

## BIOLOGICAL EVALUATION OF LOCALLY PRODUCED RECOMBINANT PHYTASE IN BROILER CHICKS

F. Sabir<sup>1</sup>, M. Tayyab<sup>1\*</sup>, A. R. Awan<sup>1</sup>, B. Muneer<sup>2</sup>, A. S. Hashmi<sup>1</sup>, M. Wasim<sup>1</sup> and S. Firyal<sup>1</sup>

<sup>1</sup>Institute of Biochemistry & Biotechnology, University of Veterinary & Animal Sciences, Abdul Qadir Jillani (Outfall) road, Lahore, Punjab, Pakistan

<sup>2</sup> Institute of Industrial Biotechnology, Government College University, Lahore, Punjab, Pakistan

\*Correspondence Author Email: muhammad.tayyab@uvas.edu.pk

### ABSTRACT

Current study deals with the production of locally characterized recombinant thermostable phytase from *Thermotoganaphthophila* (PHY<sub>TN</sub>) and its biological evaluation in poultry broiler chicks. The PHY<sub>TN</sub> was produced from 16-liter LB medium using BL21 CodonPlus cells having pET 21a containing phytase gene from *Thermotoganaphthophila*. A total of 135 birds were divided into 5 groups each having 27 birds. Group A served as negative control, group B, C and D were given feed supplemented with 500, 1000 and 1500 IU/kg of locally produced recombinant phytase while group E served as positive control. The results depicted that locally produced recombinant phytase showed significant effect on bird's weight gain, feed intake and feed efficiency ratio. Presence of 1000 IU/kg of phytase resulted in the weight gain in 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> week of trial from 504.77±1 to 533.53±1.4, 767.9±2 to 823.73±2.84 and 999.8±7.66 to 1122.8±2g respectively when compared with the control. The group B, C and D showed an average feed intake of 2710, 2780 and 2790 g as compared to 2669 g for group A. The supplementation of 1000 IU/kg of locally produced phytase in feed resulted in the improvement of feed conversion ratio from 2.6 to 2.4. The study demonstrated that PHY<sub>TN</sub> is suitable for poultry feed industry and its domestic production will contribute in the economic availability of locally produced phytase for the poultry feed industry.

**Key Words:** PHY<sub>TN</sub>, Poultry Trial, Recombinant Thermostable Phytase, *Thermotoganaphthophila*, Phytase

### INTRODUCTION

Phytate is the principle storage form of phosphorus in cereals, legumes and seeds (Reddy *et al.*, 1989). It is also called as anti-nutritional factor as it chelates the essential metal ions and minerals from the animal body (Andritotis and Ross, 2003). Monogastric animals do not have the ability to utilize phytate as phosphorus source due to non-availability of phytase enzyme responsible for the release of free phosphorus from phytate (Lie and Porres, 2003). To overcome phosphorus deficiency, the feed is being supplemented with inorganic phosphorus for sustained growth of mono-gastric animals. This increases the production cost due to high prices of inorganic phosphorus in the market. The undigested phytate is excreted as such in the faeces and cause environmental problems in the livestock intensive areas (Jongbloed *et al.*, 1992; Kornegay, 2001). Phytate also hinders the digestion and absorption of amino acids in the animal gut and act as anti-nutritional factor (Khan and Ghosh 2012). Many European countries have passed legislation to reduce the amount of inorganic phosphorus in animal feed (Kim *et al.*, 2006).

Phytase is a unique type of phosphatase, which hydrolyze phytate specifically and release inorganic phosphorus (Dvorakova, 1998). Phytases have been

characterized from plants, bacteria & fungi and are being used for supplementation in monogastric diets. The feed pelleting process takes place at 90°C that denature the mesophilic phytases. Thermostable phytases can withstand harsh conditions of feed palletization and are the ideal candidates to be used at industrial scale. A large number of such phytases have been characterized from various bacterial (Bawaneet *et al.*, 2011; Bohm *et al.*, 2010; Nugeet *et al.*, 2014; Rodriguez *et al.*, 1999) and fungal species (Mishra and Tiwari, 2013; Mullaney *et al.*, 2000; Wyss *et al.*, 1999) but still none of the phytase have been reported which can fulfill the criteria of "ideal phytase" as suggested by Lie and Porres (2003), being resistance to high temperature, catalytically active at wide range of temperature and cheap. That's why there is an ongoing surge to characterize a phytase, that can meet the ever increasing demand of industry.

Inclusion of phytase in animal feed effectively improved the animal phytate utilization and resulted in enhancement of weight gain and feed consumption. The effective hydrolysis of phytate by the exogenous phytase also increases the mineral utilization by the animals (Viveros *et al.*, 2002). Supplementation of phytase significantly improves the nutrient utilization by the monogastric animals (Liu *et al.*, 2014).

This study was designed with the aim to characterize a phytase suitable for poultry feed industry. The gene from *Thermotoganaphthophila*, a

hyperthermophile, was cloned in pET21a and expression was studied using BL21 CodonPlus cells as host. Large scale production of recombinant phytase was carried out. To check the efficacy of recombinant thermostable phytase, feed containing different concentration of the phytase was prepared and feeding trial on broiler chicks was performed.

## MATERIALS AND METHODS

**Production of recombinant phytase:** Recombinant BL21 CodonPlus cells having pET21a containing phytase gene from *Thermotoga naphthophila* were available in the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore were utilized for the production of recombinant phytase. The production of recombinant phytase was carried out under pre-optimized conditions (unpublished data). The overnight grown cells were diluted to 100 times with LB medium followed by incubation at 37°C. The cells were induced with isopropyl  $\beta$ -D-1-galactopyranoside at a final concentration of 1.4 mM, when OD reached to 0.4. The cells were further incubated for 6h at same temperature and were harvested and re-suspended in 50 mM acetate buffer pH 6 (Tayyab *et al.*, 2011)

**Cell disruption and activity assay:** The cells were lysed through sonication using ultrasonic processor (Sonics, Newtown USA). The sonicated material was centrifuged, supernatant was shifted to fresh tube and was utilized to examine the phytase activity.

PHY<sub>TN</sub> activity was measured using a modified ferrous sulfate-molybdenum blue assay (Zhang *et al.*, 2010). To examine the enzyme activity, 25  $\mu$ L enzyme solution was incubated with 475  $\mu$ L of 5mM sodium phytate in 50mM acetate buffer (pH 6.0) at 80°C for 15min. The enzyme reaction was subsequently terminated by the addition of 500  $\mu$ L of 10% (w/v) trichloroacetic acid. The released phosphate was measured at 700 nm after adding 1000  $\mu$ L of freshly prepared coloring reagent [1% (w/v) ammonium molybdate, 3.2% (v/v) sulfuric acid solution, and 7.2% (w/v) ferrous sulfate solution]. One unit of phytase activity was defined as the amount of enzyme needed to liberate 1  $\mu$ mol phosphate per min under the assay conditions. Standard curve was prepared using solutions of various concentrations of K<sub>2</sub>HPO<sub>4</sub> and was utilized for the estimation of PHY<sub>TN</sub> activity.

**Experimental Ration Formulation:** The soluble portion obtained after sonication was utilized for the supplementation in poultry feed. The feed was prepared in an automated unit available at Crescent Feed and Allied Products, Shadman, Lahore, Punjab, Pakistan. The composition of the feed was same as being utilized commercially for the poultry farmers (Table 1). Five different rations designated as A, B, C, D and E were

formulated (Table 1). The ration A was lacking phytase and served as negative control whereas ration B, C and D were supplemented with 500, 1000 and 1500 IU/kg of locally produced recombinant phytase. Ration E, served as positive control and was supplemented with 1000 IU/kg of commercially available phytase namely “Phyzyme” by Danisco Animal Nutrition (Marlborough, Wiltshire, United Kingdom).

**Feeding trial on broiler chicks:** For feeding trial, a total of 135 one-day-old broiler chicks were purchased from the local market. The birds were divided into 5 groups consisting of 27 birds. Each group was subdivided into 03 replicates having 9 birds each. Group 1 was considered as negative control and was fed with ration A. Group 2, 3 and 4 were fed with rations B, C and D (supplemented with locally produced phytase). The Group 5 (considered as positive control) was fed on ration E. The feeding trial was conducted in a room with controlled conditions at poultry farm in collaboration with the Asia Poultry Feed (Pvt.) Ltd, Lahore, Punjab, Pakistan. Water and feed were offered *ad libitum* for a period of five weeks. During feeding trial all prophylactic measures were adopted and proper vaccination were made to keep the birds healthy. The birds were weighed initially and thereafter weekly. The feed intake was recorded by each replicate during the trial. The feed conversion ratio was calculated by the formula, FCR = Feed intake/weight gain.

The collected data were analyzed through One Way Analysis of Variance (ANOVA). The obtained results were expressed as mean  $\pm$  standard deviation with values having  $P < 0.05$  was considered as significant (Khemakhem *et al.*, 2012).

## RESULTS AND DISCUSSION

Poultry trial demonstrated that the supplementation of phytase in poultry feed resulted in increased feed consumption, weight gain and improved FCR. The weight gain data obtained for the first two weeks demonstrated the same behavior for control as well as for the treatment. The feed supplemented with 500 IU/kg of PHY<sub>TN</sub> showed a growth enhancing effect on poultry birds in the 4<sup>th</sup> and 5<sup>th</sup> weeks of trial. Whereas when the birds were fed on feed having 1000 IU/kg of PHY<sub>TN</sub>, a significant effect in bird weight gain was recorded at the end of 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> week of trial (Table 2). A comparison of group A and group C in the 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> week clearly demonstrated the weight gain from 504.766 to 533.535g, 767.933 to 823.733g and 999.833 to 1120.277g respectively when the feed was supplemented with 1000 IU/kg of locally produced PHY<sub>TN</sub> (Table 2). These results are in agreement with the previous report (Santos *et al.*, 2013; Scheideler and Ferket, 2000). The increase in PHY<sub>TN</sub> units from 1000 (group C) to 1500 (group D) showed almost same effect

on weight gain and feed consumption. The observed response is might be due to reach a plateau at 1000 units of PHY<sub>TN</sub>. The same pattern was recorded by Kornegay *et al.*, (1996), which observed a plateau at much lower phytase concentration (600 IU/kg of feed). The supplementation of PHY<sub>TN</sub> in group B, C and D resulted significantly increase in feed consumption i.e. 2710, 2780 and 2790 g respectively as compared to Group A (negative control) consumed 2660 g of feed during 35 days feeding trial (Table 3). Similarly, the FCR values 2.6 of Group A and B didn't differ significantly but both of them were found significantly inferior to FCR values of 2.4 attained by C, D and E groups (Table 3).

The supplementation of feed with 1000 IU/kg of PHY<sub>TN</sub> clearly showed a significant effect on bird feed

consumption (2660 to 2780 g) and weight gain (999.83 to 1122.8 g), similarly this supplementation improved the FCR from 2.66 (group A) to 2.48 (group C). The positive control (group E) demonstrated the same behavior on feed consumption [2660 (group A) to 2920 (group E)], weight gain [999.83 (group A) to 1207.566 g (group E)] and FCR [2.66 to 2.42]. The PHY<sub>TN</sub> (group C) and commercial phytase (group E) both showed significant effect on broiler feed consumption, body weight gain and in the improvement of FCR.

The local production of recombinant thermostable phytase will be beneficial for the feed industry of Pakistan. Local production of phytase will result in cheaper availability of phytase and this will save a huge foreign exchange for the import of phytase.

**Table 1. Ingredient composition of experimental rations (%).**

	A	B	C	D	E
Corn 86	58.0	58.0	58.0	58.0	58.0
Rice Polish 14	7.50	7.50	7.50	7.50	7.50
SBM 46	29.5	29.5	29.5	29.5	29.5
Oil	2.00	2.00	2.00	2.00	2.00
CaCO <sub>3</sub>	0.95	0.95	0.95	0.95	0.95
Bone Ash	0.75	0.75	0.75	0.75	0.75
DCP	0.50	0.50	0.50	0.50	0.50
L-HCl	0.25	0.25	0.25	0.25	0.25
DLM	0.15	0.15	0.15	0.15	0.15
NaHCO <sub>3</sub>	0.17	0.17	0.17	0.17	0.17
Vitamin Premix	0.30	0.30	0.30	0.30	0.30
Phytase (IU)	000	500	1000	1500	1000

**Table 2. Effect of PHY<sub>TN</sub> supplementation on growth performance of poultry bird.**

Groups	Week 1 (P= .0959)	Week 2 P=(0.1803)	Week3 P=(.0000 ***)	Week 4 P=(.0000 **1	Week 5 P=(.0000 ***)
A (-ve Control)	118 <sup>ab</sup> ±0.46	261.67 <sup>a</sup> ±0.60	504.77 <sup>c</sup> ±1.0	767.9 <sup>d</sup> ±2	999.8 <sup>d</sup> ±7.66
B (500IU/Kg)	118 <sup>ab</sup> ±0.60	261 <sup>a</sup> ±0.40	507.30 <sup>c</sup> ±0.7	776.17 <sup>c</sup> ±0.79	1013 <sup>c</sup> ±2
C (1000IU/Kg)	119.17 <sup>a</sup> ±0.6	261 <sup>a</sup> ±0.82	533.53 <sup>b</sup> ±1.4	823.73 <sup>b</sup> ±2.84	1122.8 <sup>b</sup> ±2
D (1500IU/Kg)	117 <sup>b</sup> ±0.6	262 <sup>a</sup> ±0.41	534.9 <sup>b</sup> ±2.64	822.6 <sup>b</sup> ±2.69	1123.37 <sup>b</sup> ±2.9
E (1000IU/Kg) (Phyzyme)	118 <sup>ab</sup> ±0.6	262.9 <sup>a</sup> ±1.36	540.8 <sup>a</sup> ±1	838 <sup>a</sup> ±1.98	1207.57 <sup>a</sup> ±3.6

a-d values in the table differ significantly from each other

**Table 3. Efficacy of PHY<sub>TN</sub> in weight gain, feed intake and feed efficiency ratio**

Group	A	B	C	D	E
Overall weight gain (g)	999.8	1013.2	1122.8	1123.4	1207.6
Average feed intake (g)	2660	2710	2780	2790	2920
FCR	2.6	2.6	2.4	2.4	2.4

**Author's contributions:** Project concept and design: MT (35%), experimental design FS (35%), data analysis ARA, ASH, BM, MW & SF (30%). All authors read and approved the final manuscript.

**Acknowledgement:** The authors are thankful to the Higher Education Commission of Pakistan for providing funds.

## REFERENCES

- Andriotis, V.M. and J.D. Ross (2003). Isolation and characterisation of phytase from dormant *Corylusavellana* seeds. *Phytochemistry*, 64 (3): 689-699.
- Bawane, R., K. Tantai, L.P.S. Rajput, K.M. Bedekar, S. Kumar, I. Gontia and S. Tiwari (2011). Molecular Analysis of Phytase Gene Cloned from *Bacillus subtilis*. *Adv. Stud. Biol.* 3(3): 103-110.
- Bohm, K., T. Herter, J.J. Müller, R. Borris and U. Heinemann(2010). Crystal structure of *Klebsiella* sp. ASR1 phytase suggests substrate binding to a preformed active site that meets the requirements of a plant rhizosphere enzyme. *FEBS. J.* 277(5): 1284-1296.
- Dvorakova, J. (1998). Phytase: Sources, Preparation and Exploitation. *Folia Microbiol.* 43: 323-338.
- Jongbloed, A.W., Z. Mroz and P.A. Kemme(1992). The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *J. Anim. Sci.* 70:1159–1168.
- Khan, A. and K. Ghosh (2012). Characterization and identification of gut-associated phytase-producing bacteria in some fresh water fish cultured in ponds. *Acta. Ichthyol. Piscat.* 42(1):37–45.
- Khemakhem, A.F., M.B.Farhat, I.Boukhris, W.Bejar, K.Bouchaala, R.Kammoun, E. Maguin, S. Bejar and H.Chouayekh (2012). Heterologous expression and optimization using experimental designs allowed highly efficient production of the PHY US417 phytase in *Bacillus subtilis* 168. *A.M.B. Express*, 2:10.
- Kim, T., E.J. Mullaney, J.M. Porres, K.R. Roneker, S. Crowe, S. Rice, T. Ko, A.H.J. Ullah, C.B. Daly, R. Welch and X.G. Lei (2006). Shifting the pH profile of *Aspergillus niger* phytase to match the stomach pH enhances its effectiveness as an animal feed additive. *App. Env. Microbiol.* 72(6): 4397-4403.
- Kornegay, E., D. Denbow, Z. Yi and V. Ravindran(1996). Response of broilers to graded levels of microbial phytase added to maize–soyabean-meal-based diets containing three levels of non-phytate phosphorus. *Br. J.Nutr.* 75(06): 839-852.
- Kornegay, E. (2001). Digestion of phosphorus and other nutrients: the role of phytases and factors influencing their activity. In *Enzyme in Farm Animal Nutrition*. 237-271.
- Lei, X.G., and J.M.Porres (2003) Phytase enzymology, applications, and biotechnology. *Biotech. Lett.* 25: 1787-1794.
- Liu, S.Y., D.J. Cadogan, A. Peron, H.H. Truong and P.H. Selle (2014). Effects of phytase supplementation on growth performance, nutrient utilization and digestive dynamics of starch and protein in broiler chickens offered maize-, sorghum-and wheat-based diets. *Anim. Feed Sci. Technol.* 197, 164-175.
- Mishra, I.G. and S. Tiwari(2013). Molecular Characterization and Comparative Phylogenetic Analysis of Phytases from Fungi with Their Prospective Applications. *Food Technol. Biotechnol.* 51 (3): 313–326.
- Mullaney, E.J., C.B. Daly, K. Sethumadhavan, E. Rodriguez, X.G. Lei and A.H. Ullah(2000). Phytase activity in *Aspergillus fumigatus* isolates. *Biochem. Biophys. Res. Commun.* 275: 759–763.
- Nuge, T., Y.Z.H. Hashim, A.A. Farouk and H.M. Salleh(2014). Cloning and Expression of a Novel Phytase Gene (phyMS) from *Mycobacterium smegmatis*. *Adv. Enz. Res.* 2: 27-38.
- Reddy, N.R., M.D. Pierson, S.K. Sathe and D.K. Salunkhe(1989). Occurrence, distribution, content and dietary intake of phytate. In N.R.Reddy, M.D. Pierson, S.K. Sathe and D.K. Salunkhe (eds.) *Phytate in cereals and legumes*. CRC Press, Boca Raton, FL, USA. Pp. 39-56.
- Rodriguez, E., Y. Han and X.G. Lei(1999). Cloning, sequencing, and expression of an *Escherichia coli* acid phosphatase/phytase gene (appA2) isolated from pig colon. *Biochem. Biophys. Res. Commun.* 257: 117–123.
- Santos, D.T.T., S. Srinongkote, M.R. Bedford and C.L. Walk(2013). Effect of high phytase inclusion rates on performance of broilers fed diets not severely limited in available phosphorus. *Asian-Australas. J. Anim. Sci.* 26:227–232.
- Scheideler, S.E. and P.R. Ferket (2000). Phytase in broiler rations-effects on carcass yields and incidence of Tribal Dyschondroplasia. *J. App.Poul. Res.* 9(4):468-475.
- Tayyab, M., N. Rashid and M. Akhtar (2011). Isolation and identification of lipase producing thermophilic *Geobacillus* ssp. SBS-4S:cloning and characterization of lipase. *J. Biosci. Bioeng.* 111(3):272-278.
- Viveros, A., A. Brenes, I. Arijia and C. Centeno (2002). Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. *Poultry Science*, 81(8), 1172-1183.

- Wyss, M., R. Brugger, A. Kronenberger, R. Remy, R. Fimbel, G. Oesterheld, M. Lehmann and A.P. Van (1999). Biochemical characterization of fungal phytases (myo-inositolhexakisphosphatephosphohydrolases): catalytic prop-erties. Appl. Environ. Microbiol. 65: 367–373.
- Zhang, G.Q., X.F. Dong, Z.H. Wang, Q. Zhang, H.X. Wang and J.M. Tong (2010). Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from *Aspergillus ficuum* NTG-23. Biosci. Rep. 101(11): 4125–4131.