

Full Length Research Paper

Production and characterization of a thermostable beta-propeller phytase from *Bacillus licheniformis*

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A novel beta-propeller phytase producing thermophilic strain of *Bacillus licheniformis* was isolated. The culture conditions for production of phytase by *B. licheniformis* under shake flask culture were optimized to obtain high levels of phytase (0.267 U/ml). The optimum temperature and pH of the phytase from *B. licheniformis* (PhyC) were 55°C and 7.0, respectively. The enzyme exhibited good thermostability and pH stability in the presence of 1 mM CaCl₂. After treatment at 80°C, pH 7.0 for 10 min, the residual activity of PhyC was 57.36%. Over 80% of PhyC activity was retained after treatment by preincubation over a pH range of 6.5 to 9.0 for 1 h at 25°C. Substrate was very specific for sodium phytate and showed no activity on other phosphate esters. Its activity was greatly inhibited by EDTA and metal ions such as Cd²⁺, Mn²⁺, Cu²⁺ and Ba²⁺.

Key words: *Bacillus licheniformis*, phytase, production, optimization, characterization.

INTRODUCTION

Phosphorus is one of the necessary mineral nutrients for animals during their growth, reproduction and calcification of the bones. The major storage form of phosphorus in plant seeds is phytate (*myo*-inositol-hexakisphosphate). Monogastric animals, such as swine and poultry, as well as fish, are unable to utilize phytate due to little or no intestinal phytase activity in their digestive tracts (Reddy et al., 1982). To prevent dietary phosphorus deficiency, inorganic phosphate is commonly added to the feed for the purpose of phosphorus supplementation (Wyss et al., 1999; Mullaney and Ullah, 2005). In addition, undigested phytate reduces mineral bioavailability and impairs protein digestibility in the animal (Maenz, 2001). The undigested phytate and the unabsorbed inorganic phosphorus in monogastric derived faeces have the potential to cause phosphorus pollution in areas of intensive animal production (Ullah et al., 2003; Chen et al., 2004).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 or EC 3.1.3.26) are a

group of enzymes that catalyze the sequential hydrolysis of phytate in a stepwise manner to lower inositol phosphate, *myo*-inositol and inorganic phosphate (Mitchell et al., 1997; Farhat et al., 2008). This hydrolytic reaction plays an important role in energy metabolism, metabolic regulation and signal transduction pathways in biological system (Vats and Banerjee, 2002). More recently, increasing public concern regarding the environmental impact of high phosphorus levels in animal faeces has driven phytase application in animal feed and the biotechnological development of phytase (Lei and Stahl, 2001).

Phytases can be derived from a host of sources including plants (Maugenest et al., 1997), animals (Craxton et al., 1997) and microorganisms (Dassa et al., 1990; Ostanin et al., 1992; Rao et al., 2008), however, microbial sources are more promising for the production of phytases on a commercial scale. A wide variety of microbial phytases have been identified and characterized

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during the last decade, with a few of these phytases, from *Aspergillus* spp. and *Peniophora lycii*, being used as feed additives (Simon and Igbasan, 2002; Haefner et al., 2005). On the basis of pH profile, higher thermostability, and strict substrate specificity as well as physiological nature of phytate in digestive tract, neutral phytases from *Bacillus* species are considered ideal candidates for application in animal feed (Oh et al., 2004; Fu et al., 2008; Guerrero-Olazarán et al., 2010).

This paper describes the identification and production in submerged fermentation of thermostable beta-propeller phytase from a thermophilic strain of *Bacillus licheniformis* and characterization of the enzyme in terms of its potential application as a feed additive.

MATERIALS AND METHODS

Phytase assay

Phytase activity was determined according to the method described by Heinonen and Lahti (Heinonen and Lahti, 1980) with appropriate modification. Enzyme (100 µl) and substrate (900 µl 0.1% (w/v) sodium phytate in 0.2 M Tris-HCl buffer (pH 7.0) supplemented with 1 mM CaCl₂) were co-incubated at 55°C for 30 min. One unit of phytase activity (1.0 IU) is defined as the amount of enzyme that catalyses the production of 1 µmol inorganic phosphate per minute under the standard assay conditions. Protein concentration was measured by the dye-binding assay method of Bradford (1976), and bovine serum albumin (BSA) was used as the standard.

Isolation and identification of neutral phytase-producing strains

The local soil samples were suitably diluted and spread onto phytase screening culture medium (SCM) for selecting phytase-producing bacteria. The SCM contained (%; w/v): Calcium phytate, 0.5; NH₄NO₃, 0.5; KCl, 0.5; glucose, 3.0; MgSO₄·7H₂O, 0.05; MnSO₄·4H₂O, 0.03; FeSO₄·7H₂O, 0.03; and agar 2.0, pH 7.0. The isolates showing clear zones on phytate screening medium were further screened on submerged fermentation medium (SFM) of the following composition (%; w/v): glucose, 2.0; NH₄NO₃, 0.2; MgSO₄·7H₂O, 0.05; KCl, 0.05; FeSO₄·7H₂O, 0.001; MnSO₄·4H₂O 0.001; and CaCl₂, 0.2, pH 7.0. 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 55°C for 24 h. The fermented broth was clarified by centrifugation at 10000 rpm 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

Effect of various carbon sources on the production of phytase by B. licheniformis

The effect of various carbon sources (at 1% w/v), that is, glucose, sucrose, lactose, maltose, cellulose, dextrine, wheat bran, potato flour, corn flour and rice bran on the production of phytase by *B. licheniformis* was studied. The flasks were inoculated with actively growing culture and incubated at 55°C under shaking (120 rpm) for 48 h.

Effect of various nitrogen sources on the production of phytase by B. licheniformis

Effect of different nitrogen sources (at 0.1% w/v): (NH₄)₂SO₄, NH₄Cl, NH₄NO₃, urea, beef extract, yeast extract, peptone, soybean meal, rapeseed meal and cottonseed meal on phytase production was studied by replacing NH₄NO₃ in submerged fermentation medium.

Effect of initial pH on the production of phytase by B. licheniformis

The influence of initial pH of the medium on the production of phytase was studied between pH 4.0 and 10.0.

Effect of temperature on the production of phytase by B. licheniformis

The effect of incubation temperature on phytase production was studied between 20 and 70°C in the production medium with optimal pH of 7.5.

Effect of incubation period on the production of phytase by B. licheniformis

Various incubation periods (12, 24, 36, 48, 60, 72 and 84 h) were assayed, tested, studied and the fermentation was performed with all other parameters kept at their optimum levels.

Characterization of phytase from *B. licheniformis*

Temperature and pH optima

The temperature profile of the phytase was obtained by determining the activity on sodium phytate between 30 and 90°C at 5-degree intervals. The optimal pH was determined by measuring the activity between pH 2.0 and 9.0 (0.2 M) using glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 to 6.0) and Tris-HCl (pH 7.0, 8.0 and 9.0) buffers at 55°C.

Thermostability and pH stability

For the determination of thermostability and pH stability, the enzyme was pre-incubated (with or without 1 mM CaCl₂) at 30 to 90°C for 10 min and pH of 2.0 to 9.0 for 1 h, the residual phytase activity was assayed using sodium phytate as substrate.

Effect of metal ions and reducing compounds

The effect of metal ions and reducing compounds on phytase activity was examined with sodium phytate as a substrate after removal of Ca²⁺. Phytase was incubated in 1 and 5 mM of CuCl₂, ZnCl₂, MgCl₂, MnCl₂, LiCl₂, CaCl₂, CdCl₂, BaCl₂, EDTA, DTT and PMSF for 30 min at room temperature.

Substrate specificity

The substrate specificity of the enzyme was tested against different types of phosphate substrates (2 mM): Na-phytate, tripolyphosphate, α-Glycerophosphate, β-Glycerophosphate, p-nitrophenyl phosphate, α-Naphthyl phosphate, AMP, ADP and ATP by incubating the enzyme separately with the substrates and measuring the amount of released phosphorus as described above.

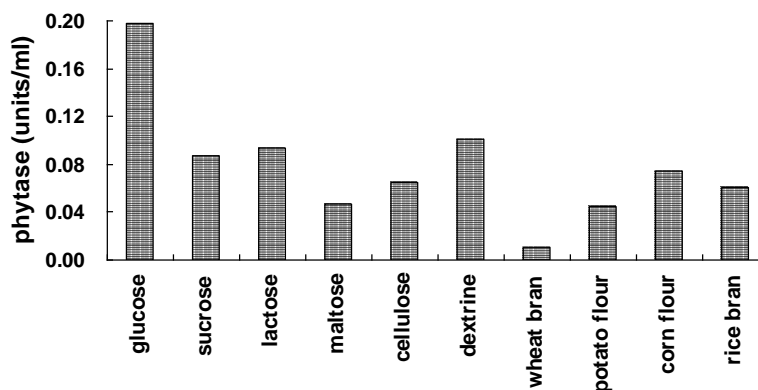


Figure 1. Effect of various carbon sources on phytase production by *B. licheniformis*.

Analytical methods

Statistical analysis

One-way ANOVA followed by LSD post-hoc test was used to determine significant differences among treatment groups. For all analysis, differences were considered to be significant at $P < 0.05$ (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Isolation and screening of phytase producing bacteria

A total of twelve phytase producing thermophilic bacteria were isolated on the basis of transparent zones formed on the opaque SCM medium containing Ca-phytate as a selective agent. The isolates showing clear zones on phytate screening medium were further screened in submerged fermentation medium, without Ca-phytate, for phytase production at 55°C. Of the twelve isolates, strain designated as ZJ-6 produced the highest phytase (0.1511 U/ml) and was identified as *B. licheniformis* on the basis of 16S rRNA sequence analysis. Several *Bacillus* species such as *Bacillus* sp. DS11 (Kim et al., 1998), *Bacillus subtilis* (Kerovuo et al., 1998), *Bacillus* sp. KHU-10 (Choi et al., 1999), *Bacillus laevolacticus* (Gulatih et al., 2007) that produce thermostability beta-propeller phytase were isolated from soil.

Optimization of phytase production under shake flask cultures

Effect of various carbon sources on the production of phytase by *B. licheniformis*

Of the various carbon sources, glucose was able to support maximal phytase expression (0.1987 U/ml) (Figure 1). Earlier reports suggest wheat bran as a

suitable carbon source for phytase production by *Bacillus amyloliquefaciens* DS11, *B. subtilis*, and *B. amyloliquefaciens* FZB45 (Kim et al., 1998; Kerovuo et al., 1998; Idriss et al., 2002), sucrose as a suitable carbon source for phytase production in *Bacillus laevolacticus* (Gulatih et al., 2007). Batal and Karem (2001) reported that adding glucose at level of 6% in solid state fermentation resulted in the highest enzyme production and higher glucose concentrations (10 and 20%) inhibited the enzyme production.

Effect of various nitrogen sources on the production of phytase by *B. licheniformis*

When NH_4NO_3 was replaced in the production medium with $(\text{NH}_4)_2\text{SO}_4$, as compared to other nitrogen sources, it showed dramatic increase in phytase production (0.218 U/ml) (Table 1). The results of the present study showed that $(\text{NH}_4)_2\text{SO}_4$ could stimulate high-phytase production.

Effect of initial pH on the production of phytase by *B. licheniformis*

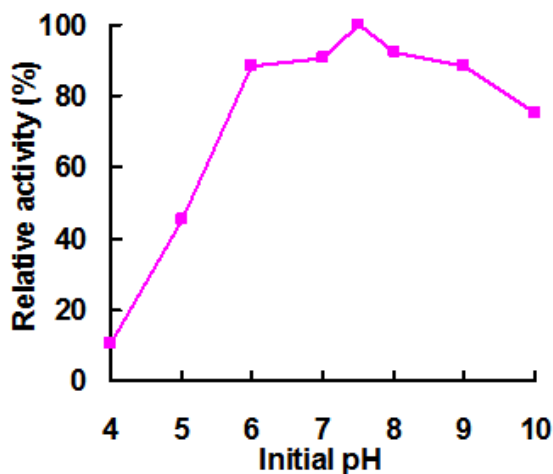
The phytase production was studied over a pH range of 4.0 to 10.0. A pH of 7.5 was found optimal for phytase production (Figure 2). The production of phytase at pH 6.0 to 10.0 was not severely affected and sharply declined when pH level was below 6.0. On the contrary, Lan et al. (2002) reported that phytase production and bacterial growth were significantly inhibited when pH level of the medium was lower than 6.8.

Effect of incubation temperature and period on the production of phytase by *B. licheniformis*

The optimal incubation temperature for phytase

Table 1. Effect of various nitrogen sources on phytase production by *B. licheniformis*.

Nitrogen sources	Phytase (U/ml)
(NH ₄) ₂ SO ₄	0.218±0.003
NH ₄ Cl	0.178±0.002
NH ₄ NO ₃	0.104±0.005
beef extract	0.126±0.003
urea	0.014±0.009
yeast extract	0.074±0.004
peptone	0.022±0.002
soybean meal	0.136±0.003
rapeseed meal	0.019±0.001
cottonseed meal	0.067±0.003

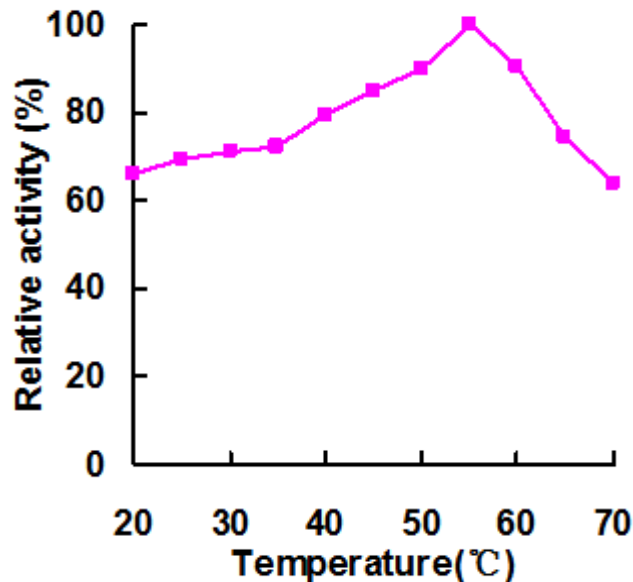
**Figure 2.** Effect of initial pH on phytase production by *B. licheniformis*.

production by *B. licheniformis* was found to be 55°C (Figure 3). This result was different from earlier reports. The optimal temperature for phytase production by *B. laevolacticus* was 50°C (Gulatih et al., 2007). The other reported *Bacillus* species such as *Bacillus* sp. KHU-10 (Choi et al., 2001), *Bacillus* sp. DS11 (Kim et al., 1998), *B. subtilis* (Kerovuo et al., 1998), produced maximal activity at 37°C. The maximal phytase activity (0.267 U/ml) under the optimized conditions was achieved during late exponential phase after 36 h of incubation at 55°C (Figure 4). Choi et al. (2001) observed that *Bacillus* sp. KHU-10 produced the highest activity of phytase after 4 days of fermentation under optimized conditions.

Characterization of the phytase from *B. licheniformis*

Temperature optimum and stability

The optimum temperature was 55°C as shown in Figure 5.

**Figure 3.** Effect of incubation temperature on phytase production by *B. licheniformis*.

To test the thermostability, the activity of the PhyC was measured after incubation at various temperatures for 10 min. The enzyme stability of the PhyC was drastically reduced above 40°C in the absence of CaCl₂. After incubation at 80°C in the presence of 1 mM CaCl₂ for 10 min, the remaining enzyme activity was 57.36% of the initial activity. These results indicated that Ca²⁺ ion has a stabilizing effect on the enzyme against thermal denaturation. This property would be very useful for the enzyme to withstand inactivation during feed-pelleting or the expansion process and decrease costly formulations to limit activity loss industrial applications.

pH optimum and stability

The phytase showed high activity at relatively broad pH range between pH 6.0 and 8.0 in the reaction mixture (Figure 6). The addition of 1 mM CaCl₂ to the incubated buffers made the enzyme more stable in the pH range of 5.0 to 9.0. In the presence of 1 mM CaCl₂, the remaining enzyme activity was above 60% after incubation at 55°C for 1 h. These results indicated that Ca²⁺ ion has an effect on enzyme activity against pH.

Effect of metal ions and reducing compounds on the PhyC

The effect of reagents and metal ions on the enzyme activity was examined using sodium phytate as a substrate after removal of calcium ions in enzyme solution by dialysis (Table 2). The enzyme activity was greatly

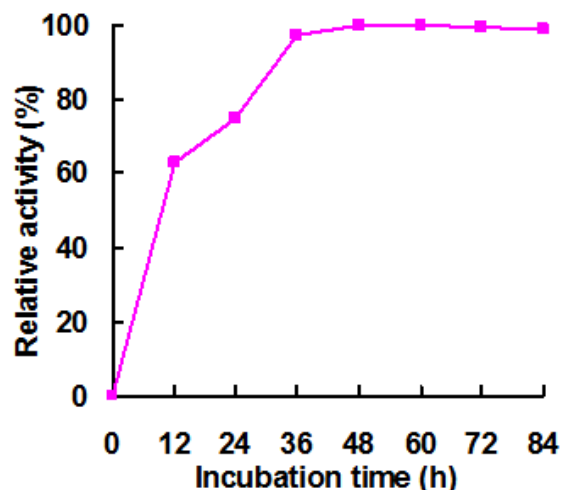


Figure 4. Effect of incubation time on phytase production by *B. licheniformis*.

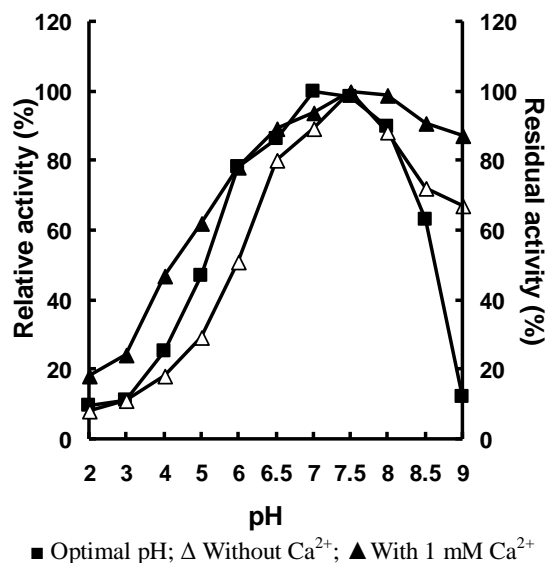


Figure 6. Effect of pH on the activity and stability of the PhyC. The enzyme was assayed at various pH buffers. For pH stability, the enzyme was pre-incubated at various pH buffers without/with 1 mM CaCl_2 for 1 h.

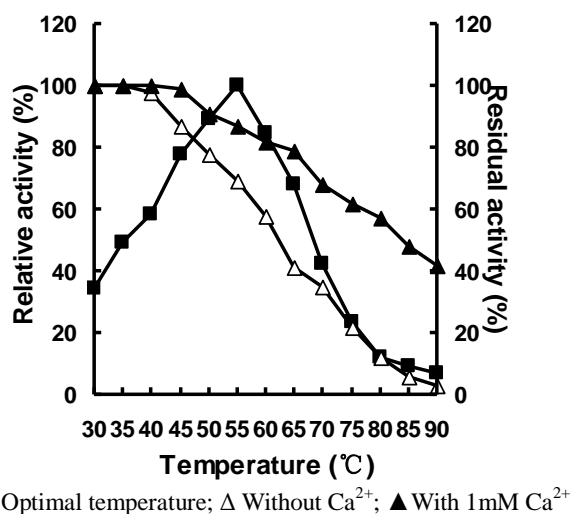


Figure 5. Effect of temperature on the activity and stability of the PhyC. The enzyme activity was assayed at various temperatures. For thermostability, the enzyme was preincubated at various temperatures without/with 1 mM CaCl_2 for 10 min.

Table 2. Effect of metal ions and reagents on the activity of the PhyC.

Reagents	Relative activity (%)	
	1 mM	5 mM
None	100	100
CuCl_2	66	49
ZnCl_2	77	69
MgCl_2	87	75
MnCl_2	66	26
LiCl_2	95	99
CaCl_2	112	98
CdCl_2	10	6
BaCl_2	72	37
EDTA	6	2
DTT	93	90
PMSF	89	88

inhibited by EDTA, Cd^{2+} , Mn^{2+} , Cu^{2+} and Ba^{2+} . PMSF and DTT showed little effect on the enzyme activity. Removal of metal ions from the enzyme by EDTA resulted in complete inactivation. The loss of enzymatic activity was most likely due to a conformational change, as the circular dichroism spectra of holoenzyme and metal depleted enzyme were different (Kerovuoto et al., 2000).

Substrate specificity

The substrate specificity of the PhyC on several phosphate

esters was tested in 0.1 M Tris-HCl buffer (pH 7.0). Controls were run for determining initial phosphorus in each substrate. As summarized in Table 3, the enzyme had high activity for sodium phytate, but no activity on other phosphorylated compounds including sodium p-nitrophenyl phosphate, a general substrate for acid phosphatase. These results imply that the PhyC is specific for inositol polyphosphate. This property made the PhyC distinct from *B. subtilis* phytase, which has hydrolytic activity on some inorganic compounds as well

Table 3. Substrate specificity of the PhyC.

Substrate (2 mM)	Relative activity (%)
Sodium phytate	100
Sodium tripolyphosphate	8
Sodium pyrophosphate	0
Sodium α -Glycerophosphate	0
Sodium β -Glycerophosphate	0
Sodium p -nitrophenyl phosphate	0
Sodium α -Naphthyl phosphate	0
ATP	0
ADP	0
AMP	0

as phytate (Shimizu, 1992).

Conclusions

In this study, a novel beta-propeller phytase producing thermophilic strain of *B. licheniformis* was isolated. The optimal fermentation parameters for producing phytase by *B. licheniformis* were determined by single factor test. Glucose and $(\text{NH}_4)_2\text{SO}_4$ were the most suitable carbon and nitrogen source for phytase production from *B. licheniformis*, respectively. The optimal fermentation conditions were: initially pH 7.5, incubation temperature 55°C. The culture conditions for production of phytase by *B. licheniformis* under shake flask culture were optimized to obtain high levels of phytase (0.267 U/ml).

The optimum temperature and pH of the phytase from *B. licheniformis* (PhyC) were 55°C and 7.0, respectively. The enzyme exhibited good thermostability and pH stability in the presence of 1 mM CaCl_2 . After treatment at 80°C, pH 7.0 for 10 min, the residual activity of PhyC was 57.36%. Over 80% of PhyC activity was retained after treatment by preincubation over a pH range of 6.5 to 9.0 for 1 h at 25°C. Substrate was very specific for sodium phytate and showed no activity on other phosphate esters. Its activity was greatly inhibited by EDTA and metal ions such as Cd^{2+} , Mn^{2+} , Cu^{2+} and Ba^{2+} . The higher temperature-stability of the rePhyCm suggests that it may be a potentially better candidate in commercial and environmental applications.

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