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Purification and characterization of an acidic, thermophilic phytase from a newly isolated *Geobacillus stearothermophilus* strain DM12

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ABSTRACT

Microbial phytases were applied mainly to animal and human foodstuffs in order to improve mineral bioavailability and food processing. In addition, phytases have potential biotechnological application in various other fields, such as environmental protection, aquaculture and agriculture. Bacillus sp. DM12, an isolate from a hot spring, produces phytase, which catalyzes the hydrolysis of phytic acid into myo-inositol and inorganic phosphates. Phytase from Bacillus sp. DM12 was purified using ammonium sulfate precipitation and dialysis, followed by anion exchange and gel filtration chromatography. Molecular weight of the purified phytase was estimated to be 28 kDa by SDS-PAGE. K_m and V_{max} values for sodium phytate were 0.177 mM and 1.126 µmol/min, respectively. The optimum temperature for phytase activity was found to be 50°C. The enzyme retained over 75% of its activity over a temperature range of 30 to 80°C. The highest phytase activity was observed at pH 4.5 and a decline of enzyme activity was observed on both sides of pH optimum. The enzyme was stable over the pH range of 3.0 to 6.0. The enzyme retained over 80% of its activity in the presence of 5 mM metal ions except $CaCl_2$. It is also indicated that the enzyme retained over 65% of its activity over a 5 mM metal ions. These properties suggest that this phytase is a suitable enzyme for the hydrolysis of phytic acid and phytates in food and feed processing industries.

Keywords: activity, characterization, phytase, purification, stability.

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Introduction

Phytic acid (*myo*-inositol hexakisphosphate) is an anhydrous storage form of phosphate, accounting for more than 80% of the total phosphorus in cereals, legumes and oilseeds (1, 2). Phytates are regarded as anti-nutritional factors that decrease feed quality, because they chelate proteins and essential minerals such as calcium, iron, zinc, magnesium, manganese, copper and molybdenum and prevent their absorption (1, 3). The organically bound phosphate of phytic acid is not metabolized by monogastric animals such as pig, poultry and fish due to lack of phytase and consequently contributes to the phosphorus pollution problems in areas of intensive livestock production (2).

Conversely, phytase (myo-inositol hexakisphosphatephosphohydrolase) catalyzes the stepwise removal of phosphates from phytic acid or its salt phytate (4). These enzymes have attracted a lot of attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology (1, 5). The scientific and practical significances of phytase are best attested by its recent distinction as one of the ten most important discoveries in swine production in the past century. The supplementation of animal feed with phytases reduces the cost of diets by removing or reducing the need for supplemental inorganic phosphate and increases the bioavailability of phosphorous in monogastric animals (2).

Several fungal, bacterial and yeast strains have been reported as the source of phytase. Some of the phytase producing microorganisms include bacteria, such as *Bacillus* (6-9), *Escherichia coli* (10), *Enterobacter* (11), *Lactobacillus* (12), *Pseudomonas* (13), *Citrobacter* (8).

Due to several biological characteristics, such as substrate specificity, catalytic efficiency and resistance to proteolysis, bacterial phytases have extensive potential in commercial applications. The increasing potential of phytase application prompts the screening for newer phytase producing microorganisms, which can meet the conditions favorable to the industrial production (2). A major drawback to the wide use of phytases and of feed enzymes in general is the constraint of thermal stability (65 to 95°C) required for these enzymes to withstand inactivation during the feed-pelleting and/or expansion processes. Therefore, the availability of heat-resistant enzymes would circumvent the aforementioned problem. In the present study, a phytase-producing bacterium was isolated from hot spring and identified by 16S rRNA sequencing, and the phytase was purified and characterized to assess its suitability for animal feed.

Materials and Methods

Isolation of phytate degrading bacteria

Bacterial strains were isolated from the water samples of the Dimand hot sprig in Jiroft, which is located in southwest of Iran. This hot spring has 78°C temperature and pH 5. Approximately 5 ml of these samples was inoculated in 50 ml of phytase specific medium [1.5% glucose, 0.5% (NH4)2SO4, 0.05% KCl, 0.01% MgSO4.7H2O, 0.01% NaCl, 0.01% CaCl2.2H2O, 0.001% FeSO4, 0.001% MnSO4, pH 6.5 with 0.5% sodium phytate (Sigma)]. These flasks were incubated at 65°C, with 170 rpm for 3 days. After that, 5 ml of this culture was added to the same fresh medium and incubated in the same conditions. Finally, 0.1 ml of this culture was streaked on phytase specific media and incubated at 65°C for 48 h.

Bacterial colonies capable of hydrolyzing sodium phytate, which can be recognized by their surrounding clear halo, were obtained by re-plating single colonies (14).

Measurement of phytase activity

Phytase activity was determined using sodium phytate as a substrate. The reaction mixture consisted of 100 µl enzyme solution and 200 µl 0.1 M sodium acetate buffer of pH 6.5 (containing 2 mM sodium phytate and 2 mM CaCl2). The reaction mixture was incubated at 65°C for 30 minnd then stopped by adding 300 µl of 10% (w/v) tri-chloroacetic acid (TCA). Then 600 µl of ammonium molybdate reagent was added (containing 1:4 mixture of 2.7% FeSO4 and 1.5% ammonium molybdate in 5.5% H2SO4) (3, 10).

After 5 min incubation at room temperature, absorbance at 700 nm was read. All the samples were assayed in triplicates. One unit of enzyme was defined as the amount of enzyme required to liberate 1 µM of inorganic phosphate per minute under the assay condition.

PCR amplification and 16S rDNA sequencing

Genomic DNA of *Bacillus* sp. DM12 was extracted according to Sambrook and Russell protocols (15) and its purity was checked by the A260/A280. Universal 16S rRNA PCR forward primer (5-AGTTTGATCCTGGCTCAG-3) and reverse primer (5-GGC/T ACCTTGTTACGACTT-3) were used for the amplification of 16S rRNA gene. PCR program was performed as follows: (1) 94°C for 5 min as initial temperature, (2) a run of 30 cycles with each cycle consisting of 45 s at 94°C, 45 s at 48°C and 90 s at 72°C, and (3) a final extension at 72°C for 5 min (16). PCR products were electrophoresed on agarose gel (0.7%) and subsequently amplified 16S rRNA bands were purified by DNA extraction kit (Cinaclone) and then DNA sequencing was performed on both strands directly by SEQ-LAB (Germany).

The phylogenetic tree was made based on the comparison of 16S rRNA sequences of *Bacillus* sp. DM12 strain with other strains of *Bacillus* sp. that were obtained from Gene Bank database (http://www.ncbi.nlm.nih.gov). All sequences were aligned with Clustal Omega that was obtained from: http://www.seqtool.sdsc.edu/ CGI/Omega.cgi (17) and phylogenetic tree was made in MEGA4 (18). The obtained 16S rRNA sequence was deposited in Gene Bank for *Bacillus* sp. DM12 strain, with KF408262 accession number.

Investigation of culture condition for phytase production

To determine the optimum condition for phytase production, *Bacillus* sp. DM12 was inoculated into 50 ml of liquid medium in a 250 ml Erlenmeyer flask and incubated in shaker at 65°C for 3 days. Samples were picked up at each 24 h interval and phytase activity was determined as described above. The parameters tested were: carbon sources such as 1.5% glucose, 1.5% galactose, and 0.75% glucose + 0.75% galactose. Nitrogen sources were 0.1% glycine, 0.1% yeast extract and 0.1% ammonium sulfate. Phosphate sources were sodium phytate (0.5%), tricalcium phosphate (0.5%), sodium phytate (0.25%) + tricalcium phosphate (0.25%), and nutrient broth. The effect of initial pH on phytase production was also investigated by using phytase specific medium with pH 5.0, 6.0, and 7.0. In addition, phytase production was investigated

in the presence of different concentration (0, 0.1, 0.2, 0.3, and 0.4%) of different ions (KCl, NaCl, MgSO4, and FeSO4).

Purification of phytase enzyme

The isolated strain, *Bacillus* sp. DM12, was cultured in phytase production media and kept in a shaker incubator (200 rpm) at 65°C for 48 h. The liquid medium used for the production of phytase was composed of 1.5% glucose, 0.5% (NH4)2SO4, 0.05% KCl, 0.01% MgSO4.7H2O, 0.01% NaCl, 0.01% CaCl2.2H2O, 0.001% FeSO4, 0.001% MnSO4, and 0.5% sodium phytate. The pH of the medium was adjusted to 7.0. The medium was inoculated at 10% (v/v) with a 16 h old culture and incubated at 65°C with 200 rpm shaking for 48 h. After incubation, culture was subjected to centrifugation at 10000 rpm for 10 min at 4°C. The supernatant was collected and used as crude enzyme for purification. The supernatant was fractionated by stepwise precipitation with ammonium sulfate powder at different saturation. The precipitate formed in each step was collected by centrifugation at 12000 rpm for 20 min, dissolved in 1 ml 0.1 M acetic acid buffer and assayed for enzyme activities. The fraction with the highest phytase activity was dialyzed overnight against Tris/HCl buffer (pH 7.5) to remove the remaining salt. The desalted samples were directly loaded onto a Q-sepharosecolumn(1.5×24 cm) previously equilibrated with 20 mMTris-HCl buffer (pH 7.5) and the column was eluted with a linear gradient of 0-1 M NaCl at a flow rate of 1 ml/min. The phytase activity in the eluted fractions was determined, active fraction from the previous step was carried out by gel filtration using Sephadex G-100 (1×100 cm). The fractions showing phytase activity were pooled and subjected to SDS-PAGE to check the homogeneity of the purified enzyme. All the steps were carried out at 4°C. Moreover, SDS-PAGE was carried out according to the method of Laemmli (19) with a 10% polyacrylamide gel, and the protein was stained with Coomassie brilliant blue. For phytasezymogram analysis, the denaturing temperature selected was 70°C rather than 100°C in order to prevent enzyme inactivation. The SDS-PAGE gel was soaked in a 0.1M sodium-acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 for a period of 1 h at room temperature and then moved to a 0.1M sodium-acetate buffer solution (pH 5.0). Phytase activity was detected by incubating the gel in a 4 mM sodium-phytate solution in 0.1M sodium-acetate buffer for a period of 30 min. Subsequent to washings with water, the phytase bands were detected by immersing the gel in a coloring reagent (freshly prepared by mixing 18 ml of 2.5N HCl, 18 ml of 2.6% (w/v) ammonium molybdate, 13 ml of dH2O and 1ml of a 0.126% (w/v) malachite-green solution) for a period of 1–2 h until the relevant visible green band(s) appeared (20).

Enzyme kinetics

The Michaelis-Menten's constant (K_m) and the maximum attainable velocity (V_{max}) were determined at different substrate concentrations [S]. The phytase activity was measured at 45°C in 0.25 M sodium acetate containing 0.125 to 4.0 mM sodium phytase. K_m and V_{max} values were obtained from a Lineweaver-Burke plot.

Enzyme characterization

The optimum temperature was determined by varying the temperature from 30 to 90^{o}C at 10^{o}C intervals. The thermal stability was also determined by assessing the residual enzyme

activity after incubating the enzyme in 0.25M sodium acetate (pH 6.5) at each temperature for 1 h and then the residual activity was measured at standard assay condition. The pH versus activity profile was determined by measuring the phytase activity at 45°C and pH 2.0-12.0 using the following buffers: glycine-HCl (0.25M, pH 2.0-3.5), sodium acetate (0.25M, pH 3.5-6.0), Tris-HCl (0.25M, pH 6.0-9.0), and glycine-NaOH (0.25M, pH 9.0-12). To study the effect of pH on enzyme stability, samples of the enzyme were pre-incubated in buffers of various pH at 45°C for 1 h and then the residual activities of the enzyme were assayed under standard conditions (pH 6.5, 45°C, 30 min). Equal volumes of metal ions or EDTA (to a final concentration of 5mM) were mixed with an enzyme solution in 0.05 M sodium acetate buffer of pH 6.5 (containing 2 mM sodium phytate and 2 mM $CaCl_2$). The mixture was incubated for 1 h at room temperature and the residual activity was then measured at standard assay condition.

Results

Screening and identification of phytase producing bacteria

In this study, phytase producing bacteria were isolated from Dimand hot spring. 67 strains showed phytase activity on PSM agar. The highest phytase producing strain, DM12, was selected based on a clear zone around the strain. This strain was grown on liquid medium containing sodium phytate as inducer at 65°C for 96 h and at each 24 h interval, the sample was picked up and the phytase activity was measured. Phytase production was sharply

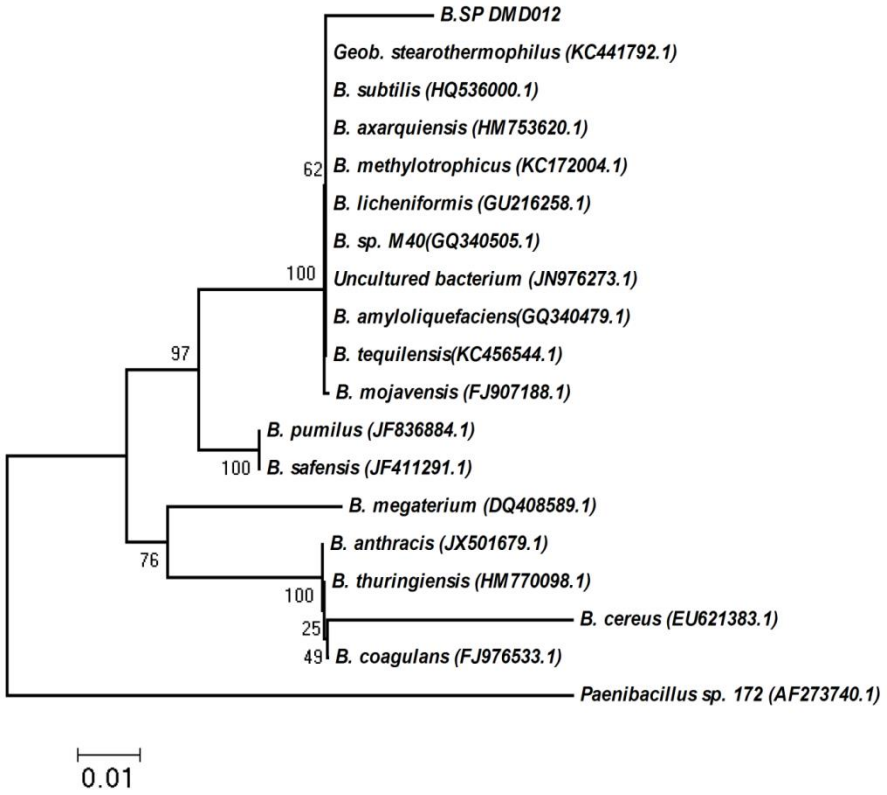

Figure 1. The phylogenetic tree of *Bacillus* sp. D12 based on 16S rRNA.NCBI accession number of each species is shown in parenethesis. Bootstrap values and scale bar depicting substitution rate per site are indicated. The phylogenetic tree constructed by the neighbor-joining method showing the position of isolate DM12.

increased up to 48 h of incubation and then gradually decreased up to 40% at 96 h. So, the highest phytase production was observed after 2 days of incubation. This strain also lowered the pH (from initial pH 6.8 to 4.0) of liquid medium as compared with the control, where it remained constant. Bacterial identification was done using its 16S rRNA gene sequence. The PCR product of 16S rRNA gene was about 1500 bp. The phylogenetic tree was created by neighbor-joining method by MEGA4 software (Fig. 1) (17). Result shows that *Bacillus* sp. DM12 is strongly related to *Geobacillus stearothermophilus* with 97% homology.

Investigation of culture condition for phytase production

To determine the optimum conditions for phytase production, the strain was inoculated into 50 ml of liquid medium in 500 ml flask and incubated on a reciprocal at 65^{o}C for 3 days. Figure 2 shows the results of different sources of carbon, nitrogen, phosphate and initial pH on phytase production. For carbon sources Glc (1.5%), Gal (1.5%) and Glc (0.75%)+ Gal (0.75%) were added to the medium and phytase activity was assayed at 24, 48 and 72 h of incubation. Results show that all three carbon sources improved phytase production at 24 and 48 h of incubation and slightly decrease at 72 h. High phytase activity was obtained in Glc (0.75%) + Gal (0.75%) medium after 48 h of incubation (Fig. 2a).

Nitrogen is considered as another energy source for phytase production. The most suitable nitrogen source for phytase production was yeast extract at 24, 48 and 72 h of incubation. Thus the yeast extract was 15 and 22% more efficient than glycine and ammonium sulfate, respectively (Fig. 2b)

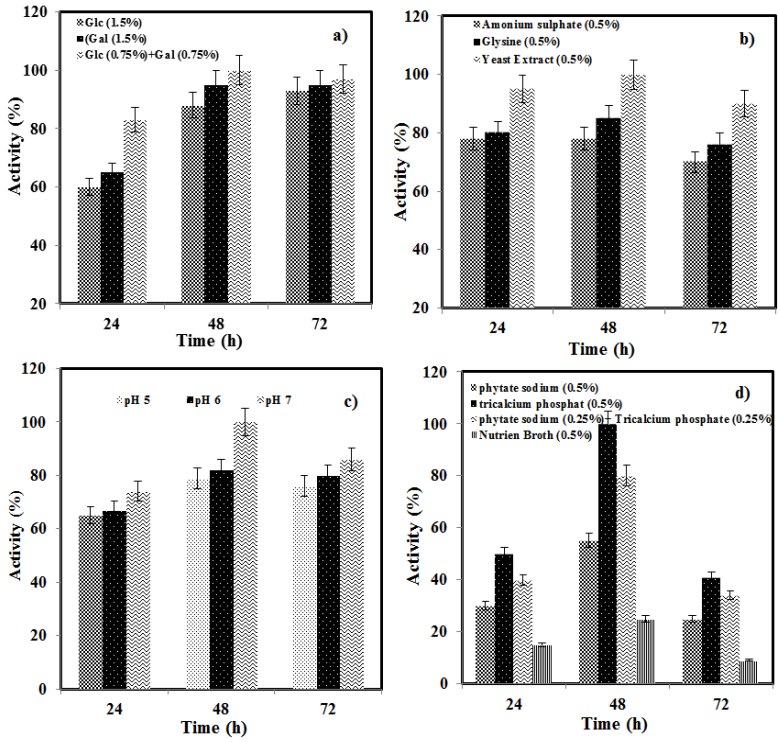

Figure 2. Effect of carbon (a), nitrogen (b), phosphate (d) sources and initial pH (c) on the phytaseproductionbyDM12 strain. Enzyme activity was measeuerd at different time of incubation (24, 48 and 72 h) after addition of compounds or incubation at indicated pH.a) Carbon sources: 1.5% glucose, 1.5% galactose, 0.75% glucose + 0.75% galactose. b) Nitrogen sources: 0.1% glycine, 0.1% yeast extract, 0.1% ammonium sulfate. c) Initial pH of phytase specific medium: 5.0, 6.0, 7.0. d) Phosphate sources: sodium phytate (0.5%), tricalcium phosphate (0.5%), sodium phytate (0.25%) + tricalcium phosphate (0.25%), nutrient broth.

The effect of initial pH on phytase production by *Bacillus* sp. DM12 was investigated. Results show that the optimum initial pH for phytase production was pH 7.0. It was 18 and 21% more efficient than pH 6.0 and pH 5.0, respectively (Fig. 2c). The effect of phosphate salts, such as sodium phytate (0.5%), tricalcium phosphate (0.5%), sodium phytate (0.25%) + tricalcium phosphate (0.25%), and nutrient broth on phytase production is shown in Figure 2d. Results show that all medium induced phytase production except nutrient broth media. Tricalcium phosphate was the best parameter for phytase production. The addition of tricalcium to the sodium phytate media results to about 25% improvement in phytase production after 48 h of incubation.

Effect of inorganic salts on phytase production

Table 1 shows the effect of inorganic salts on the phytase production by *Bacillus* sp. DM12. Phytase production was investigated in the presence of different concentration (0, 0.1, 0.2, 0.3, and 0.4%) of different ions (KCl, NaCl, $MgSO_4$, and $FeSO_4$). Results show that KCl, NaCl and $FeSO_4$ all induced a high level of phytase activity by *Bacillus* sp. DM12. The addition of 0.2% of KCl and $FeSO_4$ to the medium increased the phytase production by about 10 and 16%, respectively, when compared to the control medium. In addition, 0.1% NaCl sharply enhanced phytase production by about 22%.

Table 1. Effect of different salt concentration (%) on the phytaseactivityof *Bacillus* sp. DM12

	Enzyme activity (%)			
Salt concentration (%)	**KCl**	**NaCl**	**MgSO4**	**FeSO4**
0.0	100±1	100± 2.1	100± 2.1	100± 2.1
0.1	100± 0.93	122± 2.2	98± 1.2	100±1.2
0.2110	±2	112±1.8	97±1.6	116±1.5
0.3	100± 1.3	110± 1.1	98±0.92	110±1.6
0.4	95 ± 2.2	105±1.5	100± 0.82	105±1.0

Phytase activity was measured after 48 h of incubation as described in the material and methods. Phytase activity in medium with no salt was considered as control (100 %).

Enzyme purification

Supernatant was fractionated and concentrated with 0–100% ($(NH_4)_2SO_4$) to fraction the proteins. Results showed that phytase was precipitated in 70% ammonium sulfate. The pellet was dissolved in 50 mMTris/HCl buffer, pH 7.5 and dialyzed against the same buffer. After that, the dialysate was applied to Q-sepharose column (equilibrated with 50 mMTris/HCl buffer, pH 7.5) and fractionated by a step-wise elution using the same buffer having an increasing concentration of NaCl (0-1M). Fractions showing high phytase activity were pooled, and adjusted to gel filtration chromatography (G_{100}). Phytase was purified 57-fold from crude enzyme supernatant with 61% recovery. The purified enzyme showed a specific activity of 71.5 U/mg for sodium phytate hydrolysis (Table 2). Purified phytase migrated as a single band in SDS-PAGE under reducing conditions, suggesting its homogeneity. Molecular weight of the purified phytase was estimated to be 28 kDa by SDS-PAGE.

Table 2. Summary of purification steps of DM12 phytase

Purification steps	Total activity	Total protein (U)	Specific activity (mg)	Purification fold (U/mg)	Yield (%)
Culture supernatant	410	330	1.25	1.0	100
$(NH4)_2SO4$ precipitation (60%)	380	45	8.4	6.72	92.68
Q-Sepharose	310	14	22	17.6	75.61
Sephadex G100	250	3.5	71.5	57.2	61

Phytase activity and stability at different pH

The optimum pH of the purified phytase was 4.5 (Fig. 3a). The purified phytase was relatively stable over a pH range of 3.0 to 7.0, and retained 85% of its activity after 1 h pre-incubation in buffer solutions of acidic pHs (Fig. 3a).

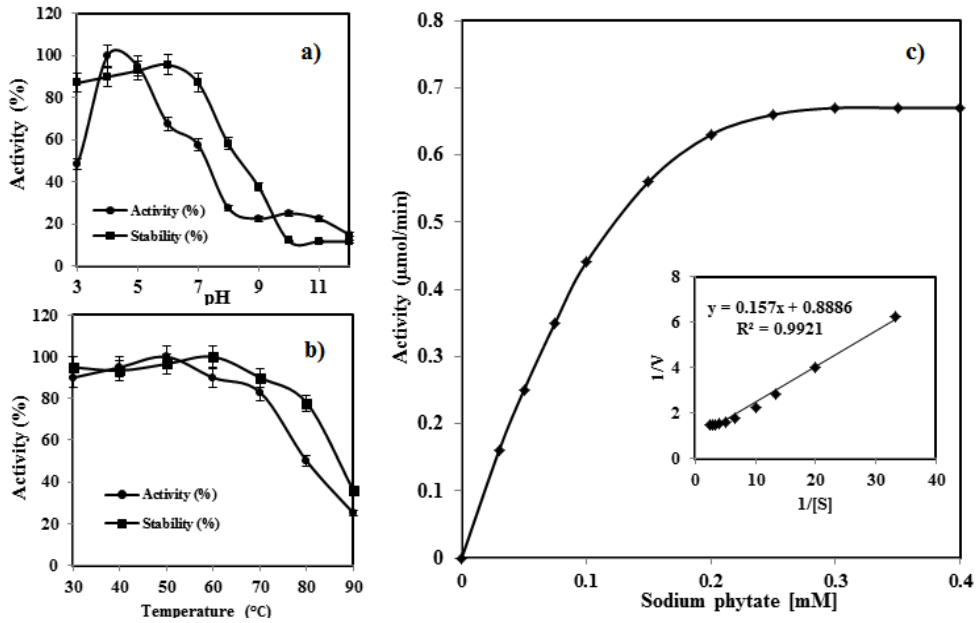

Figure 3. Characterization of phytaseactivity at different pHs, temperatures and phytate concentrations. a) Effect of pH on phytase activity and stability. b) Effect of temperatures on phytase activity and stability. c) Michaelis-Menten and Lineweaver-Burk reciprocal plots of DM12 phytase.

Phytase activity and stability at different temperatures

The optimum temperature for phytase activity was 50°C. The enzyme retained over 85% of its activity over a temperature range of 30 to 70°C and above in which there was an abrupt decrease in enzymatic activity (Fig. 3b). The enzyme was stable at temperatures below 60°C when pre-incubated at various temperatures for 1 h. However, when the incubation temperature was above 60°C, partial loss of activity was recorded at 80°C (75% activity remained) and 90°C (35% activity remained) (Fig. 3b).

Determination of kinetic parameters

The study of most phytases hitherto s follows the procedures of Michaelis-Menten kinetics. It should be noted that under the standard assay conditions (that is, 2 mM phytic acid), only the rate of the reaction from myo-inositol hexakisphosphate to pentakisphosphate is measured (2). The result of varying substrate concentrations on the phytase activity revealed that the enzyme activity follows normal Michaelis-Menten curve (Fig. 3c). From the Lineweaver-Burk reciprocal plot, the K_m and V_{max} of the DM12 phytase were established as 177 µmol and 1.126 µmol/min, respectively (Fig. 3c).

Effect of metal ions on phytase activity and stability

The effect of different metal ions on the activity of the purified phytase (Table 3) indicated that Mn^{2+} at 5 mM improved its activity by 25%. In addition, the activity of the purified phytase decreased to about 60% when 5 mM Ca^{2+} was added. However, the activity also decreased by 10% in the presence of Zn^{2+}, and Na^{1+}. Mg^{2+} was found to slightly inhibit the activity of phytase. The results in Table 3 show that this enzyme retained 65% of its activity after 1 h pre-incubation in different ions.

Table 3. Effect of metal ions on phytase activity and stability

	Activity (%)	Stability (%)
Control	100±0	100±0
$CaCl_2$	42±0.02	70±0.02
$MgCl_2$	95±0.02	65±0.04
$ZnCl_2$	90±0.04	85±0.04
KCl	85±0.05	80±0.0
NaCl	90±0.0	81±0.02
$MnSO_4$	125±0.04	98±0.03
$FeSO_4$	80±0.02	88±0.03
EDTA	80±0.03	70±0.02
SDS	70±0.01	60±0.02

Equal volumes of metal ions or EDTA were added at 5 mM final concentration.
Phytase activity with no compound addition was considered as control (100 %).

Discussion

In spite of the importance of bacterial phytase as potential biotechnological tools in various fields, only a limited number of bacterial phytases have been reported. To the best of our knowledge, this is the first report of a bacterial phytase from *Bacillus stearothermophilus* isolated from a hot spring. Medium optimization showed that high phytase activity was obtained in medium sublimentated with glucose and galactose. It was previously reported that the production of phytase by *Bacillus subtilis* using different carbon sources showed that glucose was the best carbon source (21).

The most suitable nitrogen source for phytase production was yeast extract. It was previously reported that the addition of 0.01% of yeast extract to medium, increased phosphate solubilizing ability of *Pseudomonas* sp. 2 by 44% (7). In addition, increasing the concentration of yeast extract to more than 0.05% resulted in the reduction of phosphate solubilization (7). Yeast extract was also found to be the better nitrogen source for the production of phytase using the strain BPTK4 (21). The effect of initial pH on phytase production by *Bacillus* sp. DM12 was investigated. Results show that the optimum initial pH for phytase production was pH 7.0. It was reported that optimum initial pH by

Pseudomonas sp. YH40 for phytase production was pH 6.0 (7).

Furthermore, the results of the present study show that KCl, NaCl and $FeSO_4$ all induced a high level of phytase activity by *Bacillus* sp. DM12. In addition, 0.1% NaCl sharply enhanced phytase production by about 22%. In *Bacillus* sp. C43, phytase production considerably inhibited in the presence of various concentration of metal ions except Ca^{+2} (5 mM) (2). It was previously reported that the addition of 0.2% $FeSO_4$ to the medium improved phytase activity by about 34%, in which $MgSO_4$ and KCl induced a high level phytase activity (2).

Molecular weight of the purified DM12 phytase was estimated to be 28 kDa by SDS-PAGE. Generally, the molecular weights of the phytase of *Bacillus* sp. lay within the range 40-47 kDa (13, 22-25). A variety of molecular weigth for phytases from other *Bacillus* sp. had been reported as 41 kDa *B. subtilis* (26), 47 kDa *Bacillus licheniformes*(24).

The optimum pH of the DM12 phytase was 4.5 and it was relatively stable over a pH range of 3.0 to 7.0. pH, stability of *Pseudomonas* sp. phytase was between pHs 5.0-7.0 and optimum pH of *Pseudomonas* sp. phytase was found to be pH 7.0 (8). According to the optimum pH, the produced phytase could be classified as an acidic phytase with an optimum pH range between 3.5-6.0 (10). The optimum pH activity of *B. subtillus* (natto) phytase was between 6.0-6.5 (21). It is indicated that the maximum activity of *Bacillus* phytase are in neutral pH. *E. coli* phytase (pH4.5), *Aspergillus ficuum* phytase (pH 5.0), *lactobacillus plantarum* phytase (pH 5.5) and commercially available fungal phytases (pH 4-5) were active in acidic pH (27). These acidic phytases were applied as feed additives, as they could release inorganic phosphate from feed under acidic conditions during the digestion process of animals.

The optimum temperature for phytase activity was 50^{o}C and the enzyme was stable at temperatures below 60°C when pre-incubated at various temperatures for 1 h. Power and Jagannathan reported a phytase that exhibited a maximum activity at a temperature of 60^{o}C and was stable up to a temperature of 70^{o}C (9).*Bacillus* sp. (natto) phytase had an optimum temperature of 60^{o}C (21). Thermal stability of phytase is considered to be an important and useful criterion for application as an animal additive because of its high temperature and steam process during pelleting.

However, K_m and V_{max} value of the DM12 phytase were established as 177 µmol and 1.126 µmol/min, respectively. K_m of *B. subtilis* (natto) and *Bacillus* sp. DS11 was reported as 500 and 550 µmol, respectively (28). Lower K_m of DM12 phytase suggests higher affinity for the substrate, and a higher V_{max}, suggests a higher efficiency.

DM12 phytase indicated that Mn^{2+} at 5 mM improved its activity by 25%. In addition, the activity was decreased to about 60% when 5 mM Ca^{2+} was added. *Bacillus* phytases were found to be metal ion-dependent as they required calcium for activity and stability (29). It was reported that Ca^{2+} was slightly inhibited at a 5 mM concentration and strongly inhibited (84%) at a 10 mM concentration (13). *Pseudomonas fragi* Y9451 phytase was greatly inhibited by Fe^{+3}, Cu^{+2}, Fe^{+2}, and Zn^{+2}, and moderately inhibited by Co^{+2} and Ni^{+2} (10). It was also reported that Ca^{2+} was required for the activity of *B. subtilis* phytase, and had no significant effect on *Bacillus* sp. DS11 phytase (13), and was slightly inhibitory on phytases from *E. coli* (10) and *Klebsiella terrigena*(29). Pevious research shows that

about 30% *P. fragi* Y9451 phytase was stimulated in 5 mM EDTA (13).

Finally, this enzyme has desirable activity and stability profile under acidic pH, excellent thermal stability and good ions activity and stability in the presence of metal ions. As such, this indicates that DM12 phytase has great potential for commercial interest as an animal feed additive.

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