



## RESEARCH PAPER

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## Effects of phytase supplementation on mineral digestibility in *Cirrhinus mrigala* fingerlings fed on sunflower meal-based diets

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### Abstract

Present research work was conducted to assess the effects of phytase supplementation on minerals digestibility in *Cirrhinus mrigala* fingerlings fed on sunflower meal based diets supplemented with graded levels (0, 500, 1000, 1500 and 2000 FTU kg<sup>-1</sup>) of phytase. Six experimental diets were used including reference diet and five test diets. Reference diet was fed to the fingerlings to provide appropriate nutrients for normal growth. Test diets were consisted of 70% reference diet and 30% test ingredient i.e. sunflower meal. Chromic oxide was added in the feed at 1% concentration as indigestible marker for determination of minerals digestibility. Highest digestibility values (%) of P, Mg, Na, K, Cu and Zn were observed at 1000 FTU kg<sup>-1</sup> level of phytase supplementation and these values differed significantly ( $p < 0.05$ ) from the reference diet and other test diets. Hence, it is concluded that phytase supplementation in sunflower meal based diets at 1000 FTU kg<sup>-1</sup> level is enough to release sufficient chelated minerals to improve overall performance of *Cirrhinus mrigala* fingerlings.

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## Introduction

Aquaculture industry is developing more efficiently than other food producing sectors. However, economic factors such as cost of fish feed are restraining its development (Yıldırım *et al.*, 2014). Fish meal is being used by aqua feed industry as a potential source of basic nutrients such as minerals, amino acids, fatty acids, vitamins and growth factors (Zhou *et al.*, 2004). However, increasing demand, limited and unstable supply and high cost of fish meal with the expansion of aquaculture make it necessary to find out alternative protein sources (Pham *et al.*, 2008; Lim *et al.*, 2011). Plant by-products are considered as best alternative protein and energy sources for fish growth (Gatlin *et al.*, 2007; Hussain *et al.*, 2011b; Hussain *et al.*, 2011c) and for the development of cost effective and environment friendly aqua feeds (Cheng and Hardy, 2002; Hussain *et al.*, 2011d). Sunflower meal is considered as most promising alternative to fishmeal and an economical source of important nutrients. In all over the world, it is the fourth largest source of protein contents after soybean meal, cottonseed meal and canola meal (Anjum *et al.*, 2014). It has approximately 40% protein content that mainly depends on the oil extraction and dehulling process (Mushtaq *et al.*, 2006). However, these plant protein sources have anti-nutritional factors such as phytate which make their use limited in fish feed formulation. Phytic acid or phytate is a major storage form of phosphorus (P) and has up to 80% of total P present in plant seeds, which is practically not available to agastric or mono-gastric fish species (NRC, 1993). Due to negative charge of phosphate groups present in phytate, it chelates with many cations such as magnesium (Mg), calcium (Ca), copper (Cu), iron (Fe) and zinc (Zn) and form insoluble phytate-mineral complexes (Erdman, 1979). Other than minerals, it also chelates amino acids, fatty acids and proteins and makes them unavailable to fish. Moreover, it inhibits the activities of digestive enzymes including lipases, amylases and proteases. Hence, to make plant based fish diets successful, it is compulsory to hydrolyse the phytate to release the chelated nutrients and minerals available to fish.

Various efforts have been made to release phosphorous and other chelated minerals and nutrients from phytate (Hotz and Gibson, 2005) but the most excellent results were obtained by enzymatic hydrolysis of phytate through phytase supplementation (Silva *et al.*, 2005). Phytase, an enzyme, is being used as a supplement in plant based diets to hydrolyze the phytate complexes (Cao *et al.*, 2007; Lim and Lee, 2009). Vielma *et al.* (1998) reported that phytase supplementation increased the concentration of Ca, Mg, Mn and Zn in bone, plasma and the whole body of rainbow trout (Vielma *et al.*, 1998). Nwanna *et al.* (2005) also reported improved body mineralization in African Catfish (*Clarias gariepinus*) which had phytase supplemented diets as compare to control group. However, less information is available for the formulation of artificial feeds from plant proteins for commercially important stomachless fish species including *Cirrhinus mrigala* (Iqbal *et al.*, 2014). The objective of present study was to investigate the effects of phytase supplementation in sunflower meal based diet on minerals digestibility by *Cirrhinus mrigala* fingerlings. Findings from present study will be helpful in the development of cost effective and environment friendly feed for *Cirrhinus mrigala* fingerlings.

## Materials and methods

Present experiment was conducted in Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, Government College University, Faisalabad.

### *Fish and experimental conditions*

*Cirrhinus mrigala* fingerlings were purchased from Government Fish Seed Hatchery, Faisalabad. Fingerlings were allowed to acclimatize with experimental conditions in Fish Nutrition Laboratory for two weeks in V-shaped tanks specially designed for the collection of fecal material from water media. Fifteen fish were stocked in each tank. During acclimatization period, fingerlings were fed once daily to apparent satiation on the basal diet used in subsequent digestibility study (Allan and Rowland, 1992). Water quality parameters such as pH,

dissolved oxygen (DO) and electrical conductivity were monitored through pH meter (Jenway 3510), D.O. meter (Jenway 970) and electrical conductivity meter (HANNA: HI. 8633) respectively. Aeration was provided round-the-clock to all the tanks through capillary system.

#### *Feed ingredients and experimental diets*

Feed ingredients were bought from a commercial feed mill and analyzed for chemical composition by following standard methods of AOAC (1995) before making the test diet. Feed ingredients were ground and sieved to require particle size before incorporation with test diet (Table 1). All dry ingredients were mixed in electric mixer for 10-20 minutes, there-after fish oil was gradually added, while mixing constantly. Chromic oxide (1%) was also added as an inert marker in the diet to estimate the digestibility. Ten to fifteen percent water was slowly added to prepare suitable dough of each test diet and was further processed through lab extruder for making sinking pellets. Pellets of each test diet was sprayed with five graded levels of phytase (0, 500, 1000, 1500 and 2000 FTU kg<sup>-1</sup>) resulting in the formation of 5 test diets. Phytase solution was prepared by dissolving 2g of microbial phytase (Phyzyme® XP 10000 FTU g<sup>-1</sup>; Danisco Animal Nutrition, Fin-65101 Vaasa, Finland) in powder form into 1000 ml of distilled water (Robinson *et al.*, 2002). One unit of phytase activity (FTU) is defined as the enzyme activity that liberates 1 µmol of inorganic orthophosphate min<sup>-1</sup> at pH 5.5 and 37°C at a substrate concentration (sodium phosphate) of 5.1 µmol L<sup>-1</sup> (Engelen *et al.*, 1994).

#### *Feeding Protocol and Sample Collection*

*Cirrhinus mrigala* fingerlings were fed at the rate of 5% of live wet weight on their prescribed diets. For each test diet, three replicates were assigned with stocking density of fifteen fish in each. After the feeding session of two hours, the uneaten diet was drained out from each tank. Tanks were washed completely to remove the particles of diets and refilled with water. After that, the fingerlings were stocked again in tanks. The feces were collected from

the fecal collection tube of each tank. Care was taken to avoid breaking the thin fecal strings in order to minimize the nutrient leaching. Fecal material of each replicated treatment was dried in oven at 60°C, ground and stored for chemical analysis. The experiment was lasted for 60 days.

#### *Minerals analysis of feed and feces*

Diets and feces samples were digested in boiling nitric acid and perchloric acid mixture (2:1) by following standard methods (AOAC, 1995). After appropriate dilution, mineral contents (calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn) and manganese, Mn) were estimated using Atomic Absorption Spectrophotometer (Hitachi Polarized Atomic Absorption Spectrometer, Z-8200). Calibrated standards for mineral estimation were prepared from commercially available standards (AppliChem® GmbH Ottoweg4, DE-64291 Darmstadt, Germany). The estimation of sodium (Na) and potassium (K) was done through flame photometer (Jenway PFP-7, UK). Phosphorus (P) was analyzed calorimetrically (UV/VIS spectrophotometer) using ammonium molybdate as reagent at 720 nm absorbance through standard methods (AOAC, 1995). Chromic oxide contents in diets and feces were estimated after oxidation with molybdate reagent (Divakaran *et al.*, 2002) using a UV-VIS 2001 Spectrophotometer at 370nm absorbance.

#### *Calculation of apparent digestibility coefficient (ADC)*

Apparent minerals digestibility coefficients (ADC) of test diets were calculated by the formula reported in NRC (1993).

$$ADC\% = 100 - 100 \times \frac{\text{Percent marker in diet} \times \text{Percent mineral in feces}}{\text{Percent marker in feces} \times \text{Percent mineral in diet}}$$

#### *Statistical Analysis*

Resulting data were subjected to one-way analysis of variance (ANOVA) (Steel *et al.*, 1996). The differences among means were compared by Tukey's Honesty Significant Difference test and considered significant at  $p < 0.05$  (Snedecor and Cochran, 1991). The CoStat

computer software program (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analysis.

## Results

Analysed minerals composition (%) of reference and sunflower meal based test diets and feces is presented

in table 3 and 4, respectively. It was observed that phytase supplementation reduced the amount of minerals in fish feces. Minimum minerals contents in feces was observed at 1000 FTU kg<sup>-1</sup> phytase supplementation level. However, further higher levels of phytase supplementation were unable to cause any further decrease in mineral contents in feces.

**Table 1.** Ingredients composition (%) of reference and test diets (As fed basis).

Ingredients	Reference diet	Test diets
Fish meal	20.0	14.0
Wheat flour	24.0	16.8
Corn gluten 60%	20.0	14.0
Rice polish	25.0	17.5
Fish oil	7.0	4.9
Vitamin Premix	1.0	0.7
Minerals	1.0	0.7
Ascorbic acid	1.0	0.7
Chromic oxide	1.0	0.7
Sunflower meal	-	30.0

Minerals digestibility (%) of sunflower meal based test diets in response to phytase supplementation is presented in table 5. Phytase supplementation had also resulted in improved minerals digestibility as compared to control diet. Reduced mineral excretion through fish feces was probably due to the hydrolysis of phytate contents by the phytase enzyme supplementation and so more minerals were utilized by *C. mrigala* fingerlings. Maximum digestibility

values (%) of Ca, Cu, Zn, Na, Mg and P among treatments were observed at 1000 FTU kg<sup>-1</sup> phytase level. However, Fe and K showed their maximum absorption at 500 FTU kg<sup>-1</sup> and 1500 FTU kg<sup>-1</sup> phytase levels respectively which varied significantly from other test diets and reference diet. On the other hand Mn digestibility value at 1000 FTU kg<sup>-1</sup> level and 1500 FTU kg<sup>-1</sup> were statistically at par but was higher than phytase supplemented diets.

**Table 2.** Chemical composition (%) of feed ingredients (Dry matter basis).

Ingredients	Dry matter (%)	Crude Protein (%)	Crude Fat (%)	Crude Fiber (%)	Ash(%)	Carbohydrates (%)	Gross Energy kcalg <sup>-1</sup>
Fish meal	91.63	48.15	7.16	1.07	26.73	16.89	2.69
Wheat flour	92.45	10.10	2.35	2.65	2.08	82.82	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	13.54	12.70	10.18	51.23	3.33
Sunflower meal ingredient	93.80	41.93	3.74	1.97	10.83	37.99	3.54

## Discussion

It is well known that phytate chelates with minerals and makes them unavailable to fish by reducing their digestibility (Cao *et al.* 2007; Hussain *et al.*, 2011a). In the present study, phytase inclusion at the level of 1000 FTUkg<sup>-1</sup> efficiently increased mineral contents

in *Cirrhinus mrigala* fingerlings. These findings are parallel with Yan and Reigh (2002) who determined that phytase inclusion at a level of 1000 FTU kg<sup>-1</sup> diet was sufficient to increase Ca, Mg and Mn contents in channel catfish. However, according to (Baruah *et al.*, 2005) dietary supplementation of microbial phytase

at 500 FTUkg<sup>-1</sup> in *Labeo rohita* (Hamilton) juveniles diet significantly improved bone Na, Ca, K, Mn and Fe content by 15, 12.1, 17.4, 20.4 and 40.7%, respectively. Yu and Wang (2000) and Gao *et al*

(2006) also found that in soybean meal based diet for crucian carp, 60% and 80% of phytate-P can be released by the phytase addition of 500 and 1000 FTU kg<sup>-1</sup> respectively.

**Table 3.** Analyzed minerals composition (%) in reference and test diets of *Cirrhinus mrigala* fingerlings fed on sunflower meal based test diets with graded levels of phytase Data are means of three replicates.

Diets	Phytase Levels FTU kg <sup>-1</sup>	Ca (%)	Fe (%)	K (%)	Cu (%)	Mn (%)	Zn (%)	Na (%)	Mg (%)	P (%)
Reference Diet	-	0.39±0.015	0.12±0.001	1.45±0.020	0.12±0.002	0.09±0.002	0.17±0.001	1.24±0.010	0.09±0.006	3.24±0.015
Test Diet-I	0	0.30±0.012	0.10±0.001	1.23±0.015	0.09±0.001	0.07±0.002	0.13±0.002	1.14±0.005	0.08±0.001	2.85±0.006
Test Diet- II	500	0.29±0.006	0.10±0.002	1.22±0.015	0.09±0.001	0.07±0.001	0.13±0.006	1.13±0.010	0.08±0.001	2.84±0.015
Test Diet - III	1000	0.30±0.015	0.09±0.006	1.23±0.015	0.09±0.001	0.07±0.001	0.13±0.002	1.13±0.015	0.08±0.001	2.82±0.015
Test Diet - IV	1500	0.29±0.015	0.10±0.001	1.23±0.015	0.09±0.001	0.07±0.002	0.13±0.002	1.13±0.010	0.08±0.006	2.84±0.015
Test Diet-V	2000	0.29±0.020	0.09±0.001	1.23±0.015	0.09±0.001	0.07±0.001	0.13±0.001	1.13±0.011	0.08±0.002	2.84±0.025

**Table 4.** Analyzed mineral composition (%) in the feces of *Cirrhinus mrigala* fingerlings fed on sunflower meal based test diets.

Diets	Phytase Levels FTU kg <sup>-1</sup>	Ca (%)	Fe (%)	K (%)	Cu (%)	Mn (%)	Zn (%)	Na (%)	Mg (%)	P (%)
Reference Diet	-	0.17±0.021	0.06±0.007	0.72±0.030	0.06±0.003	0.05±0.007	0.06±0.005	0.59±0.040	0.05±0.002	1.77±0.153
Test Diet o I	0	0.13±0.015	0.05±0.002	0.63±0.047	0.04±0.002	0.05±0.002	0.06±0.003	0.52±0.030	0.05±0.003	1.60±0.032
Test Diet 500 II	500	0.10±0.012	0.03±0.002	0.55±0.030	0.04±0.001	0.04±0.002	0.05±0.002	0.52±0.025	0.05±0.002	1.240±0.085
Test Diet 1000 III	1000	0.07±0.015	0.04±0.002	0.50±0.026	0.03±0.004	0.04±0.003	0.05±0.004	0.32±0.030	0.04±0.002	0.89±0.072
Test Diet 1500 IV	1500	0.09±0.015	0.04±0.004	0.32±0.015	0.04±0.002	0.03±0.001	0.05±0.003	0.47±0.055	0.04±0.002	1.17±0.046
Test Diet 2000 V	2000	0.12±0.012	0.04±0.005	0.57±0.040	0.05±0.002	0.05±0.003	0.06±0.004	0.51±0.040	0.05±0.001	1.46±0.114

Data are means of three replicates.

In present study the maximum digestibility of Fe (67%) was observed at 500 FTU kg<sup>-1</sup> level. Baruah *et al.* (2007), Debnath *et al.* (2005), Ai *et al.* (2007) and Sardar *et al.* (2007) also found that supplementation of dietary microbial phytase at 500 FTUkg<sup>-1</sup> level improved the absorption of minerals such as Fe and other minerals such as Na, K, P, Mg, Mn. Gao *et al.* (2006) found that phytase supplementation between 500 and 1000 FTU kg<sup>-1</sup> levels had significantly increased phosphorous availability to fish and

reduced the phosphorous excretion through feces. However, according to (Baruah *et al.*, 2005) dietary supplementation of microbial phytase at 500 FTUkg<sup>-1</sup> in *Labeo rohita* (Hamilton) juveniles diet significantly improved bone Na, Ca, K, Mn and Fe content, respectively. The differences in optimal doses may be due to the variation in experimental species and types of major plant sources of the experimental diets.

**Table 5.** Mineral digestibility (%) in reference and sunflower meal based test diets.

Experimental diets	Phytase levels (FTU kg <sup>-1</sup> )	Ca (%)	Fe (%)	K (%)	Cu (%)	Mn (%)	Zn (%)	Na (%)	Mg (%)	P (%)
Reference Diet	-	61.30±3.95 <sup>cd</sup>	55.76±1.26 <sup>3<sup>c</sup></sup>	54.13±1.05 <sup>1<sup>cd</sup></sup>	54.77±3.53 <sup>1<sup>cd</sup></sup>	51.01±1.48 <sup>6<sup>b</sup></sup>	63.38±2.02 <sup>1<sup>bed</sup></sup>	56.68±2.18 <sup>5<sup>c</sup></sup>	50.36±2.21 <sup>0<sup>c</sup></sup>	51.69±3.88 <sup>0<sup>d</sup></sup>
Test Diet- I	0	58.53±2.94 <sup>d</sup>	50.91±3.39 <sup>0<sup>c</sup></sup>	49.76±2.59 <sup>9<sup>d</sup></sup>	52.87±1.85 <sup>6<sup>cd</sup></sup>	34.50±2.20 <sup>0<sup>d</sup></sup>	57.43±3.59 <sup>1<sup>d</sup></sup>	55.67±3.85 <sup>0<sup>c</sup></sup>	40.25±1.89 <sup>4<sup>d</sup></sup>	45.21±3.29 <sup>0<sup>d</sup></sup>
Test Diet -II	500	67.01±2.29 <sup>bc</sup>	69.61±3.99 <sup>4<sup>a</sup></sup>	59.02±1.67 <sup>7<sup>c</sup></sup>	60.20±3.83 <sup>9<sup>bc</sup></sup>	49.42±1.16 <sup>5<sup>b</sup></sup>	65.72±0.89 <sup>4<sup>abc</sup></sup>	58.03±2.52 <sup>8<sup>bc</sup></sup>	47.24±2.75 <sup>8<sup>c</sup></sup>	59.90±2.50 <sup>4<sup>bc</sup></sup>
Test Diet -III	1000	80.92±2.6 <sup>6<sup>a</sup></sup>	66.85±3.91 <sup>8<sup>ab</sup></sup>	68.20±3.8 <sup>08<sup>b</sup></sup>	71.63±1.259 <sup>a</sup>	60.14±2.85 <sup>5<sup>a</sup></sup>	72.76±2.197 <sup>a</sup>	77.85±3.05 <sup>9<sup>a</sup></sup>	65.71±3.757 <sup>a</sup>	75.42±3.20 <sup>2<sup>a</sup></sup>
Test Diet-IV	1500	72.88±3.0 <sup>0<sup>ab</sup></sup>	65.30±2.4 <sup>67<sup>ab</sup></sup>	77.38±2.35 <sup>7<sup>a</sup></sup>	63.84±3.86 <sup>0<sup>ab</sup></sup>	64.35±2.06 <sup>1<sup>a</sup></sup>	67.97±2.85 <sup>3<sup>ab</sup></sup>	64.47±3.44 <sup>8<sup>b</sup></sup>	57.73±1.976 <sup>b</sup>	64.71±3.115 <sup>0<sup>b</sup></sup>
Test Diet -V	2000	65.30±2.5 <sup>0<sup>bed</sup></sup>	59.51±3.28 <sup>4<sup>bc</sup></sup>	60.16±2.21 <sup>0<sup>c</sup></sup>	50.33 <sup>±1.190<sup>d</sup></sup>	42.71±1.280 <sup>c</sup>	59.72±3.69 <sup>7<sup>cd</sup></sup>	61.00±1.00 <sup>3<sup>bc</sup></sup>	46.16±1.25 <sup>8<sup>cd</sup></sup>	55.55±2.38 <sup>0<sup>c</sup></sup>

Means within rows having different superscripts are significantly different

Data are means of three replicates.

From the present work it is clear that due to the supplementation of phytase at optimum level of 1000 FTU Kg<sup>-1</sup> less minerals are discharge in aquatic environment thus this minimize the chances of eutrophication. Similar suggestions came from Baruah *et al.* (2004), Nwanna *et al.* (2005), Ashraf and Goda (2007), and Gabriel *et al.* (2007) that Phytase supplementation in plant based diets may prove very helpful in developing cost effective and environment friendly feed for *Cirrhinus mrigala* fingerlings by increasing minerals digestibility and minimize the nutrient discharge into aquatic environment.

## Conclusion

In conclusion, the present study provided sufficient evidences that 1000 FTU kg<sup>-1</sup> level of phytase supplementation had a significant effect on the mineral digestibility of *Cirrhinus mrigala* fingerlings fed on sunflower meal based diets. Phytase supplementation in plant based diets may decrease the need for supplementing minerals, which will reduce the cost of fish feed and mineral's discharge through feces into the aquatic ecosystem resulting in environment friendly aquaculture.

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