormalities.

Results from the present study demonstrated that incorporation of microbial phytase at a level of 300 U kg^{-1} diet in legume based diets did not improve the overall growth performance, bone mineralization and phosphorus utilization in sea bass juveniles. This may reflect that the dosage of 300 U microbial phytase from *Aspergillus niger* added to legume diets was insufficient for the optimal growth of seabass such as weight gain (%) specific growth rate and bioavailability of phosphorus for sea bass juveniles. Previous experiments conducted by Masumoto et al (2001) and Yoo et al (2005) have reported that dietary phytase supplementation have a negative effect on the growth performance for Japanese flounder, *Paralichthys olivaceous* and Korean rockfish, *Sebastes schlegeli* respectively fed diets containing soybean meal. On the contrary, weight gain was increased in the fed phytase supplemented diets as a result of improved use of phytate-P as well as phytate-bound protein as reported in channel catfish (Jackson et al 1996), rainbow trout (Vielma et al 2002) and in striped bass (Papatryphon et al 1999). The discrepancy in the results obtained by several authors maybe associated on the level of dosage of microbial phytase supplemented to the diet, type of rearing condition and fish species.

In the present study, the highest growth rate (weight gain % =1690; SGR = 1.49%/day) and feed utilization in terms of FCR (2.10); PER (1.50) and ANPU (43.75) were obtained in the control diet and these were significantly different ($P>0.05$) from the phytase supplemented legume based diets. This could be attributed to the good quality of fish meal incorporated in the diet due to its balanced amino acid profile. Lall (1991) reported that fish meal has the richest source of phosphorus among the commercial feedstuffs used in feed formulation. Phytase supplementation in legume based diets increased the bone ash content as well as P content in the vertebra of seabass juveniles as compared to the control diet, however no significant difference was observed ($P<0.05$). It is possible that the enhanced effect of phytase would have the phosphorus digestibility in sea bass.

Conclusions. The present findings suggest that phytase supplementation at 300U/kg diet incorporated in the legume based diets did not enhance growth performance in sea bass despite a clear increase in bone ash (%) and phosphorus concentration (%) in the carcass content and vertebrae of sea bass. Further studies should be conducted to determine the different levels of dosage of phytase supplementation in this carnivorous fish to achieve optimum growth and to increase nutrient utilization.

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