Indian J. Fish., 62 (1): 78-84, 2015

Role of phytase supplementation in improving nutrient digestibility in *Labeo rohita* (Hamilton, 1822) fingerlings fed on cottonseed meal based diet

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ABSTRACT

The present research work was conducted to evaluate the role of phytase supplementation in improving nutrient digestibility in *Labeo rohita* fingerlings fed cottonseed meal-based diets having 30% protein and 4.15 k cal g^{-1} gross energy. The experimental diet comprised 70% reference diet and 30% cottonseed meal as test ingredient. The experimental diet was top sprayed with graded levels of phytase *viz*., 0, 250, 500, 750, 1000, 1250 and 1500 FTU kg^{-1} resulting in the formulation of seven test diets. Chromic oxide was used as indigestible marker in the feed to estimate nutrient digestibility. Results showed improved crude protein, crude fat and gross energy digestibility in response to phytase supplementation. Maximally improved nutrient (crude protein and crude fat) and gross energy digestibility was observed in fingerlings fed 750 FTU kg^{-1} phytase containing diet followed by the diet having 1000 FTU kg^{-1} phytase level. It was concluded that phytase supplementation to cottonseed meal-based diet at 750 FTU kg^{-1} level is adequate to release chelated nutrients from phytate for fish.

Keywords: Cottonseed meal, *Labeo rohita,* Nutrient digestibility, Phytase

Introduction

Pakistan is blessed with both inland and marine water resources which can play an important role in the development of different nutritionally and economically significant fish species (Hussain *et al*., 2011). *Labeo rohita* commonly known as rohu, is one of the major carp species found in riverine system of South Asia and is also being cultured on a large scale in Pakistan (Abid and Ahmed, 2009; Hussain *et al*., 2011a). However, pond fish productivity of the region is very low due to unavailability of balanced and cost effective feed. In recent years, limited supply and increasing fishmeal price has further intensified the problem, which has motivated the researchers to search for cost-effective alternatives to fishmeal in fish feed formulation. Replacement of fishmeal with locally available cheaper ingredients in fish feed can make it economical for fish farmers (Pham *et al.,* 2008; Lech and Reigh, 2012). Many of the plant byproducts of agriculture industry are found to be promising sources of protein and energy and may be used for the formulation of cost effective fish feeds (Cheng and Hardy, 2002; Hussain *et al*., 2011b). However, the main problem associated with the use of such plant proteins in aquafeed is the presence of antinutritional factors like phytate which negatively affects the physiology of fish digestive tract, reducing the growth performance of fish (Baruah *et al.,* 2004). Apart from minerals, phytate fish complexes with protein that ultimately decreases bioavailability of these nutrients to fish (Helland *et al.,* 2006). Phytate can bind to proteins and it has been shown to inhibit digestive enzymes (pepsin, trypsin and α-amylase) activities (Liener, 1994) leading to reduced nutrient digestibility (Kumar *et al.,* 2011). Furthermore, phytate may chelate with amino acids in the gut of different fish species and reduce availability of amino acids (Usmani and Jafri, 2002). Phytase is an enzyme that hydrolyzes the phytate. It is naturally found in microorganisms and fungi. However, mostly fishes lack phytase and are unable to use the plant based diets efficiently. Phytase is produced commercially and is being used in poultry feed. Recent trials of phytase supplementation in fish feed are also encouraging. Improved nutrient digestibility in fish fed phytase sprayed plant-based diet has been reported in several studies (Baruah *et al*., 2007; Hussain *et al*., 2011a, b, c).

Cottonseed meal is an excellent source of plant protein that has long been used in the feeds of terrestrial and aquatic animals (Gatlin III *et al.,* 2007; Lim and Lee, 2008). It is the third largest oilseed meal product in world production after soybean meal and canola meal (Lee *et al.,* 2006). It is a rich source of essential amino acid arginine, which is higher than that of fishmeal and soybean meal (EI-Saidy *et al.,* 2004). Efforts are required to study the suitability of locally available plant byproducts as major feed ingredients to enhance fish production substituting expensive fishmeal with cost effective ingredients such as cottonseed meal. The present work was undertaken with the objective of developing a cost effective feed for indigenous commercially culturable fish, *Labeo rohita.*

Materials and methods

The work was conducted in the Fish Nutrition Laboratory, Department of Zoology and Fisheries, University of Agriculture, Faisalabad.

Fish and experimental conditions

Labeo rohita fingerlings were obtained from Government Fish Seed Hatchery, Faisalabad and maintained in v-shaped tanks (specially designed for the collection of feces) having capacity to hold 70 l water, for two weeks to acclimatise them to the experimental conditions. Before start of the feeding trial, *L. rohita* fingerlings were treated with NaCl (5g l^{-1}) to make sure they are free from ectoparasites and also to prevent further fungal infections (Rowland and Ingram, 1991). During this acclimatisation period, *L. rohita* fingerlings were given reference diet once daily to apparent satiation (Allan and Rowland, 1992). Water quality parameters such as water temperature, pH and dissolved oxygen were monitored using pH meter (Jenway 3510), DO meter (Jenway 970) and electrical conductivity (EC) meter (HANNA: HI. 8633) on daily basis. Aeration (24 h) was provided to all the experimental tanks through capillary system. The range of water quality parameters (> pH 7.4-8.6, dissolved oxygen 5.8 - 7.3 mg l^{-1}, electrical conductivity 1.30-1.52 dS m^{-1} and temperature 24.9-28.7ºC) was noted.

Experimental design

Cottonseed meal (CM) was used as test ingredient to formulate experimental diet. Experimental diet comprised of 70% reference diet and 30% of test ingredient. Experimental diet was then divided into seven test diets and supplemented with graded (0, 250, 500, 750, 1000, 1250 and 1500 FTU kg^{-1}) levels of phytase. One reference diet and seven cottonseed meal based phytase supplemented test diets were fed to eight groups of fish stocked in experimental tanks. Each of the treatment and control diet had three replicates with 15 fingerlings in each replicate. Total duration of the experiment was approximately ten weeks, up to the collection of 4-5 g feces from each replicate. CM based phytase supplemented diets were compared with each other and with reference diet to determine nutrient digestibility parameters using completely randomised design (CRD). The optimum level of phytase supplementation for different nutrient digestibility parameters was determined using quadratic regression analysis. The apparent nutrient digestibility coefficient (ADCs) of crude protein, crude fat and gross energy were estimated using chromic oxide as inert marker.

Feed ingredients and formulation of experimental diets

The feed ingredients were purchased from a commercial feed mill and were analysed for chemical composition following AOAC (1995) prior to the formulation of the experimental diet (Table 1). The reference diet was prepared to supply sufficient levels of required nutrients for normal fish growth (Table 2). The feed ingredients were finely ground to pass through 0.5 mm sieve size. All ingredients were mixed in an electric mixer for 10 min and fish oil was gradually added during mixing of the diet. During mixing of ingredients, 10-15% water was also added and the mixture was then extruded using SYSLG30-IV experimental extruder to produce floating pellets (3 mm). The above procedure was followed to formulate the reference diet and seven cottonseed meal based test diets. The required concentrations (0, 250, 500, 750, 1000, 1250 and 1500 FTU kg^{-1}) of phytase (Phyzyme® XP 10000 FTU g^{-1}; Danisco Animal Nutrition, Fin-65101 Vaasa, Finland) were prepared in 25 ml distilled water and sprayed on 1 kg of test diets (Robinson *et al*., 2002). The control diet (0 FTU kg^{-1}) was sprayed with a similar amount of distilled water to maintain an equal level of moisture contents. All the prepared diets were dried and stored at 4°C until use.

Chemical analysis of feed and faeces

The samples of fish feed ingredients, experimental diets and faeces were homogenised using mortar and pestle and analysed following standard methods (AOAC, 1995). Moisture content was determined by oven drying at 105^{o}C for 12 h whereas crude protein (CP) (Nx6.25) employing

Table 1. Chemical composition (%) of feed ingredients (Dry matter basis)

Ingredients	Dry matter (%)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)	Total carbohydrate (%)	Gross energy (k cal g^{-1})
Fish meal	91.63	48.15	7.16	0.52	26.23	17.94	3.69
Wheat flour	92.45	10.10	2.35	1.65	2.08	83.82	2.96
Corn gluten 60%	92.59	59.12	4.96	1.19	1.58	33.15	4.23
Rice polish	94.09	12.35	12.31	2.71	7.90	64.73	4.33
Cottonseed meal	92.90	41.38	2.63	1.23	6.76	48.00	3.71

Table 2. Composition of ingredients (%) in reference and test diets

Ingredients	Reference diet	Test diet
Fish meal	20.0	14.0
Wheat flour	24.0	16.8
Corn gluten 60%	20.0	14.0
Rice polish	25.0	16.6
Fish oil	7.0	4.9
Vitamin premix**	1.0	1.0
Mineral***	1.0	1.0
Ascorbic acid	1.0	1.0
Chromic oxide	1.0	0.7
Cottonseed meal	-	30.0

**Each kg of Vitamin premix contains:

Vitamin A	15 M.I.U.	Vitamin D_3	3 M.I.U.
Vitamin B_1	5000 mg	Vitamin E	6000 IU
Vitamin B_2	6000 mg	Vitamin K_3	4000 mg
Vitamin B_6	4000 mg	Folic acid	750 mg
Vitamin B_{12}	9000 mcg	Calcium pantothenate	10000 mg
Vitamin C	15000 mg	Nicotinic acid	25000 mg

***Each kg mineral granules contains :

Ca (Calcium)	155 g	Mn (Manganese)	2000mg
P (Phosphorous)	135 g	Cu (Copper)	600 mg
Mg (Magnesium)	55 g	Co (Cobalt)	40 mg
Fe (Iron)	1000 mg	I (Iodine)	40 mg
Zn (Zinc)	3000 mg	Se (Selenium)	3 mg
Na (Sodium)	45 g		

micro Kjeldahl apparatus. Ether extract (EE) was extracted by petroleum ether extraction method through Soxtec HT2 1045 system (Bligh and Dyer, 1959); crude fiber (CF) as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH, whereas ash by ignition at 650°C for 12 h in electric furnace (Eyela-TMF 3100) to constant weight. Total carbohydrates (N-free extract) was calculated as: Total carbohydrate % = 100 - (CP%+ EE%+CF%+Ash%). Gross energy digestibility was determined with the help of adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, USA).

Digestibility studies

Chromic oxide was used as an inert marker at 1% inclusion level in reference diet and 0.7% in test diets, assuming that the amount of the marker in the feed and feces remains constant throughout the experimental period and that all of the ingested marker will appear in the feces. After the feeding session of 2 h, the tanks were washed completely to remove the particles of diets and refilled with freshwater. Then feces were collected from the faecal collection tube of each tank by opening the valves. Fecal material of each replicated treatment was dried in oven, ground and stored for chemical analysis. Chromic oxide content in the diets and feces was estimated after oxidation with molybdate reagent using UV-VIS 2001 Spectrophotometer at 370 nm absorbance (Divakaran *et al*., 2002). The apparent nutrient digestibility coefficients (ADC %) of crude protein, crude fat and apparent gross energy for fish were calculated indirectly at the end of the experiment using chromic oxide as inert marker.

ADC (%) of diets was calculated using the following formula (NRC, 1993):

$$\text{ADC (\%)} = 100 - 100 \times \frac{\text{\% marker in diet} \times \text{\% nutrient in feces}}{\text{\% marker in feces} \times \text{\% nutrient in diet}}$$

Statistical analysis

Data on nutrient (crude protein, crude fat) and apparent gross energy digestibility of reference diet and test diets was subjected to one-way analysis of variance (ANOVA) (Steel *et al*., 1996). The differences among means were compared by Tukey's Honestly Significant Difference Test and considered significant at $p<0.05$ (Snedecor and Cochran, 1991). The CoStat computer software (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analysis.

Results and discussion

Crude nutrient contents of test diets and feces are presented in Table 3 and 4 respectively. It is obvious from the results that in comparison with reference diet, cottonseed meal-based test diet supplemented with phytase enzyme released least amount of nutrients and gross energy through feces at 750 and 1000 FTU kg^{-1} phytase levels. This decrease in nutrient excretion through feces confirms that phytase has the property of hydrolysing phytate-nutrient complexes and make these nutrients available to fish resulting in less discharge of these nutrients in feces (Table 4).

It is apparent from nutrient digestibility data that maximum crude protein digestibility (68%) was observed

Table 3. Composition (%) of nutrients in reference and test diets

Diets	Phytase levels (FTU kg^{-1})	Crude protein (%)	Crude fat (%)	Apparent gross energy (k cal g^{-1})
Reference diet		30.21	6.65	4.26
(Test diet I)	0	29.95	4.76	4.14
(Test diet II)	250	29.79	4.75	4.13
(Test diet III)	500	29.54	4.73	4.11
(Test diet IV)	750	30.34	4.78	4.13
(Test diet V)	1000	30.03	4.75	4.14
(Test diet VI)	1250	29.89	4.76	4.12
(Test diet VII)	1500	29.78	4.77	4.13
PSE		0.1977	0.0101	0.0124
p		0.0510	0.0000	0.0000

Means within rows having different superscripts are significantly different ($p < 0.05$), Data are means of three replicates
PSE: pooled SE = √MSE/n (where MSE= mean-squared error)

Table 4. Composition of nutrients (%) in feces of *Labeo rohita* fingerlings fed on reference and test diets

Diets	Phytase levels (FTU kg^{-1})	Crude protein (%)	Crude fat (%)	Apparent gross energy (k cal g^{-1})
Reference diet		13.63^{b}	2.18^{f}	1.80^{cd}
(Test diet I)	0	14.31^{c}	1.88^{e}	2.23^{g}
(Test diet II)	250	14.04^{bc}	1.72^{d}	1.92^{de}
(Test diet III)	500	13.95^{bc}	1.46^{b}	1.76^{c}
(Test diet IV)	750	11.49^{a}	1.28^{a}	1.41^{a}
(Test diet V)	1000	13.24^{b}	1.57^{bc}	1.68^{b}
(Test diet VI)	1250	15.34^{d}	1.65^{cd}	2.02^{e}
(Test diet VII)	1500	15.78^{d}	1.88^{e}	2.10^{f}
PSE		0.1755	0.0254	0.0196
p		0.0000	0.0000	0.0000

Means within rows having different superscripts are significantly different ($p < 0.05$), Data are means of three replicates
PSE: pooled SE = √MSE/n (where MSE= mean-squared error) (*p* value = 0.000)

in the diet supplemented with 750 FTU kg^{-1} phytase concentration followed by 1000 FTU kg^{-1} level (63%). These values were significantly different from each other ($p<0.05$) as well as from the reference diet (Table 5). Similar to the present study, our previous studies with *L. rohita* fingerlings, also showed maximally improved crude protein digestibility in phytase supplemented (750 FTU kg^{-1}) sunflower (Hussain *et al*., 2011a) and corn gluten (Hussain *et al*., 2011b) meal based diets.

Furuya *et al*. (2001) observed maximum increase in crude protein digestibility in Nile tilapia (*Oreochromis niloticus*) at 700 FTU kg^{-1} level of phytase supplementation. Sugiura *et al*. (2001); Liebert and Portz (2005) and Ashraf and Goda (2007) also confirmed that phytase supplementation to plant based diets increases the protein digestibility in fish. However, crude protein digestibility showed no response to phytase supplementation in plant based diets of Atlantic salmon (Yan and Reigh, 2002) and rainbow trout (Vielma *et al.,* 2002; Cheng and Hardy, 2002; Dalsgaard *et al.,* 2009). This discrepancy in protein digestibility is probably associated with the variation in protein quality of feed ingredients, pH of fish gut, feed processing and drying procedures (Wang *et al.,* 2009). The higher apparent digestibility coefficient (ADC%) of crude

Table 5. Apparent nutrient digestibility (%) in *Labeo rohita* fingerlings fed on reference and test diets

Diets	Phytase levels (FTU kg^{-1})	Crude protein (%)	Crude fat (%)	Apparent gross energy (%)
Reference diet		61.54^{b}	71.51^{b}	62.45^{b}
(Test diet I)	0	55.47^{cd}	63.14^{e}	51.82^{d}
(Test diet II)	250	56.94^{c}	67.02^{cd}	57.23^{c}
(Test diet III)	500	57.41^{c}	72.14^{b}	62.23^{b}
(Test diet IV)	750	68.20^{a}	77.57^{a}	70.74^{a}
(Test diet V)	1000	62.84^{b}	72.11^{b}	67.11^{a}
(Test diet VI)	1250	54.90^{cd}	59.68^{bc}	56.49^{c}
(Test diet VII)	1500	52.91^{d}	64.91^{de}	54.90^{cd}
PSE		0.6219	0.6866	0.8463
p		0.0000	0.0000	0.0000

Means within rows having different superscripts are significantly different ($p < 0.05$), Data are means of three replicates
PSE: pooled SE = √MSE/n (where MSE= mean-squared error)

protein, observed in the present investigation showed the acceptability levels of the alternative plant protein based test diets supplemented with phytase enzyme (Kumar *et al.,* 2011).

Significantly higher ($p<0.05$) values of crude fat digestibility were also observed at 750 FTU kg^{-1} level when compared with reference and other test diets (Table 5). Portz and Liebert (2004), while working on Nile tilapia, reported improvement in crude fat digestibility for phytase supplemented diets. Improved crude lipid digestibility has also been reported by Ashraf and Goda (2007) in the Nile tilapia *Oreochromis nilotica* fed on phytase treated diet. In contrary, Dalsgaard *et al*. (2009) found no significant effect of phytase addition in the diet, on crude fat digestibility in rainbow trout (*Oncorhynchus mykiss*). Wang *et al*. (2009) reported reduced lipid digestibility in rainbow trout in response to phytase supplementation. They argued that phytase supplementation might inhibit the lipase activity which in turn reduced the lipid hydrolysis leading to decreased lipid digestibility in phytase supplemented diets.

Like crude protein and crude fat, significantly high ($p<0.05$) digestibility of gross energy was also observed at 750 FTU kg^{-1} phytase level as compared to reference and other phytase supplemented test diets. The findings of higher utilisation of gross energy at 750 FTU kg^{-1} level are well supported by our previous studies (Hussain *et al*., 2011a, c). In current study, maximum gross energy digestibility (71%) was at 750 FTU kg^{-1} level and after this level of phytase supplementation a decreasing trend in gross energy digestibility was observed. Comparable values of gross energy digestibility were observed when *L. rohita* fingerlings were fed on phytase supplemented sunflower (73%) (Hussain *et al*., 2011a) and corn gluten (66%) meal based test diets (Hussain *et al*., 2011b). Portz and Liebert (2004) reported significant improvement in gross energy digestibility in Nile tilapia*, O. niloticus* fed on phytase supplemented plant based diet. Similarly, Cheng and Hardy (2002) observed significant improvement in gross energy digestibility coefficient of phytase treated canola protein concentrate diet in rainbow trout. However, Lanari *et al*. (1998) did not observe positive response in phytase treated soybean meal based diet in rainbow trout. In general, phytase supplementation improves the protein bioavailability and this may lead to additional improvements in energy budget of fish resulting in higher growth (Liebert and Portz, 2005).

It is clear from the above discussion that highest apparent crude protein, crude fat and gross energy digestibility coefficients (%) of *L. rohita* fingerlings fed on cottonseed meal based test diets were observed at 750 FTU kg^{-1} level. However, it is interesting to note that further higher levels of phytase supplementation resulted in a decline in the nutrient digestibility. Similar trend in nutrient digestibility was observed in our previous studies (Hussain *et al*., 2011a, c). In another study, similar trend of crude protein, crude fat and gross energy digestibility was also reported by Baruah *et al*. (2007) while feeding phytase supplemented soybean meal-based diet to *L. rohita* fingerlings*.* They observed maximum nutrient digestibility at 750 FTU kg^{-1} phytase level and after this level of phytase supplementation, the nutrient digestibility showed a decreasing trend. The reasons for this decline in nutrient digestibility are difficult to assess but it is suggested that phytase inclusion should carefully be done within certain limits.

The results of the present study provided sufficient evidence that 750 FTU kg^{-1} level of phytase supplementation is optimum for improvement of nutrient (crude protein, crude fat) and gross energy digestibility in *L. rohita* fingerlings fed on cottonseed meal based diets (Fig. 1). Furthermore, phytase supplemented cottonseed meal based diets reduce the cost of fish feed and discharge of nutrients through faeces into the aquatic ecosystem resulting in environment friendly aquaculture.

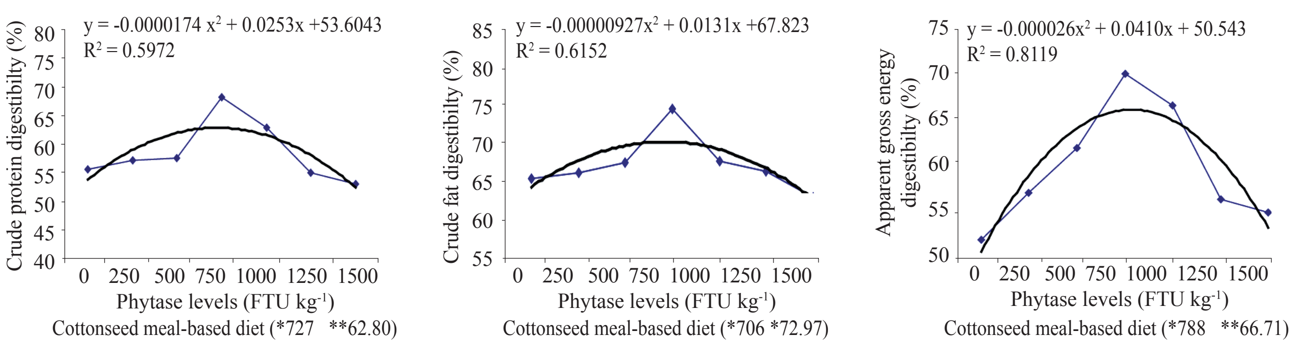

Fig. 1. Relationship between apparent nutrient digestibility (%) of reference as well as test diets and phytase levels (FTU kg^{-1})
(*Optimal phytase level FTU kg^{-1} ** Apparent nutrient digestibility %)

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Date of Receipt : 21.04.2014
Date of Acceptance : 27.09.2014