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ORIGINAL PAPER

Production and characterization of thermostable alkaline phytase from *Bacillus laevolacticus* isolated from rhizosphere soil

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Abstract A novel phytase producing thermophilic strain of *Bacillus laevolacticus* insensitive to inorganic phosphate was isolated from the rhizosphere soil of leguminous plant methi (*Medicago falacata*). The culture conditions for production of phytase by *B. laevolacticus* under shake flask culture were optimized to obtain high levels of phytase (2.957 ± 0.002 U/ml). The partially purified phytase from *B. laevolacticus* strain was optimally active at 70 °C and between pH 7.0 and pH 8.0. The enzyme exhibited thermostability with ~80% activity at 70 °C and pH 8.0 for up to 3 h in the presence/absence of 5 mM $CaCl_2$. The phytase from *B. laevolacticus* showed high specificity for phytate salts of $Ca^+ > Na^+$. The enzyme showed an apparent K_m 0.526 mM and V_{max} 12.3 μmole/min/mg of activity against sodium phytate.

Keywords *Bacillus laevolacticus* · Rhizosphere soil · Phytase production · Optimization · Characterization

Introduction

Phytase (E.C.3.1.3.8. inositol hexaphosphate phosphohydrolase) is an enzyme that hydrolyses phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis-dihydrogen phosphate) to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates. Phytic acid, as phytate is a major storage form of phosphorus in plant seeds, is an important source of phosphorus in animal feed [26]. However, phytate phosphorus is biologically unavailable to monogastric animals like pigs and poultry birds including humans, as they lack phytase in their digestive tracts. The presence of phytic acid in feed is undesirable as it chelate nutritionally important divalent cations (Ca^{2+}, Zn^{2+} and Fe^{2+}) and some proteins, thereby rendering them biologically unavailable to the animal [15]. Therefore, animal feed is supplemented with di-calcium phosphate (DCP). Because of the high proportions of phytic acid and unutilized inorganic phosphorus in monogastric derived faeces, its deposition in the environment contributes to the phosphate pollution leading to eutrophication of surface water. Therefore, the reduction of the phytic acid content of seed meals via its enzymatic hydrolysis is desirable. It is estimated that 10 kg of DCP can be replaced by 250 g of phytase enzyme. The phytases during feed formulation are subjected to steam prior to pelleting, which is detrimental to the enzyme activity and, therefore, thermostable phytases are preferred for commercial preparations. In addition, the enzyme should be active under broad pH range and specifically active against calcium phytate complex and be produced at high levels for making the biotechnological option commercially attractive [4, 14, 27].

A wide variety of microbial phytases have been discovered and characterized in the last decade, with a few of these phytases, from *Aspergillus spp.* and *Peniophora lycii*, being authorized by EU as feed additives [14, 33]. Although, on the basis of pH profile, higher thermostability, and strict substrate specificity as well as physiological nature of phytate in digestive tract, alkaline phytases from *Bacillus* sp. are considered ideal

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candidates for application in animal feed [27, 30]. However, no single phytase fit in the criteria for an ideal phytase and, therefore, search for novel sources of phytases is an area of continued research interest.

The soil plant rhizosphere bacteria, also termed as plant-growth-promoting-rhizobacteria (PGPR), are known to mobilize soil inorganic polyphosphates and/or phytate. Some of the PGPR strains of *Bacillus amyloliquefaciens*, isolated from soil rhizosphere, are known to be active phytases producers [17]. In addition to the catabolism of inositol, a product of phytate degradation, these bacteria are actively involved in the colonization of symbiotic *Rhizobium* strains and *Sinorhizobium fredii* [19]. This study reports the isolation, screening, production, and characterization of novel alkaline active thermostable phytase from a thermophilic strain of *Bacillus laevolacticus* isolated from the rhizosphere soil of leguminous plant methi (*Medicago falcata*).

Materials and methods

Organism and culture conditions

The soil samples from rhizosphere of leguminous plant (*M. falcata*) were suitably diluted and spread onto phytase screening medium (PSM) for selecting phytase-producing bacteria. The PSM screening medium contained (% w/v): Ca-phytate (Sigma, Poole, UK), 0.5; glucose, 1.0; $(NH_4)_2SO_4$, 0.03; $MgSO_4$, 0.05; $CaCl_2$, 0.01; $MnSO_4$, 0.001; $FeSO_4$, 0.001; and agar, 2.0, pH 7.0 [16]. The isolates showing clear zones on phytate screening medium were further screened on liquid production medium of following composition (%; w/v): pea flour, 0.5; sucrose, 1.0; asparagine, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4$, 0.001; and $MnSO_4$, 0.001, pH 5.5. The sterile production medium (50 ml) in 250 ml conical flasks was inoculated with 12 h old cultures grown on LB medium at 2% (v/v), and incubated under shaking conditions (120 rpm) at 45 °C for 24 h. The fermented broth was clarified by centrifugation at 11,000*g* for 10 min and assayed for phytase activity. The selected cultures were maintained on nutrient agar medium and stored at 4 °C.

Optimization of phytase production under shake flask cultures

The phytase production by *B. laevolacticus* was optimized under shake flask cultures. The effect of different production parameters, i.e. simple and complex carbon sources, nitrogen sources and their level, KH_2PO_4, pH, temperature, inoculum age, and level on enzyme production was studied.

Effect of sugars and complex carbon sources on the production of phytase by *Bacillus laevolacticus*

The effect of different sugars (at 1% w/v), i.e. as fructose, galactose, glucose, sucrose, lactose, maltose and xylose and complex carbon sources (at 0.5% w/v); pea flour, malto-dextrin, wheat bran, corn flour and coarse grounded rice, wheat, barley and corn on the production of phytase by *B. laevolacticus* was studied. The flasks were inoculated with actively growing culture and incubated at 45 °C under shaking (120 rpm) for 60 h.

Effect of nitrogen sources and level on the production of phytase by *Bacillus laevolacticus*

Effect of different nitrogen sources $(NH_4)_2SO_4$, $NaNO_3$, $NH_4H_2PO_4$, CH_3COONH_4, beef extract, corn steep liquor, malt extract, peptone, soybean meal, tryptone, urea, yeast extract on phytase production was studied by replacing asparagine in pea flour production medium. These nitrogen sources were added at concentrations equivalent to the amount of nitrogen in (0.1%) asparagine. The effect of different levels of selected nitrogen source, i.e. $NH_4H_2PO_4$ on production of phytase was studied in range of 0.1–0.5%. Furthermore, the effect of added source of phosphate as KH_2PO_4 along with different nitrogen sources was studied.

Effect of pH on the production of phytase by *Bacillus laevolacticus*

The influence of initial pH of the medium containing pea flour and 0.2% $NH_4H_2PO_4$ on the production of phytase was studied between pH 3.5 and pH 8.5.

Effect of temperature on the production of phytase by *Bacillus laevolacticus*

The effect of incubation temperature phytase production was studied between 30 and 60 °C in production medium with optimal pH of 5.5.

Effect of inoculum level and age on the production of phytase by *Bacillus laevolacticus*

The effect of different inoculum levels (1.0–5.0% v/v) on phytase production was studied. Furthermore, the effect of inoculum age (12, 24, 36, 48, 60, and 72 h) on

the production of phytase was studied at optimal inoculum level of (2% v/v).

Enzyme purification and characterization

The culture was grown under optimized culture conditions and the enzyme extract was recovered by centrifugation (11,000*g*, 10 min). To the extract was added sodium azide (0.001%), and concentrated using lyophilizer (Drywinner-3; HETO, Denmark).

The concentrated sample was loaded on to DEAE-Sepharose (Fast Flow) column (24 × 2.6 cm^2; Pharmacia, Phoenix, AZ, USA), equilibrated with sodium acetate buffer (20 mM, pH 5.0). The column was eluted first with the above said buffer (2-bed volume) followed by linear gradient of 0–0.5 M NaCl in acetate buffer (20 mM, pH 5.0) at a flow rate of 1 ml/min. The bound phytase was eluted using NaCl gradient buffer. The active fractions containing phytase activity were pooled, desalted, and concentrated using PD-10 column. Phytase was further purified using poly-buffer exchanger (PBE-94) column (15 × 1.0 cm^2). The column was equilibrated with sodium acetate buffer (20 mM, pH 5.0). The sample containing active fractions of phytase from DEAE-sepharose column was applied to the column. The column was eluted under isocratic conditions (3-bed volumes) using 20 mM acetate buffer and followed by a linear gradient of buffer containing 0–1.0 M NaCl at a flow rate of 0.2 ml/min. Fractions containing phytase activity were pooled for biochemical characterization. All purification steps were carried out using AKTA prime protein purification system (Amersham Biosciences, Freiburg, Germany).

Enzyme assay

Phytase activity was measured in an assay mixture containing 100 µl of sodium phytate (0.5 w/v) prepared in 0.2 M sodium acetate buffer pH 5.5 [1, 5, 7, 8] and 100 µl of suitably diluted enzyme. The reaction was stopped by adding an equal volume (200 µl) of 15% trichloroacetic acid after 30 min of incubation at 50 °C [3]. The liberated phosphate ions were quantified by mixing 100 µl of assay mixture with 900 µl of developing reagent containing 0.76 M H_2SO_4—2.5% ascorbic acid—0.06% ammonium molybdate (3:1:0.1). After 20 min of incubation at 50 °C, absorbance was measured at 820 nm using Novospec II spectrophotometer (Pharmacia). One unit of phytase activity was defined as the amount of enzyme required to liberate 1 µmole of phosphate per min under the assay conditions. The specific phytase activity was defined as U per mg of protein. Protein concentration in the enzyme preparations and during the purification steps was determined by the method of Bradford [2] using bovine serum albumin as standard.

SDS-PAGE

The partially purified phytase was fractionated by SDS-PAGE using 10% SDS-PAGE gels by the method of Laemmli using Mini–protein II electrophoresis unit (BIO RAD, Mississauga, ON, Canada). The proteins in the resolved gel were detected by silver staining.

Temperature and pH optima

The temperature profile of the phytase was obtained by determining the activity on sodium phytate between 30 and 90 °C. The optimal pH was determined by measuring the activity between pH 2.0 and pH 10.0 (0.2 M) using glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0–6.0), Tris–HCl (pH 7.0 and 8.0), and glycine-NaOH (pH 9.0 and 10.0) buffers at 50 °C.

The temperature and pH stability of phytase

For the determination of temperature and pH stability, the enzyme was pre-incubated (with or without 5 mM $CaCl_2$) at 60, 70 and 80 °C and pH of 6.0, 7.0 and 8.0, for 0–4 h. the residual phytase activity in the samples at different intervals was assayed using sodium phytate as substrate.

Effect of metal ions, salts and reducing compounds

Phytase was incubated in 5 mM of $MnSO_4$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $CuSO_4$, $CaCl_2$, $BaCl_2$, NaCl, KCl, SDS, EDTA, and DTT for 30 min at room temperature and the phytase activities in the aliquots were determined using sodium phytate as substrate.

Substrate specificity

The substrate specificity of phytase enzyme was tested against different type of phosphate substrates (0.5% w/v), i.e. Na-phytate, Ca-phytate, riboflavin phosphate, AMP, ADP, ATP, p-nitrophenyl phosphate, $NaDPH_2.Na_4$, phenyl phosphate, phosphoenol phosphate by incubating the enzyme separately with the substrates and measuring the amount of released phosphorus as described above.

Kinetic parameters

The V_{max} and K_m of phytase was determined against sodium phytate as substrate using Line weaver–Burk plot.

All experiments in the study were performed in triplicate and SE at 5% level was calculated.

Results and discussion

Isolation and screening of phytase producing bacteria

Twelve phytase producing thermophilic bacteria were isolated from the rhizosphere of leguminous plant methi (*M. falcata*) on the basis of transparent zones (Fig. 1) formed on the opaque PSM solidified medium containing Ca-phytate as a selective agent [16]. These cultures were further screened on pea flour-sucrose liquid medium [11], without Ca-phytate, for phytase production at 45 °C. Of the twelve isolates, strains designated as Tj1, Tj3, Tj4, and Tj6 produced 0.158, 0.216, 0.202, and 0.283 U/ml phytase, respectively. The best producing strain Tj6, a gram positive rod spore forming bacteria, was identified as *B. laevolacticus* on the basis of 16S rRNA sequence analysis. Idriss et al. [17] have reported the ability of some phytase producing root colonizing *B. amyloliquefaciens* strains to make the phytate phosphorus in soil available for plant nutrition under phosphate-starving conditions and thus contribute to their plant-growth-promoting activity. Few Gram-positive and Gram-negative soil bacteria e.g. *Bacillus subtilis* [20], *B. amyloliquefaciens* DS11 [22], *Klebsiella terrigena* [12], *Pseudomonas* sp. [29], and *Enterobacter* sp. 4 [37] that produce extracellular phytase have been isolated from soil near the root of leguminous plants.

Fig. 1 Growth and clearzone of *Bacillus laevolacticus* on phytase screening medium

Optimization of phytase production by *Bacillus laevolacticus* under shake flask culture

Effect of sugars and complex carbon sources

Of the various sugars (monosaccharides and disaccharides), sucrose was able to support maximal phytase expression (0.106 U/ml). Upon combining sucrose (1.0%) and pea flour (0.5%), the phytase production increased to 0.35 U/ml as compared to 0.299 U/ml when pea flour was the sole carbon source (Fig. 2). Earlier reports suggest wheat bran as a suitable carbon source for phytase production by *B. amyloliquefaciens* DS11, *B. subtilis*, and *B. amyloliquefaciens* FZB45 [17, 20, 22]; however, it poorly supported phytase production in *B. laevolacticus.* The observed high expression of phytase on pea flour medium by *B. laevolacticus* can be ascribed to it being a good source of oligosaccharides, polysaccharides (starch, lignin), and phytic acid [11]. The presence of sucrose that positively supported the phytase production has previously been used by Kim et al. [22] as optimal source of carbon during studies on the culture conditions for a new phytase producing fungus.

Effect of nitrogen sources

When asparagine was replaced in the production medium (Table 1) with $NH_4H_2PO_4$, as compared to other nitrogen sources, it showed dramatic increase in phytase production (0.975 U/ml). The phytase production was further enhanced to 1.76 U/ml when the level of $NH_4H_2PO_4$ was increased to 0.2%. The results of present study showed that $NH_4H_2PO_4$, a composite source of nitrogen and inorganic phosphorus, could stimulate high-phytase production. Similar observations were made when the other nitrogen sources included in the study were supplemented with inorganic

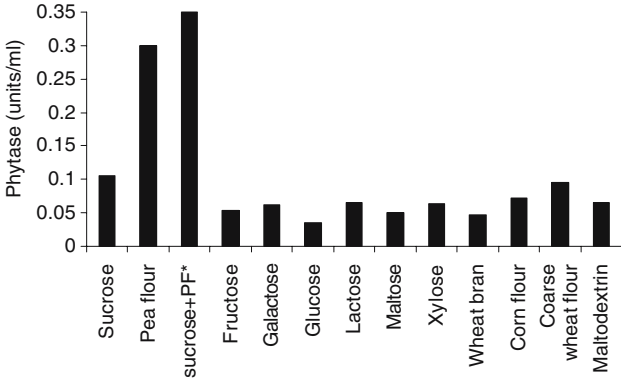


Fig. 2 Effect of sugars and complex carbon sources on phytase production by *Bacillus laevolacticus* (*asterisk* Pea flour)

Table 1 Effect of nitrogen sources on phytase production by *Bacillus laevolacticus*

Nitrogen sources	Phytase (U/ml)
$NH_4H_2PO_4$	0.975 ± 0.002
CH_3COONH_4	0.016 ± 0.0017
$(NH_4)_2SO_4$	0.018 ± 0.002
$NaNO_3$	0.004 ± 0.0016
Beef extract	0.236 ± 0.002
CSL	0.015 ± 0.003
Malt extract	0.011 ± 0.002
Peptone	0.004 ± 0.002
Soybean meal	0.014 ± 0.002
Asparagine	0.335 ± 0.0017
Tryptone	0.004 ± 0.0016
Urea	0.022 ± 0.0024

phosphorus (KH_2PO_4), although the observed phytase levels were maximal when $NH_4H_2PO_4$ was used in the medium (data not shown), though addition of the phosphate did not have any effect on the growth, monitored as O.D and biomass. The presence of inorganic phosphorus is an essential ingredient of phytase production medium [10, 31]; however, it negatively regulates the phytase expression when added at concentrations above 50 mg/l [22]. In case of *B. subtilis*, minimal medium containing inorganic phosphate and phytate did not induce phytase production but defined medium containing phytate as sole source could [20]. Similarly, phytase production was strongly inhibited in *Schwanniomyces castellii* when phosphorus levels exceeded 0.132% [23]. The observed ability of *B. laevolacticus* to tolerate high levels of phosphate, like that of previously observed in phytase producing rumen bacterium *Mitsuokella jalaludinii* [24], may result in much more efficient hydrolysis of phytate.

Effect of pH and temperature

The phytase production was studied over a pH range of 3.5–8.5. A pH of 5.5 was found optimal for phytase production (1.80 U/ml). The production of phytase at other pH was also not severely affected (data not shown). On the contrary, Lan et al. [24] reported that phytase production and bacterial growth were significantly inhibited when pH level of the medium was lower than 6.8. The optimal temperature for phytase production by *B. laevolacticus* was found to be 50 °C where maximal phytase activity (2.34 U/ml) after 60 h of incubation was achieved (Fig. 3). The other reported bacteria such as *Escherichia coli*, *M. jalaludinii*, *Lactobacillus amylovorus*, and *Bacillus* spp. produce optimally in mesophilic temperature range of 37–39 °C [24, 34, 35].

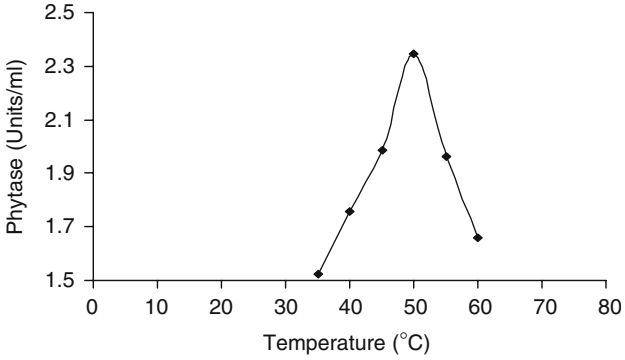


Fig. 3 Effect of temperature on the production of phytase by *Bacillus laevolacticus*

Effect of inoculum (level and age)

Maximal phytase yield (2.402 U/ml) was obtained with 2% (v/v) inoculum; however, phytase activity decreased at lower and higher inoculum concentrations. The respective phytase activities obtained at 1, 3, 4, and 5% (v/v) inoculum levels was 1.884, 1.858, 1.006, and 0.542 (U/ml). The inoculum age too had a significant influence on phytase production; it was found that 24 h old actively growing culture (2% v/v) resulted in increased phytase activity (2.95 U/ml; Fig. 4). However, use of 12 and 36 h old culture as inoculum resulted in decreased phytase activities, 2.502 and 1.961 (U/ml), respectively. The maximal phytase activity under the optimized conditions was achieved during late exponential phase after 60 h of incubation at 50 °C (Fig. 5). Yoon et al. [37] also observed that the phytase activity reached a maximum at 72 h when the culture entered the stationery phase and then decreased slightly. The observed production pattern was ascribed to either a nutrient or energy limitation known to occur in the stationary phase as trigger for phytase production [9].

Fig. 4 Effect of inoculum age on the production of phytase by *Bacillus laevolacticus*

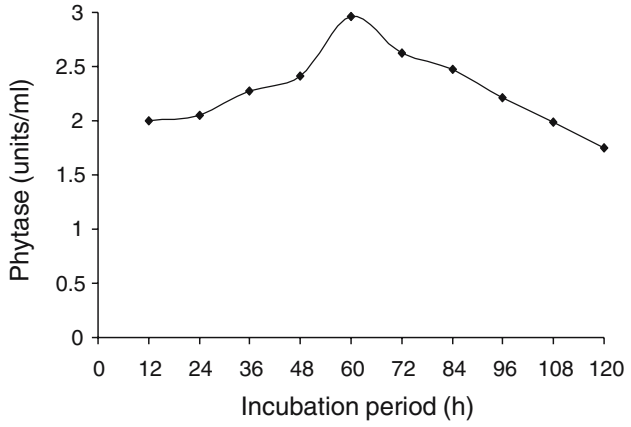


Fig. 5 Profile of phytase production by *Bacillus laevolacticus*

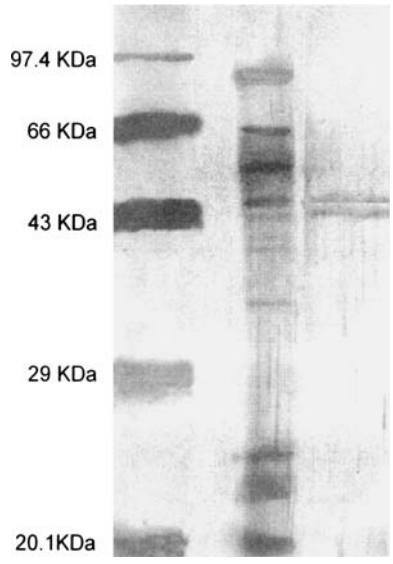


Fig. 6 SDS-PAGE analysis of phytase from *Bacillus laevolacticus*—lane 1: molecular mass marker (Phosphorylase b, 97,400; Bovine serum albumin, 66,000; ovalbumin, 43,000; Carbonic anhydrase, 29,000; Soybean trypsin inhibitor 20,100); lane 2: Crud; lane 3: partially purified phytase

Characterization and purification of phytase from *Bacillus laevolacticus*

Phytase was partially purified to 6.5-fold with 24.4% yield using two steps ion exchange chromatography. The enzyme exhibited a specific activity of 12.69 U/mg proteins (Table 2). The partially purified preparation on SDS-PAGE showed two bands corresponding to 41 and 46 kDa (Fig. 6). Earlier reports on the purified phytase from *Bacillus* sp. KHU-10, *B. subtilis* (natto) N-77, *B. subtilis, B. amyloliquefaciens* had estimated the molecular weight (Mr) of 44, 33.8, 36, and 44 kDa [9, 22, 28, 32], respectively.

The partially purified phytase was optimally active at pH 7.0 and 70 °C (Figs. 7, 8) and was catalytically active under alkaline pH range (8–10) showing 90% of the optimal activity. The phytase was thermostable at 60 °C and showed ~80% of residual activity after 3 h of incubation at pH 8.0 in presence/absence of $CaCl_2$. However, in the presence/absence of 5 mM $CaCl_2$, phytase was also stable at 80 °C and showed ~100% of residual activity for 30 min at pH 8.0 (Fig. 9) and then declined sharply thereafter. Simon and Igbasan [33] has reported that the stability of *Bacillus* phytase is strongly dependent on the presence of calcium though *B. laevolacticus* did not show such strong dependence on calcium. The observed stability of phytase from *B. laevolacticus* was higher then the reported temperature of 50–60 °C for *Enterobacter* sp.4 [37], 60 °C for *B. subtilis* (natto) N-77 [32]. Thermostability is particularly an important trait since feed pelleting is commonly performed at temperatures between 65 and 95 °C and, therefore, the enzyme should withstand inactivation due to high temperatures.

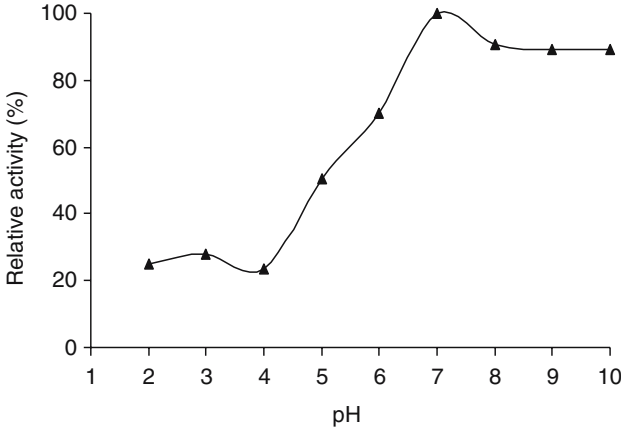


Fig. 7 Effect of pH on partially purified phytase activity

Table 2 Purification procedures for the phytase from *Bacillus laevolacticus*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	67.5	131	1.94	1	100
DEAE-sepharose	30	83	2.77	1.43	63.45
PBE-94	2.5	31.73	12.69	6.50	24.4

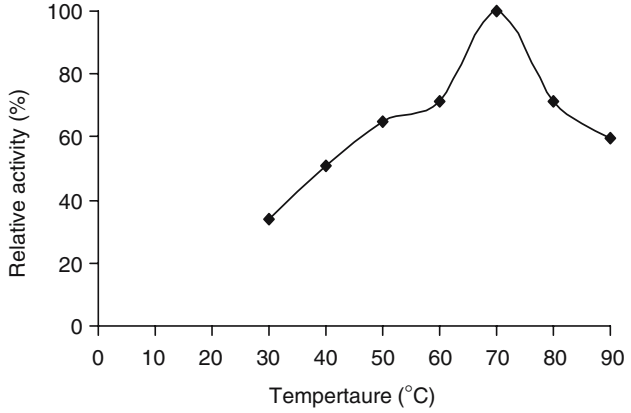


Fig. 8 Effect of temperature on partially purified phytase activity

The presence of $CuSO_4$ and $ZnSO_4$ moderately inhibited the phytase activity; however, $MnSO_4$, $CaCl_2$, $MgSO_4$, NaCl, KCl, $BaCl_2$ and $CoCl_2$ showed insignificant effect on the enzyme activity. The presence of EDTA, at 1 mM inhibited the enzyme activity up to 90% and completely at 5 mM concentration, indicating the role of metal ions in the enzyme activity. The phytases from *B. subtilis* [21, 32] and *B. amyloliquefaciens* [22] were also reported to be Ca^{+2} dependent which under alkaline conditions formed metal-phytate complex with divalent metal cations [6, 25].

The alkaline active phytase from *B. laevolacticus* showed high selectivity for calcium phytate with 1.33-fold higher activity as compared to sodium phytate. The alkaline phytases of *Bacillus* sp. KHU-10, *Bacillus* DSII, *B. subtilis, Klebsiella pneumoniae* subsp. *Pneumoniae* XY-5, and *E. coli* exhibited strict substrate specificity for phytate esters [9, 13, 18, 22, 28, 36].The phytase from *B. laevolacticus*, however, showed relaxed substrate specificity and registered activities between (5.08–40%) against p-nitro-phenyl-phosphate, AMP, ATP, phenyl phosphate, and $NaDPH_2.Na_4$ (Table 3). The apparent K_m and V_{max} for the hydrolysis of partially purified phytase against sodium phytate was determined to be 0.526 mM and 12.3 µmole/min/mg, respectively. The K_m values of other phytases for sodium phytate from *B. subtilis* (natto) [32], *Bacillus* sp. DSII [22], and *Bacillus* KHU-10 [9] has also been reported in the same range. On the basis of these results, the alkaline phytase from *B. laevolacticus* can be classified as *PhyD* [27].

Bacillus laevolacticus is a good source of thermostable alkaline phytases insensitive to the presence of inorganic phosphate in the medium that produces high levels of phytase as compared to other reported wild type strains of *Bacillus* sp. (Table 4).The partially purified enzyme showed properties that are suitable for animal feed applications.

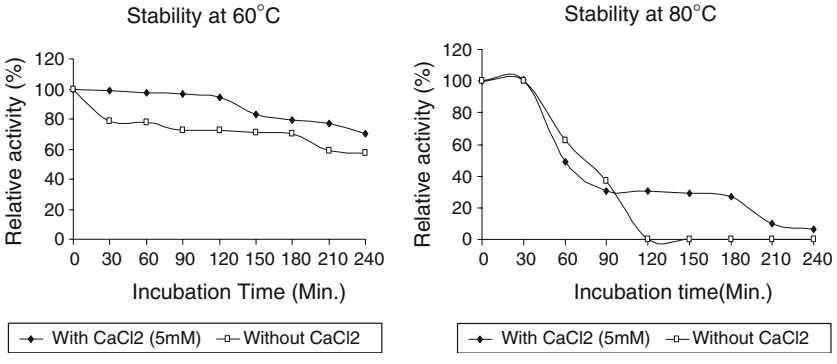
Table 3 Substrate specificities of phytase from *Bacillus laevolacticus*

Substrates (0.5% w/v)	Relative activity (%)
Na-phytate	100
Ca-phytate	133.29
Riboflavin phosphate	0
AMP	5.08
ADP	102.96
ATP	41.33
p-NPP	5.08
$NaDPH_2 \cdot Na_4$	8.89
Phenyl phosphate	32.44

Table 4 Comparative phytase activity of different *Bacillus* strains

Bacillus strains	Phytase (U/ml)	References
Bacillus	0.024	Power and Jagannathan [28]
Bacillus sp. KHU-10	0.160	Choi et al. [9]
Bacillus amyloliquefaciens (Recombinant)	2.0	Kim et al. [22]
Bacillus subtilis VTTE-68013 (Recombinant)	27.1	Kerovuo et al. [20]
Bacillus laevolacticus	2.95	This work

Fig. 9 Stability of partially purified phytase at alkaline pH 8.0 and at different temperature (60 and 80 °C), presence/absence of $CaCl_2$ (5 mM)



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