

Cloning, Overexpression, and Characterization of a Metagenome-Derived Phytase with Optimal Activity at Low pH ^S

Hao Tan^{1,2}, Xiang Wu^{1,2}, Liyuan Xie^{1,2}, Zhongqian Huang^{1,2}, Bingcheng Gan^{1,2}, and Weihong Peng^{1,2*}

¹Institute of Soil and Fertilizer, Sichuan Academy of Agricultural Sciences, Chengdu 610066, P.R. China

²R&D Department, JINDI Agricultural Biotechnology Inc., Chengdu, P.R. China

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*Corresponding author

Phone: +86-28-84504712;

Fax: +86-28-84787971;

E-mail: pwh424@163.com

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A phytase gene was identified in a publicly available metagenome derived from subsurface groundwater, which was deduced to encode for a protein of the histidine acid phosphatase (HAP) family. The nucleotide sequence of the phytase gene was chemically synthesized and cloned, in order to further overexpress the phytase in *Escherichia coli*. Purified protein of the recombinant phytase demonstrated an activity for phytic acid of 298 ± 17 $\mu\text{mol P/min/mg}$, at the pH optimum of 2.0 with the temperature of 37°C. Interestingly, the pH optimum of this phytase is much lower in comparison with most HAP phytases known to date. It suggests that the phytase could possess improved adaptability to the low pH condition caused by the gastric acid in livestock and poultry stomachs.

Keywords: Phytic acid, phytase, metagenome, pH optimum, acidophilic

Phosphorus (P) is a key mineral element for the growth, reproduction, and metabolism of animals. In animal feed made from leguminous and cereal materials, a high proportion (40% to 70%) of the total P could be present in phytic acid (*myo*-inositol hexakisphosphate) [4, 25]. The phytic acid is a stable compound that is difficult to be chemically hydrolyzed, thereby releasing inorganic phosphate unless catalyzed by phytase, an enzyme that could cleave the multiple phosphoric ester bonds in phytic acid. Since most animals lack the ability to produce phytase in their guts, animal feed is usually supplemented with phytase. Owing to the presence of gastric acid, the stomach environment of most livestock and poultry is very likely to have a pH of below 3.0 [2, 5, 17–19, 22, 24]. The pH condition is too acidic for most phytases to work at their highest efficiency: the biochemical characteristics of the phytases reported to date showed that a majority of the histidine acid phosphatase (HAP) family phytases are likely to demonstrate the optimum catalytic efficiency around pH 4.0 to 5.5 [12, 13, 21, 33], whereas only a few derived mainly from the *Aspergillus* genus are adapted to

much lower pH (1.0–3.0) [9, 11, 14, 29, 30]. However, the activity of those acidophilic phytases (pH optimum <3.0) is usually not very ideal. For instance, a phytase derived from *A. ficuum* NTG-23 with a pH optimum of 1.3 [34] has an activity 18 times lower than the AppA phytase of *Escherichia coli* with a pH optimum of 4.5 [8, 32]. Thus, there is ongoing interest in the exploitation of novel phytases with desirable enzymatic properties, including improved adaptability to low pH condition [28]. In this study, a novel HAP phytase was obtained from a publicly available metagenome of subsurface groundwater and overexpressed in *E. coli*. The enzymatic properties of this phytase were characterized.

For this, the amino acid sequences of the conserved motifs possessed by HAP phytases (RHGX₂XP and HDTN) [20, 21] were used to manually search for putative HAP phytase genes among the publicly available metagenomes in the IMG\M database [15, 16]. A putative phytase gene consisting of 1,074 base pairs (bp) was identified in a metagenome derived from subsurface groundwater that was contaminated by coal-tar waste in the South Glens

Falls, NY, USA [10] (under the IMG Gene Accession ID of JGI1357J11328_100303543). The deduced peptide sequence of this putative phytase gene consists of 357 amino acids, containing typical conserved motifs (RHGNRTP and HDTN) possessed by HAP phytases. Using SignalP 4.0 [6, 23], the first to the twenty-fifth amino acids from the N-terminal was predicted as a putative signal peptide of this phytase. It suggests that the phytase might be a secreting enzyme located in the extracellular or periplasmic space of the host. Compared with other known HAP phytases using BLASTP, the amino acid sequence of this phytase has the maximum similarity of only 35% to a predicted acid phosphatase of *Francisella tularensis* subsp. *holarctica* FTNF002-00 [1]. It indicates that the phytase is much dissimilar to all HAP phytases known to date. The novel phytase gene was given the name *appA_Gw*.

In order to confirm the phytase activity of the protein product of *appA_Gw* and further characterize the biochemical properties of this enzyme, a plasmid was constructed for overexpressing the recombinant protein of AppA_Gw in *E. coli*. The nucleotide sequence of the region from the 76 to 1,074 bp of the *appA_Gw* gene, excluding the section encoding for the predicted signal peptide, was chemically synthesized. The nucleotide sequence was subjected to codon optimization for improved performance of expression in *E. coli* (Fig. S1). The synthesized fragment was cloned in-frame between the *EcoRI* and *SalI* sites of a pET26b(+) vector, which allows the PelB signal peptide and the poly-histidine tag of the pET26b(+) vector to respectively fuse in-frame to the N-terminal and C-terminal of the recombinant protein rAppA_Gw, for extracellular localization and affinity-chromatographic purification. The constructed plasmid was named pET-rAppA_Gw.

For overexpression of rAppA_Gw, the constructed plasmid was chemically transformed into *E. coli* BL21(DE3) cells. A 2YT medium with a modified recipe (containing 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 10% (w/v) D-sorbitol, 1.25% (w/v) NaCl, 0.2% (w/v) D-glucose, and 40 mM Tris-HCl of pH 7.4) was used. The transformed *E. coli* cells containing pET-rAppA_Gw were grown in the modified 2YT medium until the optical absorbance at 600 nm reached 0.6–0.8, and then induced by adding isopropyl- β -D-1-thiogalactopyranoside to a final concentration at 0.1 mM. The induced cells were allowed to further grow at 18°C for 16 h, and subsequently the overexpressed protein rAppA_Gw was purified from the medium supernatant by affinity chromatography, using a Ni-NTA agarose column as previously described by Böhm *et al.* [3] (Table S1). A protein of homogeneity was obtained, with a

molecular mass of 42 kDa, as determined by SDS-PAGE (Fig. S2). It is in accordance with the theoretically predicted molecular mass of rAppA_Gw. A gel filtration chromatographic analysis using a Superdex 200 10/300 GL column revealed that rAppA_Gw was present as a monomer (data not shown). To determine the biochemical characteristics of rAppA_Gw, the phytase activity of the purified protein was measured under various pH and temperature conditions, using an AMol Colorimetric Assay as previously described by Liao *et al.* [14]. The amount of rAppA_Gw protein was quantified using a Bradford Assay (Bradford, 1976). One unit (U) of the phytase activity is defined as releasing 1 μ mol inorganic phosphate from phytic acid per minute. The activity value is the mean of three replicates, with standard deviation. For comparison, the rAppA phytase derived from *E. coli* (Ec_rAppA) was used. The *E. coli* Ec_rAppA was assayed under the same pH and temperature conditions. In order to obtain the purified protein of Ec_rAppA, the nucleotide sequence of the *appA* phytase gene of *E. coli* strain K-12 substr. W3110 (GenBank Accession No. JF274478) was chemically synthesized and cloned into the pET26(+) vector. The *E. coli* Ec_rAppA was overexpressed in *E. coli* BL21(DE3) and purified using the same procedures that were used for rAppA_Gw, as described above.

The pH condition that is optimal for the catalysis efficiency of rAppA_Gw under the usual physiological temperatures of livestock and poultry was determined. For this, the phytase activity of rAppA_Gw was measured at 37°C, which is close to the gut temperature of most livestock and poultry, and at different pH points from 0.0 to 6.0 with 0.5 intervals. The results showed that at 37°C, rAppA_Gw demonstrated the maximum activity of 298 ± 17 U/mg at pH 2.0 (Fig. 1A), indicating that rAppA_Gw has a very acidic pH optimum. The phytase activity of rAppA_Gw was still present at even lower pH (pH 0.5 to 1.5), whereas at the pH above 5.0 the activity decreased significantly. In comparison, at 37°C the Ec_rAppA derived from *E. coli* demonstrated the maximum activity of 867 ± 38 U/mg at pH 4.5 (Fig. 1A) and a lower activity of 204 ± 13 U/mg at pH 2.0. Altogether, the results indicated that rAppA_Gw was an acidophilic phytase. In comparison with other HAP phytases characterized to date, particularly those broadly commercialized low pH phytases such as the PhyB derived from *A. niger*, the pH optimum of rAppA_Gw is indeed very acidic [3, 11, 13, 31, 33]. Although a phytase derived from *A. ficuum* NTG-23 [34] was reported as having a more acidic pH optimum (pH 1.3), its activity (150.1 U/mg) is not as high as rAppA_Gw. Since the pH in the stomach of most livestock and poultry is very acidic, usually at a pH

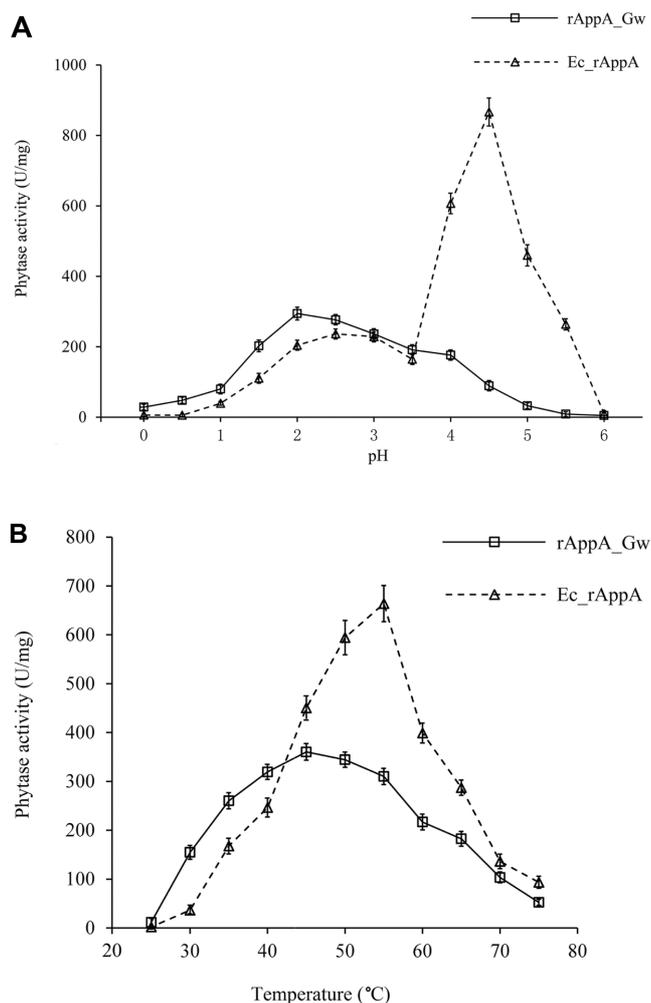


Fig. 1. pH and temperature profiles of rAppA_Gw.

The phytase activity was measured at (A) 37°C and different pH points ranging from 0.0 to 6.0, (B) at pH 2.0 and different temperatures ranging from 25°C to 70°C. The phytase of *E. coli* (Ec_rAppA) was used as a comparison.

value below 3.0 [2, 5, 17–19, 22, 24], rAppA_Gw could have a better catalyzing performance in stomach as benefited from its good adaptability to extremely low pH. It suggests that rAppA_Gw could be promising for the development of feed additives with high acid resistance.

The kinetic characteristics of rAppA_Gw were determined at pH 2.0 and 37°C, using the approach previously described by Vats and Banerjee [30]. The K_m and k_{cat}/K_m values of rAppA_Gw were determined to be 0.51 ± 0.03 mM and 10.91 ± 0.40 / $\mu\text{M}/\text{s}$, respectively, suggesting that rAppA_Gw possesses a moderate affinity for the substrate phytic acid and a moderate catalytic efficiency, in comparison with other phytases characterized to date [13, 33]. The

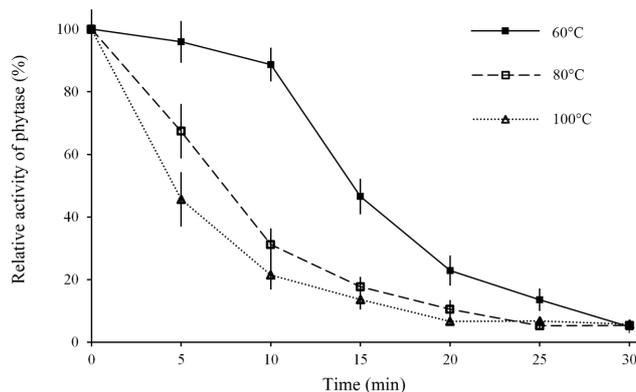


Fig. 2. Relative activity (in percentage) of rAppA_Gw that remained after different durations of heat treatment (0–30 min) at 60°C, 75°C, or 90°C, respectively.

After the heat treatment, the phytase activity was measured at 37°C and pH 2.0. The full activity (100%) is defined as the activity of rAppA_Gw without any prior heat treatment.

Table 1. Effects of metal ions on the relative phytase activity of rAppA_Gw.

Metal ions	Relative phytase activity (%)			
	1 mM	5 mM	10 mM	50 mM
Ca ²⁺	108	121	92	86
Mg ²⁺	102	60	37	11
Al ³⁺	17	10	0	0
Cu ²⁺	85	57	22	3
Zn ²⁺	113	28	17	0
Fe ²⁺	94	36	29	n/a*
Ni ²⁺	77	80	71	19
Mn ²⁺	115	13	2	0
Co ²⁺	98	84	51	15

n/a*: Fe²⁺ at high concentrations interferes with the colorimetric assay reagents. The phytase activity was measured at pH 2.0, 37°C, and is expressed as a percentage (mean of three replicates) of the activity level in the absence of the metal ions. For all the metal ions in the table, the anion of the chemicals used is Cl⁻.

influence of metal ions on the phytase activity of rAppA_Gw was also estimated, by measuring the phytase activity (at pH 2.0, 37°C) while each tested metal ion was present in the catalytic reaction at different concentrations (1, 5, 10, and 50 mM) (Table 1). The determined phytase activity is shown by a percentage in comparison with the activity level with no metal ion supplemented, which is defined as the 100% activity. The results generally showed that the tested metal ions are likely to inhibit the phytase activity of rAppA_Gw at high concentrations. Among these metal

Table 2. Substrate specificity of rAppA_Gw, shown by the relative activity of rAppA_Gw for different substrates.

Substrate	Relative activity (%)
Phytic acid	100
<i>para</i> -Nitrophenyl phosphate	25
AMP	0
ADP	0
ATP	0
GTP	0
NADP	0
Glucose 1-phosphate	2
Glucose 6-phosphate	3

The activity for each tested substrate was determined at pH 2.0, 37°C, and is expressed as a percentage (mean of three replicates) of the activity level for phytic acid.

ions, Al³⁺ demonstrated a severe inhibitory effect at all the four tested concentrations. The inhibitory effect of Mg²⁺, Cu²⁺, Fe²⁺, Mn²⁺, and Co²⁺ rose greatly while the concentrations of these metal ions were increased. In comparison, Ca²⁺ demonstrated no significant inhibitory effect upon the phytase activity at the concentrations up to 50 mM. It means that rAppA_Gw is likely to possess a tolerance to the presence of calcium ion, suggesting that rAppA_Gw is potentially suitable for application in animal feed containing an extra supplement of calcium nutrient. Moreover, the substrate specificity of rAppA_Gw was tested for several organic phosphate compounds at pH 2.0 and 37°C. The results showed that rAppA_Gw has a high preference for phytic acid while its activity for other substrates is much weaker in comparison (Table 2). It means rAppA_Gw is likely to be a true phytase rather than an acid phosphatase possessing a contingent activity for phytic acid.

The temperature that is optimal for the catalysis efficiency of rAppA_Gw was also determined, at the pH optimum of 2.0. The phytase activity of rAppA_Gw was measured at different catalyzing temperatures, from 25°C to 70°C with 5°C intervals. The results showed that the phytase activity of rAppA_Gw reached the maximum value of 363 ± 17 U/mg at 45°C (Fig. 1B), and declined significantly while the temperature went above 55°C. In comparison, the *E. coli* Ec_rAppA was most active around 55°C (664 ± 36 U/mg) under the tested pH condition (pH 2.0), but was less active than rAppA_Gw in the temperature range below 40°C (Fig. 1B). It means that rAppA_Gw is most active at medium temperatures while lacks the ability to catalyze

more efficiently at high temperatures. Although rAppA_Gw is not a thermophilic phytase, it could be suitable for working in animal stomach since its temperature-optimum of 45°C and pH-optimum of 2.0 are very close to the physiological temperature and gastric pH of most livestock and poultry [2, 5, 17–19, 22, 24]. For a potential application as an additive to animal feed, there is a need to estimate the ability of rAppA_Gw to undergo industrial treatments at high temperatures without losing its phytase activity and to subsequently restore the activity after the heat-processes. Therefore, the resistance of rAppA_Gw to heat-denaturation was determined. For this, rAppA_Gw was incubated at 60°C, 75°C, and 90°C respectively for up to 30 min and the decline of phytase activity was monitored. The results showed that the phytase activity could remain stable during the first 10 min at 60°C, however the activity declined at a much faster rate at 75°C and 90°C (Fig. 2). It indicated that rAppA_Gw was unlikely to be a very thermostable enzyme, yet its heat-tolerance is still acceptable as compared with the broadly commercialized low-pH phytases derived from *Aspergillus* microorganisms. It means that heating processes such as thermo-pelleting should be avoided if this phytase would be applied as an additive enzyme in animal feed. Also, protein engineering approaches such as amino acid substitution and direct-evolution might be used to enhance the thermostability of rAppA_Gw in order to make this phytase more applicable in feed industries [7, 26, 27], which would be a further focus of our study.

In all, this study identified a novel phytase and has thereby demonstrated the significance of a culture-independent way for exploiting useful enzymes from environmental microbial communities. In comparison with most of the known phytases, the newly reported phytase could work more efficiently at very low pH that is typical in the stomach environment of most livestock and poultry. Moreover, the phytase activity is maximized near the physiological temperatures in livestock and poultry stomachs. Thus, the novel phytase has the potential for an update or replacement of current commercialized phytases for its good adaptability to the physiological pH and temperatures in livestock and poultry stomachs. Future efforts will be required for improving the thermostability of this enzyme.

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References

- Barabote RD, Xie G, Brettin TS, Hinrichs SH, Fey PD, Jay JJ, et al. 2009. Complete genome sequence of *Francisella tularensis* subspecies *holarctica* FTNF002-00. *PLoS One* **4**: e7041.
- Bilik K, Strzetelski J, Furgal-Dierzuk I, Sliwinski B. 2014. Effect of supplementing TMR diets with artificial saliva and acid buf on optimizing ruminal pH and fermentation activity in cows. *Ann. Anim. Sci.* **14**: 585-593.
- Böhm K, Herter T, Müller JJ, Borriss R, Heinemann U. 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**: 1284-1296.
- Ding Q, Yang P, Huang H, Wang Y, Yao B. 2010. Development status and research trends of phytases. *J. Agric. Sci. Technol.* **12**: 27-33.
- Dono ND, Sparks NH, Olukosi OA. 2014. Association between digesta pH, body weight, and nutrient utilization in chickens of different body weights and at different ages. *J. Poult. Sci.* **51**: 180-184.
- Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* **2**: 953-971.
- Fu D, Huang H, Meng K, Wang Y, Luo H, Yang P, et al. 2009. Improvement of *Yersinia frederiksenii* phytase performance by a single amino acid substitution. *Biotechnol. Bioeng.* **103**: 857-864.
- Golovan S, Wang G, Zhang J, Forsberg CW. 2000. Characterization and overproduction of the *Escherichia coli* *appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. *Can. J. Microbiol.* **46**: 59-71.
- Greiner R, da Silva LG, Couri S. 2009. Purification and characterization of an extracellular phytase from *Aspergillus niger* 11T53A9. *Braz. J. Microbiol.* **40**: 795-807.
- Hanson BT, Yagi JM, Jeon CO, Madsen EM. 2012. Role of nitrogen fixation in the autecology of *Polaromonas naphthalenivorans* in contaminated sediments. *Environ. Microbiol.* **14**: 1544-1557.
- Kostrewa D, Wyss M, D'Arcy A, Van Loon APGM. 1999. Crystal structure of *Aspergillus niger* pH 2.5 acid phosphatase at 2.4 Å resolution. *J. Mol. Biol.* **288**: 965-974.
- Lei X-G, Porres JM. 2003. Phytase enzymology, applications, and biotechnology. *Biotechnol. Lett.* **25**: 1787-1794.
- Lei X-G, Weaver JD, Mullaney EJ, Ullah AH, Azain MJ. 2013. Phytase, a new life for an "old" enzyme. *Annu. Rev. Anim. Biosci.* **1**: 283-309.
- Liao Y, Li C-M, Chen H, Wu Q, Shan Z, Han X-Y. 2013. Site-directed mutagenesis improves the thermostability and catalytic efficiency of *Aspergillus niger* N25 phytase mutated by I44E and T252R. *Appl. Biochem. Biotechnol.* **171**: 900-915.
- Markowitz VM, Ivanova NN, Szeto E, Palaniappan K, Chu K, Dalevi D, et al. 2008. IMG/M: a data management and analysis system for metagenomes. *Nucleic Acids Res.* **36**: D534-D538.
- Markowitz VM, Mavromatis K, Ivanova NN, Chen I-MA, Chu K, Kyrpides NC. 2009. IMG ER: a system for microbial genome annotation expert review and curation. *Bioinformatics* **25**: 2271-2278.
- Moessler A, Kottendorf S, Liesner VG, Kamphues J. 2010. Impact of diets' physical form (particle size; meal/pelleted) on the stomach content (dry matter content, pH, chloride concentration) of pigs. *Livestock Sci.* **134**: 146-148.
- Moessler A, Wintermann M, Sander SJ, Kamphues J. 2012. Effect of diet grinding and pelleting fed either dry or liquid feed on dry matter and pH in the stomach of pigs and the development of gastric ulcers. *J. Anim. Sci.* **90**: 343-345.
- Mohammed R, Hunerberg M, McAllister TA, Beauchemin KA. 2014. Characterization of ruminal temperature and its relationship with ruminal pH in beef heifers fed growing and finishing diets. *J. Anim. Sci.* **92**: 4650-4660.
- Mukhametzhanova AD, Akhmetova AI, Sharipova MR. 2012. Microorganisms as phytase producers. *Microbiology* **81**: 267-275.
- Mullaney EJ, Ullah AH. 2003. The term phytase comprises several different classes of enzymes. *Biochem. Biophys. Res. Commun.* **312**: 179-184.
- Olukosi OA, Dono ND. 2014. Modification of digesta pH and intestinal morphology with the use of benzoic acid or phytobiotics and the effects on broiler chicken growth performance and energy and nutrient utilization. *J. Anim. Sci.* **92**: 3945-3953.
- Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**: 785-786.
- Qadis AQ, Goya S, Ikuta K, Yatsu M, Kimura A, Nakanishi S, Sato S. 2014. Effects of a bacteria-based probiotic on ruminal pH, volatile fatty acids and bacterial flora of Holstein calves. *J. Vet. Med. Sci.* **76**: 877-885.
- Reddy NR, Sathe SK, Salunkhe DK. 1982. Phytates in legumes and cereals. *Adv. Food Res.* **28**: 1-92.
- Shivange AV, Dennig A, Schwaneberg U. 2014. Multi-site saturation by OmniChange yields a pH- and thermally improved phytase. *J. Biotechnol.* **170**: 68-72.
- Shivange AV, Serwe A, Dennig A, Roccatano D, Haefner S, Schwaneberg U. 2012. Directed evolution of a highly active *Yersinia mollaretii* phytase. *Appl. Microbiol. Biotechnol.* **95**: 405-418.
- Tomschy A, Brugger R, Lehmann M, Svendsen A, Vogel K, Kostrewa D, et al. 2002. Engineering of phytase for improved activity at low pH. *Appl. Environ. Microbiol.* **68**: 1907-1913.
- Ullah AH, Cummins BJ. 1987. Purification, N-terminal amino acid sequence and characterization of the pH 2.5

- optimum acid phosphatase (E.C. 3.1.3.2) from *Aspergillus ficuum*. *Prep. Biochem.* **17**: 397-422.
30. Vats P, Banerjee UC. 2005. Biochemical characterisation of extracellular phytase (*myo*-inositol hexakisphosphate phosphohydrolase) from a hyper-producing strain of *Aspergillus niger* van Teighem. *J. Ind. Microbiol. Biotechnol.* **32**: 141-147.
31. Wyss M, Pasamontes L, Remy R, Kohler J, Kuszniir E, Gadiant M, *et al.* 1998. Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. *Appl. Environ. Microbiol.* **64**: 4446-4451.
32. Yao MZ, Wang X, Wang W, Fu YJ, Liang AH. 2013. Improving the thermostability of *Escherichia coli* phytase, *appA*, by enhancement of glycosylation. *Biotechnol. Lett.* **35**: 1669-1676.
33. Yao MZ, Zhang YH, Lu WL, Hu MQ, Wang W, Liang AH. 2012. Phytases: crystal structures, protein engineering and potential biotechnological applications. *J. Appl. Microbiol.* **112**: 1-14.
34. Zhang GQ, Dong XF, Wang ZH, Zhang Q, Wang HX, Tong JM. 2010. Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from *Aspergillus ficuum* NTG-23. *Bioresour. Technol.* **101**: 4125-4134.