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Characterization of Recombinant Thermostable Phytase from *Thermotoga naphthophila*: A Step for the Fulfilment of Domestic Requirement of Phytase in Pakistan

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Authors' Contribution
FS did experimental work. MT planned and supervised the study and guided for manuscript write-up and editing. ASH, NR and SF facilitated the student for the conduction of experiments. ARA, BM and MW helped during writing the manuscript.

Key words
PHYTN, Thermotoga naphthophila, Recombinant phytase, Poultry feed, Phytate.

ABSTRACT

Supplementation of feed with phytase is the most suitable strategy for the availability of free phosphorus for the growth of monogastric poultry birds. Current study deals with the production and characterization of recombinant thermostable phytase from *Thermotoga naphthophila* (PHY_{TN})**.** This study was an initial step for the fulfilment of domestic industrial requirement of phytase in Pakistan. The PCR resulted in the amplification of 1.8 kb phytase gene. SDS-PAGE confirmed the size of recombinant protein as 70 kDa. The optimization studies demonstrated the maximal production of recombinant phytase, when the recombinant cells were induced with 1.4 mM IPTG with the post induction time of 6 hours. PHY_{TN} showed maximal activity at 80°C in 50 mM sodium acetate buffer pH 6. Presence of Fe^{3+} or Cu^{2+} showed an enhancing effect on the PHY_{TN} activity. Thermostability studies demonstrated that PHY_{TN} retains 88% residual activity when the protein was incubated at 80°C for 1.5 h in the presence of 1.5 mM Fe^{3+}. The enzyme exhibited K_m and V_{max} values of 50 mM and 2500 µmole/min respectively when sodium phytate was used as substrate. The stability of enzyme at a wide range of temperature and pH makes it a potential candidate to be used in the poultry feed industry.

INTRODUCTION

Phytate (myo-inositole hexakisphosphate) is the storage form of phosphorus in plants especially in cereal, legumes and oilseeds which are being used as major component of animal feed (Reddy *et al*., 1982). Monogastric animals like poultry, pigs and humans doesn't have ability to hydrolyze phytate (Lie and Porres, 2003) present in their feed and passed it as such in their manure which results in the phosphorus pollution and water eutrophication in periphery of intensive livestock population area (Kim *et al*., 2006). Due to presence of six reactive phosphate groups, phytate chelates important minerals such as Fe^{3+}, Ca^{2+}, Zn^{2+} and Mg^{2+} (Andritotis and Ross, 2003) or form irreversible complexes with amino acids or proteins and make digestive enzymes ineffective (Pallauf and Rimbach, 1996). Addition of inorganic phosphorus in the animal feed was a common practice being performed for the fulfillment of the phosphorus requirement that increases the overall cost and contribute to environmental pollution (Kim *et al*., 2006).

Supplementation of poultry feed with phytase results in the hydrolysis of phytate and the availability of free phosphorus for the growth of monogastric animals (Dvorakova, 1998). Production of phytases have been reported from bacteria (Bawane *et al*., 2011; Bohm *et al*., 2010; Nuge *et al*., 2014; Rodriguez *et al*., 1999), fungi (Mishra and Tiwari, 2013; Mullaney *et al*., 2000; Wyss *et al*., 1999), plants (Zhang *et al*., 2013) and animals (Cho *et al*., 2006). Microbial phytases are being preferred on plants or animals due to the ease in production. The poultry feed palletization process takes place at high temperature where most of the mesophilic enzymes become denatured, so there is need of thermostable phytase that can withstand high temperature of palletization.

Current study involves the domestic production of

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recombinant thermostable phytase from *Thermotoga naphthophila*. This locally produced phytase was purified and characterized. The phytase was also utilized to examine its efficacy in poultry birds (not part of this studies) with the aim to fulfill the local industrial demand of phytase in Pakistan and to save the huge foreign exchange for the import of phytase.

MATERIALS AND METHODS

Cloning of phytase gene

The genomic DNA of *Theromotoga naphthophila* was purchased from Leibniz institute DSMZ-German collection of microorganisms and cell cultures, Germany. The phytase encoding gene was amplified using PHY-TN-F (ATTGAGACATATGGAGTCAAAACCC) and PHY-TN-R (ATTACTAAAGCTTACTCTTCTGCCTTC) as forward and reverse primers, respectively. The forward primer contained the unique restriction site of *Nde*I. The amplified PCR product was purified using DNA extraction kit (Thermoscientific, Life Sciences, USA) and the purified PCR product was ligated in pTZ57R/T using T_4 DNA ligase (Thermoscientific, Life Sciences, USA). The ligated vector was utilized for the transformation of *E.coli* DH5α competent cells and positive clones were selected on the basis of blue white screening (Sambrook and Russel, 2001). The plasmid DNA was isolated and the presence of insert in the vector was confirmed by single and double digestion using *Nde*I and *Hind*III.

Expression studies of recombinant phytase

The phytase gene was subcloned in the pET21a and was utilized for the transformation of BL21 CodonPlus cells. Overnight grown culture of transformed BL21 CodonPlus cells were diluted to 1% with fresh LB media and was incubated at 37°C till the OD reached 0.4. The cells were induced with 1mM IPTG followed by further incubation at 37°C for 4.5h. The cells were harvested by centrifugation, resuspended in 50mM sodium acetate buffer pH 6 and were lysed by sonication using ultrasonic processor (Sonics, Newtorn, USA). The soluble part after lysis was incubated at 80°C for 1h in the water bath. The production of recombinant protein was examined by analyzing the heat treated samples through SDS-PAGE (Lamilli, 1970).

Optimization for the production of recombinant phytase

Maximal production of PHY_{TN} was examined by varying the IPTG concentration from 0.1 to 1.5mM and post induction incubation time from 1 to 10h. The sample were withdrawn after every 1 h and the production was analyzed on SDS-PAGE.

Purification of recombinant phytase

The recombinant protein was purified using various chromatographic techniques. The samples after heat treatment was applied on pre equilibrated DEAE sephadex A-50 column using 50 mM sodium acetate buffer pH 6. The unbound proteins were washed away and the elution of bound protein was done with the NaCl gradient in the same buffer. The fractions after ion exchange chromatography were examined by SDS-PAGE. The partially purified fractions with phytase activity were pooled and was loaded to pre equilibrated Sephadex G-50 column using same buffer. The purity of the fraction was analyzed through SDS-PAGE.

Activity assay

Phytase activity was measured using a modified ferrous sulfate-molybdenum blue assay (Zhang *et al*., 2010). The 25 µl enzyme solution was incubated with 475 µl of sodium phytate (5mM) in 50mM acetate buffer (pH 6.0) at 80°C for 15 min. The enzyme reaction was subsequently terminated by the addition of 500 µl of 10% (w/v) trichloroacetic acid. The released phosphate was measured at 700 nm after adding 1000 µl 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 required to liberate 1 µmol phosphate per min under the assay conditions. Phytase activity units were calculated using standard curve of K_2HPO_4.

Effect of temperature on PHY_{TN} activity

Effect of temperature was analyzed by examining the PHY_{TN} activity at various temperature ranging from 40 to 100°C. Regarding the thermostability studies, recombinant protein was incubated at 80°C in water bath in the presence or absence of metal ions. The samples were withdrawn after every 10 min and were utilized to examine the residual activity (Ali and Taj, 2016).

Effect of pH on PHY_{TN} activity

The activity was examined at various pH ranging from 2 to 9 using 50 mM of each of Glycine-HCl buffer (2-4), sodium acetate buffer (4-7) and Tris HCl buffer (7-9). The activity was recorded and the data was utilized to find out the optimum pH.

Effect of metal ion on PHY_{TN} activity

The activity was examined in the presence of 1mM of each of EDTA, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Ca^{2+} and Zn^{2+}.

Effect of substrate concentration

PHY_{TN} activity was observed by increasing the

substrate concentration from 1 to 20 mM. The obtained data was utilized to plot the Lineweaver-Burk plot and for the estimation of the kinetic parameters.

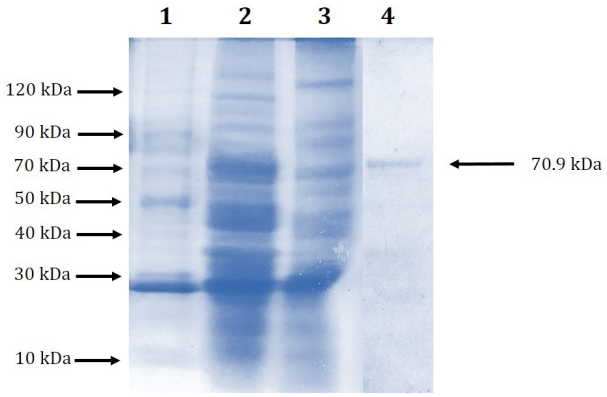

Fig. 1. Comassie brilliant blue stained acrylamide gel showing purification of phytase. Lane 1, molecular weight protein ladder (NOVEX, Life Technolgies); Lane 2, soluble portion after lysis of cells having PHY-pET; Lane 3, negative control (soluble portion after lysis of cells containing pET21a without insert); Lane 4, purified PHY_{TN}.

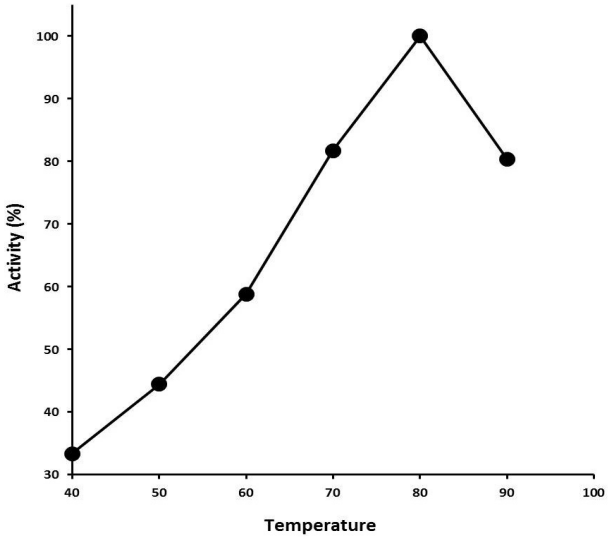

Fig. 2. Effect of temperature on PHY_{TN} activity. Activity assay was performed at various temperature ranging 40 to 80 °C using 50 mM acetate buffer (pH 6) using 5 mM sodium phytate as substrate. X axis shows the temperature while Y axis shows the activity (%).

RESULTS

The PCR resulted in the amplification of 1.8 kb phytase gene. The presence of gene in recombinant vector was confirmed by restriction digestion. Single restriction with *Nde*I resulted in the linearization of the vector while the double digestion resulted in the liberation of the insert from recombinant vector. The deduced nucleotide sequence of phytase gene showed maximum homology of 46% with *Oryza sativa*, 45.5% with *Triticum aestivum* (Li *et al*., 2013), 43.4% with *Aspergillus fumigatus* (Suresh and Das, 2014), 35.12% with *Glycine max* (Singh *et al*., 2013), 30.9% with *Bacillus subtilis* (Bawane *et al*., 2011) and 29.64% with *Obesumbacterium proteus* (Zinin *et al*., 2004).

SDS-PAGE determined the production of recombinant phytase as intracellular protein having molecular weight of 70 KDa (Fig. 1). The optimal PHY_{TN} production was observed when BL21 CodonPlus cells harboring pET21a containing phytase gene were induced with 1.4 mM IPTG with post induction incubation period of 6h at 37°C.

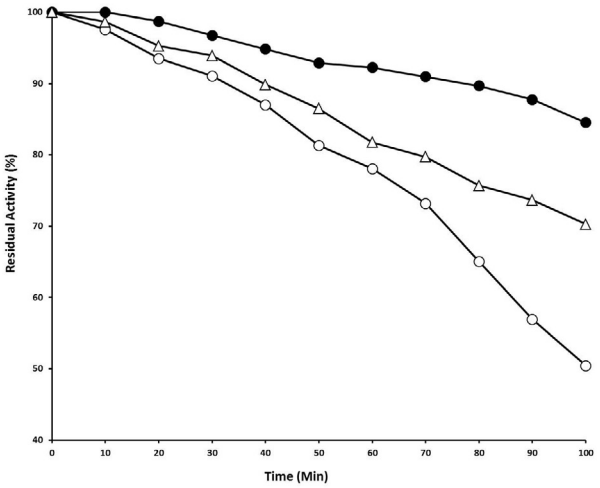

Fig. 3. Thermostability studies of phytase. The protein was incubated at 80 °C in the absence or presence of metal ions (Cu^{2+} or Fe^{3+}) separately in 50 mM acetate buffer at pH 6. Unfilled circles shows the stability in the absence of any metal ion, while filled circles and unfilled triangles shows the stability in the presence of Fe^{3+} and Cu^{2+}, respectively.

Increase in temperature from 40 to 80°C revealed a linear increase in PHY_{TN} activity. Maximal activity was recorded at 80°C while further increase in temperature resulted in the decreased activity (Fig. 2). The protein showed stability and retains 57% residual activity when the PHY_{TN} was incubated for 1.5 h at 80°C in the absence of metal ions (Fig. 3, unfilled circle). The incubation of protein in the presence of Cu^{2+} or Fe^{3+} was also studied as these ions were involved in the enhancement of PHY_{TN} activity. The presence of Cu^{2+} or Fe^{3+} showed a stabilizing effect on the PHY_{TN} structure. In the presence of Cu^{2+} (Fig.

3, unfilled triangle) or Fe^{3+} (Fig. 3, filled circle) the protein retained 74% or 88% residual activity respectively with an incubation of 1.5h at 80 °C. The PHY_{TN} activity was increased with the increase in pH from 2 to 6 with optimal activity between pH 6 to 7 in 50 mM sodium acetate buffer (Fig. 4). Further increase in pH showed a decline in activity.

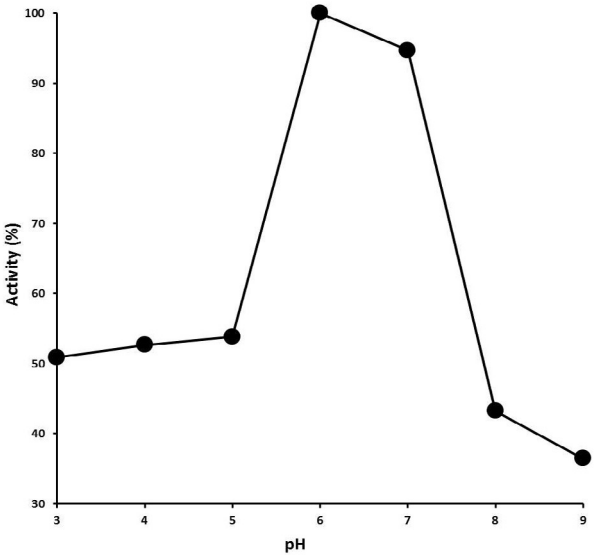

Fig. 4. Effect of pH on PHY_{TN} activity. Activity assay was performed as various pH using 50 mM of each, Glycine-HCl buffer (3-5), acetate buffer (5-6) and Tris-HCl Buffer (6-9) at 80°C using 5 mM substrate. X axis in figure shows pH whereas Y axis shows PHY_{TN} activity (%).

Abolishment of activity in the presence of 1mM EDTA confirmed the metal dependence of PHY_{TN}. Presence of Cu^{2+} or Fe^{3+} showed an enhancing effect on the PHY_{TN} activity when used at a final concentration of 1mM. 1.5 mM Fe^{3+} was recorded to be optimal concentration for the maximal activity of PHY_{TN}. Kinetic studies demonstrated the K_m and V_{max} of 50 mM and 2500 µmole/min, respectively (Fig. 5) when sodium phytate was used as substrate.

DISCUSSION

The study was conducted for the domestic production of recombinant thermostable phytase for the poultry feed industry of Pakistan. In the current study, the recombinant phytase was produced and characterized. Poultry trials were conducted to examine the efficacy of this phytase in poultry birds (not part of this study). The local production of recombinant thermostable phytase will be beneficial for the feed industry of Pakistan as this will result in cheaper availability of phytase in near future and will save a huge foreign exchange for the import of phytase.

The comparison of amino acid sequence of PHY_{TN} with the reported phytase showed that PHY_{TN} showed maximum identity of 46 and 45.5 % with various purple acid phosphatases *i.e. Oryaza sativa* and *Triticum aestivum,* respectively (Li *et al*., 2013). The PHY_{TN} sequence homology analysis with reported purple acid phosphatase demonstrated that PHY_{TN} didn't share various conserved domains "DXG and GDXXY" which are specialized for the incorporation of metal ion and for enzyme function (Hegeman and Grabau, 2001). The PHY_{TN} required Fe^{3+} as cofactor that is a unique characteristic of purple acid phosphatases (Dionisio *et al*., 2011). PHY_{TN} is a unique phytase as sequence similarity, dependence on iron for activity keep it close to purple acid phosphatase but the absence of conserved catalytic domains make this phytase unique.

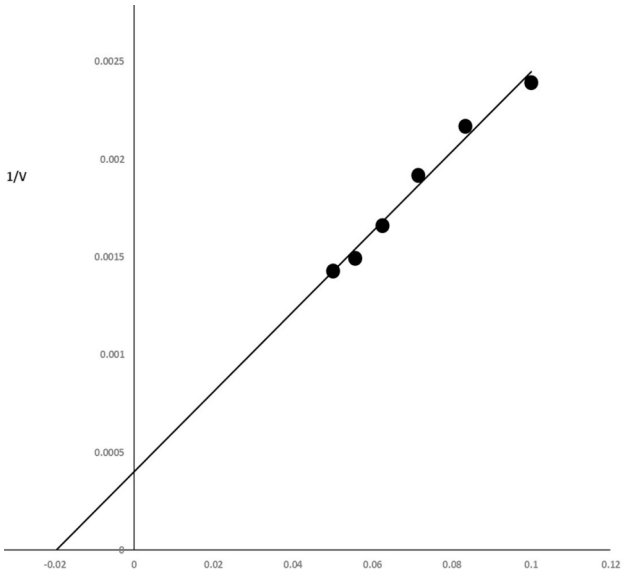

Fig. 5. Lineweaver-Burk plot. X axis shows the values for 1/substrate while Y axis shows the values for $1/V_0$. The plot was utilized to calculate the kinetic parameters.

PHY_{TN} showed optimal activity at 80°C which is higher as compared to 70°C for phytase from *Bacillus subtilis* (Lei *et al*., 2013) while the same behavior was shown by fungal phytase from *Aspergillus* sp. L117 (Lee *et al*., 2005). The PHY_{TN} showed maximum activity in the presence of 50 mM acetate buffer pH 6 that is different as compared to 4.5 for phosphatases from Glycine max while fungal phytase from *Aspergillus niger* while *Triticum aestivum* showed similar pH optima (Lei *et al*., 2013). PHY_{TN} is a metal dependent enzyme that require Cu^{2+}

or Fe^{3+} for its activity. Previous reports demonstrate that purple acid phosphatase from wheat, barley maize and rice also require Fe^{2+} for their activity (Dionisio *et al*., 2011). The presence of Mn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+} showed an inhibitory effect on the activity of PHY_{TN} (Table I). Similar behavior was reported for the activity of phytase from metagenome derived phytase (Tan *et al*., 2015) and a phytase from *Pseudomonas putida*, whose activity was also inhibited in the presence of Zn^{2+} (Dharmsthiti *et al*., 2005).

Table I.- Effect of Metal Ions on PHY_{TN} activity.

Divalent cation or EDTA or detergent	Relative activity (%)
None	100
EDTA	0
Metal Ions*	
Zn^{2+}	62
Mg^{2+}	75
Ca^{2+}	78
Mn^{2+}	80
Co^{2+}	86
Cu^{2+}	115
Fe^{3+}	158

*Metal chlorides were used in the activity assay.

Thermostability studies demonstrated that PHY_{TN} was found to be stable with 88% residual activity even after an incubation of 1.5 h at 80°C. This stability is higher as compared to reported phytase from *A. niger* (Wyss *et al*., 1998); whereas, the phytase from *Aspergillus* sp. L117 showed higher level of stability (Lee *et al*., 2005). It was examined that 1.5 mM Fe^{3+} concentration is required for the maximal activity of PHY_{TN} that is quite higher than 0.1 mM Fe^{3+} for optimal phytase activity from *Talaromyces thermophilus* (Singh and Satyanarayana, 2013).

Poultry trials demonstrated that the supplementation of PHY_{TN} in poultry feed resulted in increased feed consumption, weight gain and improved FCR as compared to negative control. The supplementation of feed with PHY_{TN} (1000 IU/kg) clearly exhibited a significant effect on bird feed consumption (2660 to 2780 g), weight gain (999.83 to 1122.8 g) and in the improvement of FCR from 2.66 to 2.48.

CONCLUSION

Current study deals with the successful characterization of recombinant thermostable phytase from *T. naphthophila*. The characterization studies demonstrated enzyme stability at a wide range of temperature and pH that makes it a potential candidate to be used in the poultry feed industry of Pakistan. However, experiments at large scale will be essential for the utilization of this phytase for poultry feed supplementation.

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Statement of conflict of interest

The authors have no conflict of interest.

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