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Enzymes produced by agro-industrial co-products enhance digestible values for Nile tilapia (*Oreochromis niloticus*): A significant animal feeding alternative.

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ABSTRACT

The use of enzymes in animal feed is common practice nowadays and its utilization has shown good results for improving the efficiency of nutrient utilization. In this sense, the goal of this work was to add low cost fungal phytases and proteases, with promising biological characteristics, produced by Solid State Fermentation using agro-industrial co-products to improve nutritional availability of plant based diets for Nile tilapia. Considering the enzymes chosen for the digestibility trail, *Aspergillus niger* produced acid phytases, as *A. oryzae*, produced proteases withoptimum pH over 8. The enzymes were stable at high temperatures (90 °C for phytase and 50 °C for protease) and showed very distinct behavior when different substrates were tested. Subsequently, the upscale production of *A. niger* phytase and *A. oryzae* protease with 7000 U g^{-1} and 2500 U g^{-1}, respectively, were applied as additive in a plant protein based fish diet. They increased protein, mineral, energy and lipids availability ($P < 0.05$), showing that these new enzymes can improve animal production and performance. Additionally, these low cost enzymes are an alternative as additives to improve soybean meal digestibility in fish plant based diets.

1. Introduction

The intensification of fish production requires improved production technology and low cost and low pollution feeding systems. Due to high cost and limited availability of animal protein sources; its replacement by plant protein has been encouraged. The utilization of soybean meal as feed ingredient has some advantages like availability, amino acid profile and environmental sustainability (Hardy, 2010; Hassaan et al., 2015). However, plant protein ingredients contain anti-nutritional factors that reduce the availability of nutrients and minerals, increasing waste output (Hardy, 2010). Phytic acid is the major anti-nutritional factor, which is the phosphorous storage compound in most plant protein sources (Cheryan, 1980). Phytate (IP6) is a salt of *myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate, which is present in several portions in plant seeds. Most phosphorous bound to phytate (P-IP6) is excreted by fish into the water due to its low availability, this can make phosphorous less available for fish utilization on its metabolism. In addition, this molecule binds to metal ions and also to cationic groups in proteins and amino acids. It has been demonstrated that IP6 can inhibit pepsin and other proteases activity in vitro by the formation of binary protein-IP6 complexes (Morales et al., 2011).

Phytases (E.C. 3.1.3.8 and E.C. 3.1.3.26) are a special class of phosphatases that catalyze the sequential hydrolysis of IP6 to less phosphorylated *myo*-inositol derivatives and inorganic phosphate. One of the main commercial phytases used in animal nutrition is produced from *Escherichia coli* and has two pH optima at 2.5 and 4.5 (Elkhalil et al., 2007). However, some latest studies demonstrate the utilization of new fungal phytases in fish diets with promising results (Liebert and Portz, 2005; Dalsgaard et al., 2009). It has been demonstrated that the addition of phytase to plant ingredients used in fish nutrition improves phosphorous availability and also prevents binding of IP6 to protein, this resulting in increased nutrient utilization (Storebakken et al., 1998). It has been also suggested that a number of factors such as pH, nature of the protein source and the presence of digestive proteases may determine the effect of phytase on protein and minerals bioavailability within fish digestive tract (Morales et al., 2011). Details of the specific effects of such factors on the action of phytase efficacy have not been elucidated yet and more research is needed to obtain a better insight

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Table 1
Formulation (g kg^{-1}) and proximate composition (% dry matter) of the experimental diets with two plant protein based ingredients and enzyme addition.

Diets	Reference	SB[a]	CG[a]	SBF[a]	SBP[a]	SBFP[a]	CGF[a]	CGP[a]	CGFP[a]
Ingredients									
Soybean	561.61	692.95	392.95	692.95	692.95	692.95	392.95	392.95	392.95
Corn	367.69	257.27	257.27	257.02	256.27	256.02	257.02	256.27	256.02
Corn gluten			300.00				300.00	300.00	300.00
Soybean oil	14.20	9.94	9.94	9.94	9.94	9.94	9.94	9.94	9.94
L-Lysine	1.35	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94
DL-Methionine	3.49	2.44	2.44	2.44	2.44	2.44	2.44	2.44	2.44
Threonine	3.08	2.16	2.16	2.16	2.16	2.16	2.16	2.16	2.16
Dicalcium phosphate	39.58	27.71	27.71	27.71	27.71	27.71	27.71	27.71	27.71
Premix[b]	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Vit C[c]	0.50	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
BHT[d]	0.20	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
NaCl	1.00	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Coline	1.30	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91
Cr_2O_3[e]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Phytase				0.25		0.25	0.25		0.25
Protease					1.00	1.00		1.00	1.00
Analyzed composition									
Dry matter	93.0	94.2	94.3	92.5	93.6	93.4	93.0	93.6	92.9
Protein	34.8	40.6	46.0	40.4	40.4	38.9	45.4	45.2	45.4
Lipids	3.7	3.2	2.2	3.2	3.5	2.8	1.3	2.2	1.8
Energy (kJ g^{-1})	18.8	19.2	20.7	19.0	18.9	19.0	20.7	20.5	20.5
Ash	6.4	5.8	5.1	5.4	5.8	6.6	5.7	5.7	5.5
Cr_2O_3[e]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phosphorous	1.2	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0
Magnesium	0.45	0.41	0.29	0.38	0.37	0.44	0.27	0.29	0.31
Calcium	1.25	0.98	0.87	0.94	1.07	1.08	0.82	0.98	0.85
Manganese	0.008	0.004	0.006	0.005	0.008	0.008	0.006	0.008	0.006

[a] SB: Soybean meal test diet without enzyme; CG: Corn gluten test diet without enzyme; SBF: Soybean meal diet with phytase; SBP: Soybean meal diet with protease; SBFP: Soybean meal diet with phytase and protease; CGF: corn gluten diet with phytase; CGP: Corn gluten diet with protease; CGFP: Corn gluten diet with phytase and protease.
[b] Vitamin and mineral supplement (levels per kg of product): vitamin A = 1200,000 IU; vitamin D3 = 200,000 IU; vitamin E = 12,000 mg; vitamin K3 = 2400 mg; vitamin B1 = 4800 mg; vitamin B2 = 4800 mg; vitamin B6 = 4000 mg; vitamin B12 = 4800 mg; folic acid = 1200 mg; calcium pantothenate = 12,000 mg; vitamin C = 48,000 mg; biotin = 48 mg; choline = 65,000 mg; nicotinic acid = 24,000 mg; Mn = 4.000 mg; Zn = 6.000 mg; *I* = 20 mg; Co = 2 mg; Cu = 4 mg e Se = 20 mg.
[c] Vitamin C Rovimix@ Stay-35, DMS Nutritional Products, Switzerland.
[d] Butyl-hydroxytoluene.
[e] Chromium oxyde.

into the mechanisms underlying phytase–protein interactions and subsequent availability of proteins and amino acids after digestion in fish. Moreover, the right phytase, regarding especially pH and temperature, used as nutritional additive can have different effects in nutrient digestibility.

Proteases (EC 3.4.23.18) hydrolyze peptide bonds, so protein becomes more available for the animal. These enzymes constitute an important group of enzymes commercially produced with different industrial applications and they are being used as animal feed additive recently. Nowadays, proteases constitute > 25% of biomolecules produced for industrial usage (Uyar and Baysal, 2004). Protein based food with several anti-nutritional factors and low nutritional availability are common in the animal feed industry. High levels of proteases inhibitors are found, mainly in soybean meal, which is one of the most important protein ingredients in animal fed. Studies with vegetable ingredients and enzyme addition in diets for animals showed promising results (Denstadli et al., 2011; Morales et al., 2011). Additionally, proteases can also help with reduction of environmental pollution as they increase nutrient availability and reduce losses to the environment.

The aim of this study was to determine the effect of supplemental low-cost homemade fungal phytase and protease on protein, energy and mineral availability by juvenile Nile-tilapia fed plant based protein diets.

2. Materials and methods

2.1. Enzyme production

The enzymes were obtained by solid fermentation using two different substrates provided by the Department of Animal Breeding and Nutrition, University of São Paulo State: 1) wheat middling and 2) soybean meal; and fungal samples from lyophilized strains of *Aspergillus niger* (INCQS 40018) and *Aspergillus oryzae* (INCQS 40068) from Osvaldo Cruz Institute (FIOCRUZ), according Novelli et al. (2016). The crude extract enzyme activities were determined as described below.

The scale up of the process was done increasing the number of Erlenmeyer flasks and, at the same time, using flasks with double the size maintaining the proportion of substrate and sterilized water.

2.2. Enzyme activity

The phytase activity was determined using *p*-nitrophenyl phosphate as substrate at pH 5.0 and 37 °C. The phytase activity unit was the quantity of enzyme necessary to release one μmol of *p*-nitrophenol per reaction minute at 410 nm. The control sample was measured with denaturized crude enzyme extract (Stockmann et al., 2003).

The protease activity was measured using azocasein as substrate, with modifications at pH 7.0 and 37 °C (Charney and Tomarelli, 1947). The protease activity unit was defined as the quantity of enzyme necessary to increase 0.1 of absorbance at 428 nm in the assay conditions.

2.3. Biochemical characterization

For biochemical characterization, the optimal activity and stability of enzymes produced at different pH and temperature were tested.

2.3.1. Effect of temperature on activity and stability

The optimum temperature was determined as described for each enzyme activity (pH 5.0 to phytase and pH 7.0 to protease) at different temperatures, as follows: 20, 30, 40, 50, 60, 70, 80, and 90 °C (Novelli

et al., 2016).

The temperature stability was determined by incubating the crude enzyme extract at different temperatures for 1 h, followed by determining the residual activity as described in the section above for enzymes activity.

2.3.2. Effect of pH on activity and stability

Optimum pH was determined as described for each enzyme activity (37 °C) using buffer solutions at different pH values as follows: 0.1 M acetate buffer pH 4.0 and 5.0; 0.1 M sodium phosphate buffer pH 6.0 and 7.0; and 0.1 M borax-boric acid buffer pH 8.0 and 9.0 (Novelli et al., 2016).

The pH stability was determined by incubating the crude enzyme extract in buffer solutions at different pH values at 30 °C for 24 h, followed by determining the residual activity as described in the section on protease activity.

2.4. Diets

Two diets were prepared with the test ingredients (soybean meal and corn gluten) added to a reference diet formulated as fish nutritional requirements (NRC, 2011) in a proportion of 30% (m/m), as proposed by Pezzato et al. (2004). Table 1 shows the calculated formulation and analysis of the experimental diets.

The diets were mixed with water (20% of dry weight) and extruded (5.0 mm pellet) at approximately 120 °C in a single-screw laboratory extruder (20 kg h^{-1} of the feed; Esteec®, Ribeirão Preto, SP, Brazil). The pellets were air dried overnight, enzymes were sprayed over the diets and the soybean oil was added at the end of the process. Then, it was stored at 4 °C until further use. The diets were added with 0.1% of chromic oxide (Cr_2O_3) as an inert marker to determine nutrient availability.

Phytase from *Aspergillus niger* produced by Solid State Fermentation (SSF) at the Bioprocess Laboratory, Institute of Biosciences São Paulo State University was added to the diet in a ratio of 2000 U kg^{-1}, as recommended by Portz and Liebert (2004) and, for protease produced by SSF with *Aspegillus oryzae*, 1500 U kg^{-1} was added, as recommended by Mahmoud et al. (2014). The determination of phytase activity in the experimental diets was performed using *p*-nitrophenyl phosphate as substrate, aliquot of 1000 μL of 5 mmol L^{-1} *p*-nitrophenyl phosphate were added to 500 μL of acetate buffer pH 5.0 (0.1 mol L^{-1}) and 100 mg of the experimental diet. The system was incubated for 10 min at 37 °C. The reaction was stopped with 2 mL of sodium hydroxide (0.1 mol L^{-1}). The protease activity was determined using azocasein as substrate. The reaction media was 0.5 mL of azocasein 0.5% (m/v) and 0.5 mL of diet solution 1:5 (m/v) in borax-boric acid buffer pH 9.0 (0.1 mol L^{-1}), they were incubated for 40 min at 37 °C. The reaction was stopped with 0.5 mL TCA 10% (m/v) and centrifuged at 6000 rpm for 15 min at 15 °C; 1 mL of potassium hydroxide (5 mol·L^{-1}) was added to 1 mL of the supernatant.

2.5. Culture conditions, fish and feeding

The experiment was evaluated by the Ethic Commission for Animal Utilization of the Veterinary and Animal Science College, University of São Paulo State "Julio de Mesquita Filho" under protocol number 20/2013-CEU and was implemented at AquaNutri, FMVZ, São Paulo State University – UNESP, Botucatu, SP, Brazil.

The apparent availability coefficient was determined according to Pezzato et al. (2004). Nine 250 L tanks were used for the feeding procedure, and five conical 300 L tanks were used to collect faeces via a settlement column. Both systems were connected to a biological filter and an electronic thermostat (27.0 ± 0.5 °C.). The water was circulated at 6.59 L min^{-1} through the aquaria. Groups of ten Nile tilapia/aquarium (150 ± 5.0 g) were used in the trail and diets were randomly assigned to each tank. Fish were fed seven days prior to the beginning of the faecal collection (acclimatization period). Fish were hand fed until apparent satiation from 8:00 am to 5:00 pm. At 6:00 pm, the cages were transferred to the collecting faeces aquaria. For this collection the first five groups of fish were transferred to collecting faeces aquaria and on the consecutive day the remaining four groups were transferred and faeces collected. This procedure was carried out until representative volume of faeces for each replication of chemical analysis was collected. The acclimatization and faecal collection process (round) were repeated three times to obtain triplicate measurements per treatment (test diets). Faeces were dried at 55 °C, ground and stored at − 20.0 °C until chemical analysis.

Chemical analysis of feedstuffs, diets and faeces were determined according AOAC (1995) protocols. Chromium oxide content of diets and faeces were determined according Bremer-Neto et al. (2005) and gross energy content was determined in an adiabatic calorimetric bomb (Parr Instrument Company, Moline-IL, EUA). Calcium, magnesium, manganese and phosphorous were mineralized with nitro-perchloric acid solution for further analyses. Phosphorous was determined by phospho-vanado-molybdate colorimetric method according Moraes et al. (2009); calcium, magnesium and manganese were determined using a SHIMADZU AA-6800 atomic absorption spectrometer equipped with a background absorption correction with a deuterium lamp and self-reverse (SR) system.

2.6. Apparent digestibility coefficients (ADC) calculation

Below Eq. (1) was used to assess the apparent digestibility coefficients (ADC, %) of dry matter protein, energy lipid and availability of phosphorous, calcium, manganese and magnesium of the experimental diets (Cho et al., 1982), as:

$$ADC = 100 - [100\,[\%Cr_2O_3r/Cr_2O_3f] \times [\%Nf/\%Nr]] \quad (1)$$

where:

ADC = Apparent digestibility coefficient (%);

$\%Cr_2O_3r$ = diet chromium oxide III percentage;

$\%Cr_2O_3f$ = faeces chromium oxide III percentage;

%Nf = crude energy or percentage of dry matter, protein, lipids or minerals in the faeces;

%Nr = crude energy or percentage of dry matter, protein, lipids or minerals in the diet.

The ADC of the experimental diets was used to calculate the apparent digestibility coefficient of dry matter, energy, protein and minerals availability of the test ingredients when enzymes were added according to Bureau et al. (1999) as follows (Eq. (2)):

$$ADC_{test\ ingredient} = ADC_{test\ diet} + (ADC_{test\ diet} - ADC_{ref\ diet}) \times ((0.7 \times N_{ref\ diet})/(0.3 \times N_{test\ ingredient})) \quad (2)$$

2.7. Experimental design

Nine compound diets were evaluated, eight containing the two plant test ingredients (soybean meal and corn gluten) and the addition or not of the supplemental enzymes (phytase, protease, both and no enzyme), and one reference practical diet, with three replicates for each combination. The digestibility was determined indirectly using 0.1% (m/m) of chromium oxide III as an inert marker. Analysis of variance test by SAS statistics was used and, when there was difference between averages, it was applied the Duncan test with 5% significance.

3. Results and discussion

Aspergillus oryzae show no satisfactory performance when fermented in soybean bran. *A. niger* produced acid phytases in both substrates, as for *A. oryzae*, optimum pH was basic; optimum temperature was 37 °C for *A. niger* and 30 °C for *A. oryzae* (Fig. 1). Phytase produced by *A. niger*

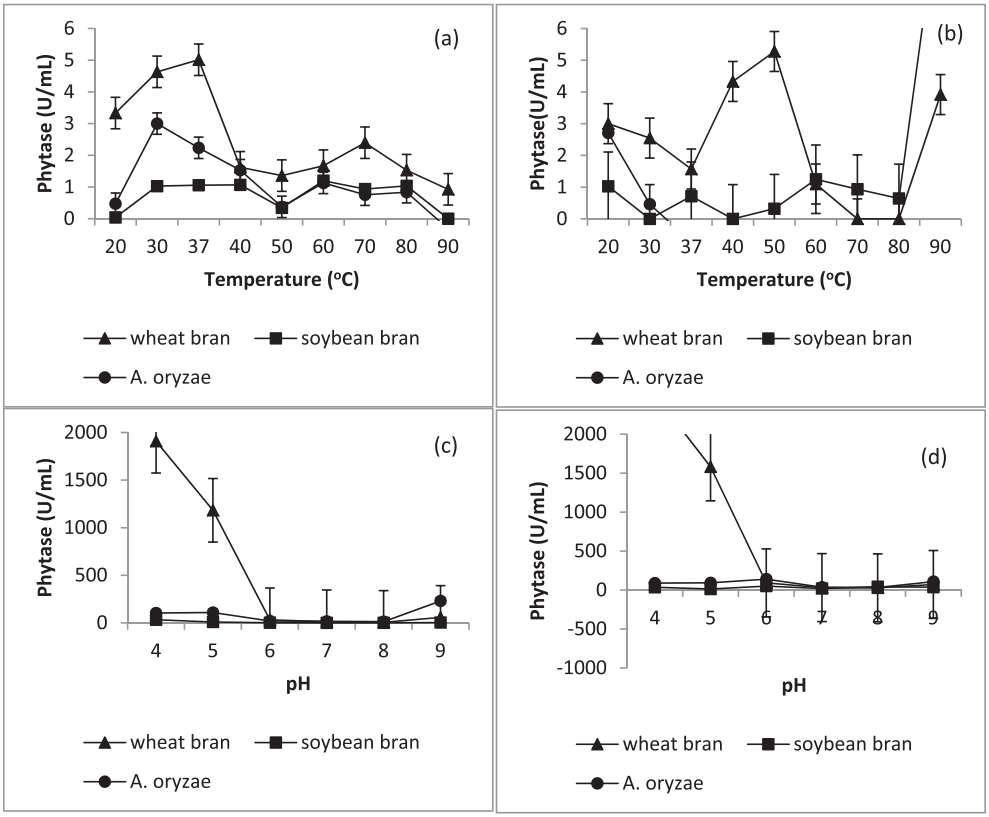

Fig. 1. Influence of temperature (a – optimum, and b – stability) and pH (c – optimim, and d – stability) on phytase activity produced by solid fermentation with *Aspegillus niger* 40018 using wheat bran (-▲-) and soybean bran (-■-) as substrates and *Aspergillus oryzae* using wheat bran as substrate (-●-).

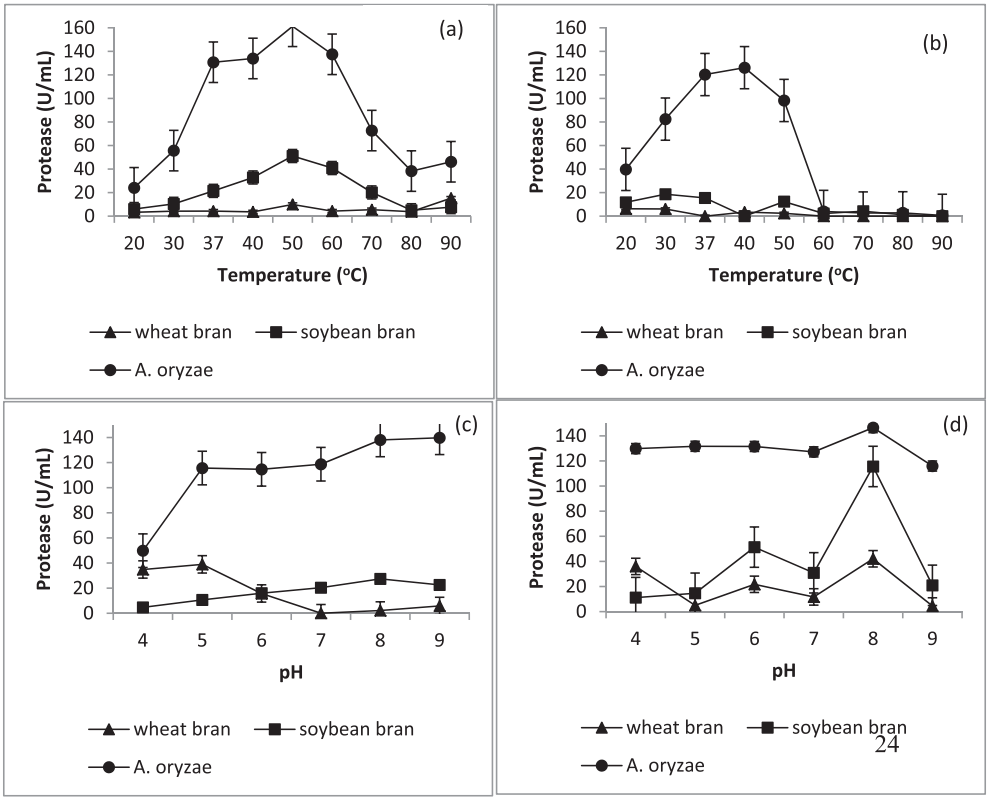

Fig. 2. Influence of temperature (a – optimum, and b – stability) and pH (c – optimim, and d – stability) on protease activity produced by solid fermentation with *Aspegillus niger* 40018 using wheat bran (-▲-) and soybean bran (-■-) as substrates and *Aspergillus oryzae* using wheat bran as substrate (-●-).

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with both substrates showed different behavior for temperature stability at 50 °C and 90° C and 60 °C and 90 °C, for fermentation in wheat bran and soybean bran, respectively (Fig. 1b). Besides this characteristic present isoforms of the enzyme, it also demonstrates the enzyme capacity to support high temperatures, that is essential in some industrial processes. Protease from *A. niger* showed very distinct behavior when different substrates were tested. Moreover, proteases from *A. oryzae* were stable at all pHs and produced higher yields (Fig. 2).

Table 2
Apparent digestibility coefficients (ADC) of dry matter, protein, energy, lipids of plant ingredients, soybean meal and corn gluten, with fungal enzymes addition.

Treatment	Soybean meal				Corn gluten			
	No enzyme	Phytase	Protease	Phytase + protease	No enzyme	Phytase	Protease	Phytase + protease
Dry matter	72.62 c	93.48 ab	94.39 ab	92.37 ab	94.43 ab	80.22 bc	84.57 abc	97.92 a
Protein	93.92 bcd	98.47 a	97.36 ab	97.51 ab	95.17 abc	91.47 d	91.96 cd	96.43 ab
Energy	78.39 b	94.87 a	94.87 a	93.33 a	95.60 a	85.75 ab	88.52 ab	96.97 a
Lipid	95.53 ab	104.83 a	105.02 a	110.83 a	77.23 bc	33.61 e	64.73 cd	54.81 d

Values with different letters within a row indicate significant difference ($P < 0.05$).

In this study, we aimed to investigate the enzyme production and the nutritional availability of two types of plant protein based diets with and without enzymes addition for Nile tilapia. To this end, we tested eight diets including two protein sources, soybean meal and corn gluten, and the addition or not of phytase, protease and both enzymes. The addition of enzymes significantly affected nutrients digestibility when soybean meal was used as plant protein test ingredient; as for corn gluten, when compared to soybean meal with enzyme utilization, showed no difference.

There were significant differences in the apparent digestibility coefficient (ADC) of dry matter, protein, energy and lipids when phytase was added to soybean meal as plant based ingredient (Table 2). The digestibility varied significantly with or without phytase utilization, and was significantly lower when soybean meal was used as test ingredient alone. The availability of phosphorous was also affected by fungal phytase addition (Table 3). A significant effect of phytase supplementation on protein and energy utilization rate was observed by Liebert and Portz (2005), recommending this additive for maximum growth performance for Nile tilapia. Most microbial phytase act efficiently under conditions present in the stomach (acid pH), however, not all phytase have the same pH profile (Morales et al., 2011). In this study, *Aspergillus niger* phytase showed optimum pH at 5. Also, as Nile tilapia stomach pH is acid, phytase activity increase in low pH and protein digestion occurs in the stomach; this condition may help to improve protein digestibility.

Vielma et al. (2004) reported positive effects of supplemented phytase on protein digestibility in rainbow trout (*Oncorhynchus mykiss*); however, protein utilization was not significantly increased. Researchers have shown enhanced amino acid and protein availability due to supplemental phytase for land animals (Sebastian et al., 1997; Martin et al., 1998) and fish (Guimarães et al., 2009). Likewise, plant protein utilization has been reported to increase by enzymes utilization in several studies (Storebakken et al., 1998; Vielma et al., 2004; Sugiura et al., 2001). Hien et al. (2015) showed that the soybean meal supplemented with phytase, lysine, threonine and methionine can substitute fish meal by 40% for *Channa striata* and *Channa micropeltes* fingerlings, without affecting performance, feed efficiency and survival.

As expected, the supplementation effect of microbial phytase and/or protease on phosphorous, magnesium, calcium and manganese utilization was significant when added to soybean meal (Table 3). Apparent phosphorous availability and bone mineralization are considered as the most sensitive conditions for assessing the influence of phytase on phosphorous utilization (Cao et al., 2007). Currently, the capacity of phytase to increase total phosphorous availability in fish has been demonstrated. Sugiura et al. (2001) observed that for rainbow trout fed diet with 50% soybean meal pretreated with 1000 U phytase kg^{-1}, the ADC of phosphorous reached 93%. Sajjadi and Carter (2004) reported that phosphorous availability after phytase supplementation was significant higher for Atlantic salmon (*Salmon salar*) when compared to control diets without phytase. Additionally, the positive effect of phytase on ADC of phosphorous has also been observed in Nile tilapia fed soybean meal based diets (Liebert and Portz, 2005).

Moreover, the digestibility of energy and lipid where increased with addition of fungal protease to soybean meal (Table 2). The availability of phosphorous also ranged from 36.39 to 82.63%, with no enzyme and protease addition in soybean meal, respectively (Table 3). The enzyme activity is linked to the animal feeding habits (Sabapathy and Teo, 1993), this may modify the enzyme addition effect on the ingredient, as it can be more efficient for some fish species than others. Likewise, exogenous protease added to diet of *Cichla* sp., a carnivorous fish, affected body characteristics and growth performance (Soares et al., 2008), however, these authors did not studied the effect of protease on nutrients digestibility. Ng et al. (2002) observed reduction of anti-nutrients effect and increase of nutritional value of palm bran for Nile tilapia; and the use of an enzyme complex, including protease, for Nile tilapia diets improved the digestibility of protein, fat and gross energy digestibility (Guimarães et al., 2009). The exogenous protease could be responsible for increase and complement the enzyme-substrate machinery, improving even carbohydrates and lipids digestion. And more, similarly to phytase, protease can degrade some proteins that act as anti-nutritional factors and improve nutrients digestibility.

Otherwise, there were no differences for corn gluten treatments with enzymes addition (Tables 2 and 3). Corn gluten nutritional composition, as amino acids, minerals or other components, may have caused an inhibition on the activity site of the enzyme produced and, for this reason, enzyme addition did not work for this ingredient. Even more, when the enzymes were combined in the treatments, they did not show improvement on ingredients digestibility. This may show that the fungal protease produced can degrade the phytase and increase the negative effect on gluten digestibility and its anti-nutrients. In this sense, the stability of phytase during stomach digestion in the presence of pepsin may be a limiting factor for its efficiency and more studies simulating the fish stomach conditions and interaction with ingredients might be needed. On the other hand, calcium availability improved

Table 3
Phosphorous (P), Magnesium (Mg), Calcium (Ca) and Manganese (Mn) availability of plant based ingredients, soybean meal and corn gluten, and enzymes addition.

Treatment	Soybean meal				Corn gluten			
	No enzyme	Phytase	Protease	Phytase + protease	No enzyme	Phytase	Protease	Phytase + protease
Phosphorous	36.39 c	80.22ab	82.63 a	75.03 ab	66.55 abc	48.08 bc	54.41 abc	68.70 abc
Magnesium	66.09 c	79.45 b	78.51 bc	92.05 a	73.71 bc	49.74 d	66.22 c	76.78 bc
Calcium	18.03 cd	38.91 bc	73.28 a	62.50 ab	5.29 d	15.32 cd	56.23 ab	52.92 ab
Manganese	− 281.78e	37.17 d	70.86 b	94.41 a	47.00 cd	46.51 cd	65.68 bc	51.55 bcd

Values with different letters within a row indicate significant difference ($P < 0.05$).

with protease addition in the diet for both plant ingredients. Calcium chemical characteristic may be responsible for that improvement, as it is a metal ion that can also bound to phytate and anti-nutritional factors. Many studies reported increase in protein digestibility with microbial phytase supplementation in plant based diets using soybean bran (Liebert and Portz, 2005; Vielma et al., 2004), on the other hand, corn gluten needs further considerations. Likewise, Gonçalves et al. (2007) identified that phosphorous availability varies according the vegetable ingredient used and defined that mineral availability for corn gluten diets with phytase addition was improved only with high levels of enzyme addition, also due to its natural phytase occurrence or the amount of phytate present in the corn gluten. Furthermore, high levels of plant ingredients in the diet result in high content of insoluble carbohydrates, as well as elevated levels of anti-nutritional ingredients like phytates (Francis et al., 2001; Riche et al., 2001). According to Tacon (1993), oilseed meals contain many thermostable anti-nutrients and most importantly enzyme inhibitors. Whether these or other anti-nutrients factors were totally inactivated was not determined in this study. Also, it is important to remember that the enzymes are substrate specific and the corn gluten has different composition than soybean meal, so this might affect in the nutrients utilization as well.

The lipids digestibility was over estimated when both enzymes were added to the ingredients; this may indicate the presence of other enzymes, like lipases in the fungal enzyme complex produced, since the homemade enzymes were used as a crude extract and they were not purified. Furthermore, because of lipase presence, lipids release in the faeces can be more than what should be expected. Supplementation of phytase may also improve the bioavailability of protein and might lead to additional improvements in growth and energy deposition or lipids excretion. The reduction of phytate–protein complexes in the gut and increased nutrient availability could be an explanation for this observation. In vitro studies of Singh and Krikorian (1982) demonstrated negative effects of phytates on protein utilization.

In addition, the optimum supplemental doses of the enzymes for each ingredient may need further research. Also, the interaction between enzymes and sources need more attention to encounter an ideal way to maximize enzyme efficacy. Further investigations are necessary to clarify the influence of supplemental fungal enzymes on nutrients and minerals utilization in fish. Thereby, this additive may reduce environmental pollution by phosphorous and other minerals excreted, as they increase mineral availability, reducing the mineral input and water pollution.

4. Conclusion

In conclusion, the substrate, as well as, the microorganism species can affect the biochemical character of the enzyme produced. Additionally, both microbial enzymes demonstrated to be efficient for increasing nutritional utilization of soybean meal for Nile tilapia; and more, these low cost enzymes are alternative additives to improve soybean meal digestibility for a cost-effective and environmentally friendly fish diet.

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