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# Purification and characterization of phytase from *Bacillus lehensis* MLB2

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**Abstract:** A potent phytase-producing bacterium *Bacillus lehensis* MLB2 was isolated from bean-grown soil. The optimum conditions recorded after optimization were 24 h incubation time, pH 5.5, 37 °C, 2% inoculum level, 0.5% rice bran and 0.5% potassium nitrate. An overall 3.144-fold enhancement in phytase production was achieved after optimization. The use of an inexpensive substrate rice bran and short incubation period make the phytase production cost effective. The purified phytase (152.9 U/mg) had a molecular mass of approximately 98.686 kDa as determined by sodium dodecyl sulphate-polyacryalamide gel electrophoresis and confirmed by liquid chromatography-mass spectrometry, optimum pH of 4.5, and temperature of 37 °C. It maintained maximum stability in the acidic region from pH 2.0 to 6.0 and retained 100% at 60 °C or below. It showed an enhanced activity in the presence of 5 mM $K^+$ and $Na^+$. $Ca^{2+}$, $Mg^{2+}$, and $Ba^{2+}$ did not have any effect or slightly activate the phytase. Group-specific reagents indicated the presence of cysteine and tryptophan in or near the active site of the enzyme. Better pH and temperature broad range adaptability, strict sodium phytate specificity and low $K_m$ value of 0.1232 mM, and *in vitro* release of a significant amount of orthophosphate from feedstuffs, and thus reduction of environmental phosphorus pollution, make the *B. lehensis* MLB2 phytase a good candidate for feed additive applicability.

**Key words:** *Bacillus lehensis* MLB2; phytase; rice bran; inorganic phosphate; feed additive.

**Abbreviations:** DDT, dithiothreitol; EDTA, ethylenediaminetetraacetatic acid; NAI, *N*-acetyl imidazole; NBS, *N*-bromosuccinimide; NEM, *N*-ethylmaleimide; PAGE, polyacryalamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; TPTZ, 2,4,6-Tris(2-pyridyl)-*s*-triazine.

## Introduction

Phytate is the principal form of phosphorus occurring mainly in oilseed meals, cereal grains, and legumes, accounting for 18–88% of total P content (Reddy et al. 1982; Nayini & Markakis 1984). Phytate was found to be an anti-nutritional factor, since it significantly chelates various metal ions such as $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Fe^{3+}$, $Na^+$, $Cu^{2+}$, and $Zn^{2+}$, leading to mineral deficiency. It also affects animal digestion by forming complexes with proteins (Wodzinski & Ullah 1996; Vohra & Satyanarayana 2003). Phytases catalyze the stepwise dephosphorylation of phytate to orthophosphate and inositol. They are obtained from microorganisms since microbial phytases possess better stability compared to animal or plant phytases (Dvořáková 1998; Vohra & Satyanarayana 2003). Based on the catalytic mechanism, phytases are classified into histidine acid phytases, $\beta$-propeller phytases, cysteine phytases and purple acid phytases (Nayini & Markakis 1984; Vohra & Satyanarayana 2003; Vats & Banerjee 2004). Phytases have been often used as feed additive in fish, swine, or poultry diets. Numerous animal studies have shown the importance of the supplemental microbial phytase in maximizing the utilization of phosphate from phytate, and in reducing the environment pollution that may result from excessive manure phosphorus runoffs (Ahmad et al. 2000; Cho et al. 2003; Park & Cho 2011).

The industrial demand of phytase with better properties continues to stimulate the researchers. For both economic and environmental reasons, the availability of inorganic P of phytate is necessary to reduce the total cost of inorganic P additive for animal diets (poultry, fish and swine diets) and to limit the total P levels in manure (Reddy et al. 1982; Vohra & Satyanarayana 2003; Vats & Banerjee 2004). Isolation and optimization of culture conditions for phytase production in a significant amount is a continuous exercise. Enzyme purification and characterization must also be carried out in order to discover new phytases with de-

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sired properties. Various bacterial phytases have been purified to homogeneity, using cation and anion exchange chromatography, gel filtration chromatography, or a combination of these methods (Cho et al. 2003; Hong et al. 2011; Escobin-Mopera et al. 2012, Roy et al. 2012; El-Toukhy et al. 2013). Although various bacterial and fungal phytases have been purified, none possesses all the desired properties that can be commercially exploited. There is thus a continuous demand of novel industrial phytases with better properties destined for use as feed supplements.

The phytase of *Bacillus lehensis* MLB2 has never been purified and characterized, and tested as feed additive. The objectives of the present study were to purify and characterize the phytase of *B. lehensis* MLB2 as well as to ascertain its suitability as feed supplement by analyzing the *in vitro* inorganic phosphate liberated by its action on the major animal feedstuff ingredients, *viz.* wheat bran and rice bran.

## Material and methods

*Chemical and reagents*

Sodium phytate (phytic acid dodecasodium salt), phenylmethylsulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), *N*-bromosuccinimide (NBS), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), dithiothreitol (DTT), and *N*-acetyl imidazole (NAI) were procured from Sigma-Aldrich Co. (St Louis, MO, USA). The other chemicals, such as molecular weight markers, sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), urea, sodium azide ($NaN_3$), acrylamide, and bisacrylamide were brought from Hi-Media Laboratories (Mumbai, India). All the chemicals and reagents used were of analytical grade.

*Isolation and identification of phytase-producing bacteria*

The soil sample was collected from bean-growing field under sterile conditions, serially diluted ($10^{-1}$–$10^{-5}$) and spread plated on the modified specific sodium phytate agar screening medium (Cho et al. 2003). The medium (pH 5.5) contained (g/L): sodium phytate (as the sole C and P source) – 5.0, sodium chloride – 0.1, ammonium nitrate – 5.0, magnesium sulphate – 0.1, potassium chloride – 0.5, ferrous sulphate – 0.01, calcium chloride – 0.1, manganese sulphate – 0.01, and agar – 17.5. The incubation was carried out at 30 °C for 72 h. The production of phytase was indicated by clear zones around them. The bacterial isolates with higher clear zones were re-plated twice, grown in phytase production medium with shaking at 100 rpm for 24 h at 30 °C, and activity determined. The isolate with higher phytase activity was identified by morphological and physiological aspects, as described in the Bergey's manual of systematic bacteriology. It was sent to Bangalore Genei Pvt. Ltd. (Karnataka state, India) for strain level identification.

*Bacterial inoculum preparation and submerged fermentation*

The isolate was grown on the nutrient agar for 24 h at room temperature (30 °C). Nutrient broth (25 mL) was then inoculated with a loopful from agar slant bacterial culture, incubated with shaking at 100 rpm (Model S150, Stuart, India) for 24 h at 30 °C. The resulted broth served as inoculum. The inoculum (1 mL) was taken from the culture broth and inoculated into 100 mL of the fermentation medium contained in 250-mL Erlenmeyer flask. The fermentation medium used was the same as the one used for isolation except that it does not contain agar. After 24 h of incubation on a shaker at 100 rpm and 30 °C, the broth was centrifuged with cooling centrifuge (C-30 BL Remi, India) at 10,000 rpm for 10 min at 4 °C. The clear supernatant served as crude phytase and was used in the phytase activity determination.

*Phytase assay and protein estimation*

Extracellular phytase activity was assayed by the modified method of Holman (1943) using sodium phytate as substrate. Sodium phytate (0.95 mL, 5 mM) in acetate buffer (50 mM, pH 5.5) was mixed with 0.05 mL enzyme, and incubated at 37 °C for 15 min. The reaction was arrested by addition of 1 mL of 10% (w/v) trichloroacetic acid. Two mL of freshly prepared colouring reagent [sulphuric acid (3.2%, v/v), ammonium molybdate (1%, w/v), and ferrous sulphate (7.2%, w/v)] was added and the absorbance was measured at 700 nm (UV spectrophotometer, Model SL-159, Elico, India) after a proper dilution. One unit of phytase activity is the amount of enzyme, which releases 1 µmol of inorganic phosphate per min per mL under the assay conditions. The protein content was estimated by Lowry et al. (1951) using the bovine serum albumin as standard.

*Optimization of culture conditions*

The important factors affecting the phytase production were investigated, optimizing each parameter at a time keeping the other nutritional and physicochemical parameters unchanged. Time course for maximum phytase production was first studied for 3 days with shaking at 100 rpm, recording enzyme activity every 12 h. The effect of different inoculum level ranging from 1% to 5% on enzyme production was evaluated at pH 5.5 by incubating for 24 h at 30 °C. The effects of pH and temperature in the 4.5–7.5 and 20–55 °C ranges, respectively, were also evaluated. The effect of nutritional factors, such as carbon sources (glucose, sucrose, rice bran, and sodium phytate as control) and nitrogen sources (peptone, yeast extract, sodium nitrate, potassium nitrate, and ammonium nitrate as control) on phytase production were also evaluated at 37 °C for 24 h. The phytase activity in all cases was assayed as described earlier.

*Purification of phytase from B. lehensis MLB2*

The bulk enzyme production was performed under optimized conditions and the bacterial culture broth was centrifuged as before. The phytase in the supernatant was subjected to partial purification by acetone precipitation as per the modified method of Niyonzima & More (2015). The chilled acetone (2.5 volumes) was mixed with the supernatant (1 volume), incubated at –20 °C for 4 h, and then centrifuged at 10,000 rpm for 10 min. After discarding the supernatant, the remaining pellet served as partially purified phytase. The pellet was re-dissolved in a small amount of 0.1 M acetate buffer (pH 5.5) and subjected to the lyophilization (Freeze dryer, Model LY3TTE, Snijders Scientific, Tilburg Holland).

The partially purified lyophilized sample was then purified by affinity chromatography as per the method of Spivak et al. (1977) with slight modifications. An agarose-concalvin A column was used to purify the phytase. The column was first equilibrated with 0.1 M acetate buffer (pH 5.5). The sample was loaded and the unbound fraction collected. A sucrose solution of 1 M strength was utilized to collect the bound fraction. The protein content and phytase activity of bound and unbound samples were evaluated as before. The bound fraction having higher protein content and very higher phytase activity was lyophilized. The resulted sample was used for the phytase characterization.

*SDS-polyacrylamide gel electrophoresis (PAGE) and molecular weight estimation*
The SDS-PAGE was carried out according to Laemmili et al. (1970) under non-reducing conditions to determine the molecular weight of the purified phytase of *B. lehensis* MLB2. A separating gel and a stacking gel of 12.5 and 6% acrylamide, respectively, were used. After electrophoresis the gel was Coomassie Brilliant Blue R-250 stained for 2 h and destained. After destaining the molecular mass of the phytase was determined by comparing with the marker proteins. Liquid chromatography-mass spectrometry was also used to determine the molecular weight of the purified phytase of *B. lehensis* MLB2, following the procedure of Niyonzima & More (2014).

*Characterization of the phytase of B. lehensis MLB2*
*Effect of pH on the B. lehensis* MLB2 *phytase activity and stability.* The effect of pH on the phytase activity was studied over the range of 2.0–9.0 using different buffers of 0.1 M glycine-HCl buffer (pH 2.0–3.0), acetate buffer (pH 4.0–6.0), and Tris-HCl buffer (pH 7.0–9.0). The assay was performed as previously mentioned by pre-incubating 0.05 mL of phytase with 0.95 mL of each of the buffers at room temperature (30 ℃) for 30 min. For pH stability, the residual phytase activity was recorded as per the assay procedure but after a pre-incubation of 24 h in different buffers.

*Effect of temperature on the B. lehensis* MLB2 *phytase activity and stability.* The influence of temperature on phytase activity was studied over the temperature range of 0–90 ℃. The phytase (0.05 mL) was pre-incubated with 0.95 mL of 0.1 M acetate buffer (pH 4.5) at the respective temperatures for 30 min and the phytase activity recorded as before. For temperature stability, the residual phytase activity was determined as per the standard assay but after a pre-incubation of 24 h at different temperatures.

*Effect of metal ions on phytase activity.* The effect of different metal ions on phytase activity was evaluated using the chlorides of $Fe^{2+}$, $Hg^{2+}$, $Ca^{2+}$, $Ba^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Mn^{2+}$, $K^{+}$ and $Na^{+}$ of 5 mM concentration. The assay was performed by pre-incubating 0.05 mL of phytase with 0.95 of each additive at 37 ℃ for 30 min. The residual phytase activity was evaluated by the standard assay procedure and was expressed as percentage of the initial activity without metal ion, taken as 100%.

*Effect of group-specific reagents and potential inhibitors on phytase activity.* The effect of inhibitors on enzyme activity was investigated using EDTA, urea, SDS, sodium azide, PMSF, NEM, NBS, TPTZ, DTT, and NAI. The assay was carried out by pre-incubating 0.05 mL of phytase with 0.95 of each inhibitor at 37 ℃ for 30 min. The residual phytase activity was determined as previously described, and was expressed as percentage of the initial activity without inhibitor, considered as 100%.

*Substrate specificity and determination of $K_m$ and $V_{max}$.* The substrate specificity of the purified phytase of *B. lehensis* MLB2 was studied by adding separately each substrate in the standard assay mixture in place of sodium phytate. The substrates used were sodium phytate, $\alpha$-glycerophosphate, fructose-6-phosphate, $\beta$-glycerophosphate, glucose-6-phosphate, and 4-nitrophenylphosphate, and the assay was carried out as described earlier in 0.1 M acetate buffer (pH 4.5). The $K_m$ and $V_{max}$ for the phytase were estimated by an Eadie-Hofstee plot using different concentrations of sodium phytate ranging from 0 to 0.15 mM (in 0.1 M acetate buffer, pH 4.5) at 37 ℃.

*Inorganic phosphate release from feed substrates by B. lehensis phytase treatment.* Wheat bran and rice bran were finely ground and autoclaved for 15 min in order to inactivate the endogenous phytases. Each substrate (5 g) was dissolved in 40 mL of acetate buffer (0.1 M, pH 4.5). Incubation in a shaking water bath was then carried out at 37 ℃ for 300 min after mixing each suspension with 550.5 U *B. lehensis* MLB2 phytase per kg of feed substrate. After every 50 min, an aliquot (1 mL) was removed, and centrifuged with a cooling centrifuge (REMI C-30 BL) at 10,000 for 2 min at 4 ℃. The suspensions incubated without enzymes served as control (Park & Cho 2011). The released inorganic phosphate in the supernatant was determined as mentioned previously.

*Statistical analysis*
To evaluate the phytase activity, three independent experiments for each treatment were carried out. One way analysis of variance (ANOVA) was utilized to compare the means, whereas Duncan's multiple range test was used to find out the means for groups in homogeneous subsets at the significance level of 5%. The statistical analyses were automatically given by SPSS statistical package (PASW Statistics 18).

## Results and discussion

*Isolation and identification of the organism*
In the present study, a phytase-producing bacterium was isolated from soil fields of bean plants. It was identified as *Bacillus* based on morphological and physiological characteristics. It was further confirmed as *B. lehensis* MLB2 by Bangalore Genei Pvt. Ltd. with the GenBank accession number AY793550. The bacterial phytase production was indicated by the clear zones around the organism, which was attributed to the liberation of *myo*-inositol and inorganic phosphates from the phytate substrate (Kumar et al. 2011). Similarly, various bacterial strains were screened from soil and phytase production was shown by clear zones around the culture (Cho et al. 2003; De Angelis et al. 2003; Hong et al. 2011; Roy et al. 2012; Sasirekha et al. 2012; El-Toukhy et al. 2013; Lee et al. 2014).

*Optimization of culture conditions for maximum phytase production*
The various factors affecting the phytase production by *B. lehensis* MLB2 were studied, investigating one factor at a time, keeping other parameters unchanged. The time course for maximum phytase production was analyzed every 12 h for 72 h, and maximum production (220.7 U/mL) was observed after 24 h. A decrease in enzyme production was observed thereafter (Table 1). Similarly, the optimal time for phytase production (98.76 U/mL) by *Pseudomonas aeruginosa* was 24 h (Sasirekha et al. 2012). The studies carried out by Hong et al. (2011) with *Bacillus subtilis* CF92 and Kumar et al. (2011) with *Serratia marcescens* showed the phytase production to a maximum after 48 h of incubation. The optimal time for phytase production by *Shigella* sp. CD2 (Roy et al. 2012), *Bacillus* sp. T4 (Lee et al. 2014), and *B. subtilis* MJA (El-Toukhy et al. 2013) was 72, 84 and 96 h, respectively. The main advantage of the phytase production by *B. lehensis* MLB2 is the

Table 1. Effect of incubation time, inoculum level, initial pH, incubation temperature, carbon and nitrogen sources on phytase activity (U/mL) by *Bacillus lehensis* MLB2.[a]

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Incubation time (h) | 12 | 24 | 36 | 48 | 60 | 72 | |
| Enzyme activity (U/mL) | $49.7 \pm 5.0^{b}$ | $70.2 \pm 3.0^{a}$ | $56.0 \pm 6.5^{b}$ | $49.0 \pm 1.0^{b}$ | $36.7 \pm 7.6^{c}$ | $28 \pm 2.6^{d}$ | |
| Inoculum load (%) | 1 | 2 | 3 | 4 | 5 | | |
| Enzyme activity (U/mL) | $72.5 \pm 1.3^{c}$ | $161.0 \pm 7.8^{a}$ | $121.0 \pm 5.6^{b}$ | $70.7 \pm 3.8^{c}$ | $41.7 \pm 3.0^{d}$ | | |
| Initial pH | 4.5 | 5 | 5.5 | 6 | 6.5 | 7.0 | 7.5 |
| Enzyme activity (U/mL) | $125.3 \pm 7.8^{c}$ | $141.7 \pm 1.5^{b}$ | $166.7 \pm 1.5^{a}$ | $140.7 \pm 4.5^{b}$ | $118.7 \pm 5.0^{c,d}$ | $114.3 \pm 4.2^{d}$ | $102.7 \pm 2.1^{e}$ |
| Incubation temperature (℃) | 20 | 30 | 37 | 45 | 55 | | |
| Enzyme activity (U/mL) | $160.0 \pm 6.0^{b}$ | $170.7 \pm 7.5^{b}$ | $188.0 \pm 8.2^{a}$ | $170.3 \pm 2.5^{b}$ | $138.0 \pm 6.1^{c}$ | | |
| Carbon source (0.5%, w/v) | Glucose | Sucrose | Rice bran | Sodium phytate | | | |
| Enzyme activity (U/mL) | $86.1 \pm 8.7^{b}$ | $81.0 \pm 4.4^{b}$ | $195.7 \pm 5.1^{a}$ | $192.0 \pm 4.4^{a}$ | | | |
| Nitrogen source (0.5%, w/v) | Yeast extract | Peptone | Potassium nitrate | Sodium nitrate | Ammonium nitrate | | |
| Enzyme activity (U/mL) | $202.7 \pm 1.5^{b}$ | $203.0 \pm 4.4^{b}$ | $220.7 \pm 7.5^{a}$ | $193.3 \pm 2.9^{b}$ | $195.3 \pm 5.0^{b}$ | | |

[a] The values with different letters for each treatment are significantly different at 5% significance level.

short incubation time, which makes the submerged fermentation cost effective. After optimal time, the phytase production decreased with prolonged incubation time, which can be attributable to the depletion of some nutrient in the production medium. The decrease in phytase production by *Bacillus* sp. T4 was ascribed to the enzyme denaturation resulted from the interaction with other medium ingredients or pH medium change (Lee et al. 2014).

The inoculum level plays a vital role in the production of phytase from both bacteria and fungi (Ahmad et al. 2000). The phytase production by the isolate was optimum at 2% inoculum level (Table 1). The inoculum level of 1% led to a significant yield for phytase production by *B. subtilis* MJA (El-Toukhy et al. 2013). High inoculum level of 4% and 5% were observed for the phytase production by *Bacillus* sp. T4 (Lee et al. 2014) and *Serratia marcescens* (Kumar et al. 2011), respectively. The decrease in MLB2 phytase secretion observed at higher inoculum levels may be ascribed to the rapid utilization of the nutrients by the isolate. Similarly, a decline in phytase production by *Sporotrichum thermophile* BJTLR50 was attributed to the competition for nutrients by fungal cells (Singh & Satyanarayana 2012).

The initial pH is an important factor affecting phytase production, since it plays an important role in bacterial or fungal growth. The phytase production by *B. lehensis* MLB2 was maximally observed at pH 5.5 (Table 1). Likewise, Roy et al. (2012) has reported 5.5 as the optimum pH for the production of phytase from *Shigella* sp. CD2. The initial pH of 6.0 was optimal for phytase production by *P. aeruginosa* (Sasirekha et al. 2012), whereas the neutral pH favours the production of phytase by *Bacillus* species (El-Toukhy et al. 2013, Lee et al. 2014). The optimum temperature for the production of phytase was found to be at 37℃ (Table 1). Similarly, 37℃ was optimum incubation temperature for phytase production by *B. subtilis* CF92 (Hong et al. 2011), *Shigella* sp. CD2 (Roy et al. 2012), and *P. aeruginosa* (Sasirekha et al. 2012). Earlier studies by Kumar et al. (2011) and El-Toukhy et al. (2013) reported the production of phytase from bacterial species at the temperature of 30℃.

Effect of carbon sources on phytase production was analyzed. The isolate was able to use all the tested carbon sources with the maximum phytase production in the presence of rice bran at par with sodium phytate (Table 1). Likewise, sodium phytate was the best carbon source for phytase production by *Lactobacillus sanfranciscensis*i CB1 (De Angelis et al. 2003). A moderate enzyme production was observed with carbohydrates, *viz.* glucose and sucrose (Table 1). Contrastingly, glucose and/or sucrose led to a higher yield for phytase production by bacterial species (Sasirekha et al. 2012; El-Toukhy et al. 2013). The phytase production by *B. lehensis* MLB2 using an inexpensive waste product of rice-processing industry (rice bran) makes the production cost-effective.

Peptone, yeast extract, sodium nitrate, potassium nitrate and ammonium nitrate were used to study the effect of nitrogen sources on phytase production by *B. lehensis* MLB2. The highest phytase production was attained with potassium nitrate (Table 1). Similarly, an inorganic nitrogen source (nitrate) was the best for phytase production by *Bacillus* sp. T4 (Lee et al. 2014). In contrast, organic nitrogen sources led to better phytase yields in *Pseudomonas syringae* MOK1 (Cho et al. 2003), *P. aeruginosa* (Sasirekha et al. 2012), and *B. subtilis* MJA (El-Toukhy et al. 2013). Nampoothiri et al. (2004) and Escobin-Mopera et al. (2012) have used the organic nitrogen sources, such as beef extract and yeast extract in their modified phytase production liquid medium, and observed a high protein concentration with low yield of enzyme. The use of organic nitrogen sources increases the protein concentrations, which may interfere with the purification procedures. In the present study, the utilization of inorganic nitrogen sources led to higher phytase yield and simplified the purification procedures.

*Purification of phytase of B. lehensis MLB2*

The phytase of *B. lehensis* MLB2 was purified by acetone precipitation and affinity chromatography. A recovery of 33.9% in phytase activity was achieved, with 4.1 purification fold and a specific activity of 152.9 U/mg (Table 2). El-Toukhy et al. (2013) reported 3.95-purification fold for the phytase of *B. subtilis* MJA with

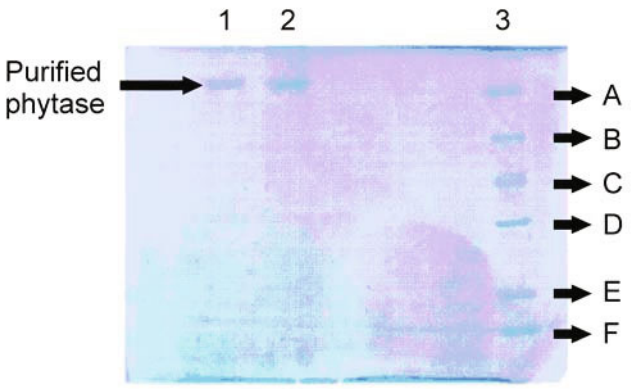


Fig. 1. SDS-PAGE of phytase of *Bacillus lehensis* MLB2. Acetone purified enzyme (lane 1), affinity chromatography purified enzyme (lane 2), protein molecular weight markers (lane 3). Proteins in the marker lane (kDa): phosphorylase b (97.4), bovine serum albumin (66.0), ovalbumin (43.0), carbonic anhydrase (29.0), soybean trypsin inhibitor (20.1) and lysozyme (14.3).

4.224 U/mg and 57.7% yield. Roy et al. (2012) recovered 41% for phytase of *Shigella* sp. CD2 with a specific activity of 780 U/mg and 133.7-purification fold. A low recovery of 2% was recorded for phytase of *K. pneumonia* 9-3B with 240-purification fold (Escobin-Mopera et al. 2012).

The purified phytase of *B. lehensis* MLB2 was subjected to SDS-PAGE under non-reducing conditions and a single band was observed. By comparison with the standard molecular weight markers, the apparent molecular weight of the phytase was found to be 98 ± 1 kDa (Fig. 1). This was similar to the molecular weight of 98.686 kDa found by liquid chromatography-mass spectrometry (Fig. 2). The phytase of *B. lehensis* MLB2 was thus a homogenous monomeric protein. Earlier studies reported low molecular weight bacterial phytases ranging from 38 kDa to 50 kDa (Cho et al. 2003; De Angelis et al. 2003; Hong et al. 2011; Escobin-Mopera et al. 2012; Park et al. 2012; Roy et al. 2012; El-Toukhy et al. 2013). This is the first bacterial phytase showing a high molecular weight. However, a low molecular weight of 14 kDa was reported for the fungal phytase of mushroom *Lentinus edodes* (Zhang et al. 2013), whereas a higher molecular weight of 3699 kDa was reported by Oh et al. (2004) for the plant phytase of *Lilium longiflorum*.

Table 2. Summary of purification of phytase from *Bacillus lehensis* MLB2.

| Purification step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| Crude extract | 1625.0 | 43.3 | 37.5 | 1.0 | 100.0 |
| Acetone precipitation | 812.5 | 11.4 | 71.3 | 1.9 | 50.0 |
| Affinity chromatography | 550.5 | 3.6 | 152.9 | 4.1 | 33.9 |

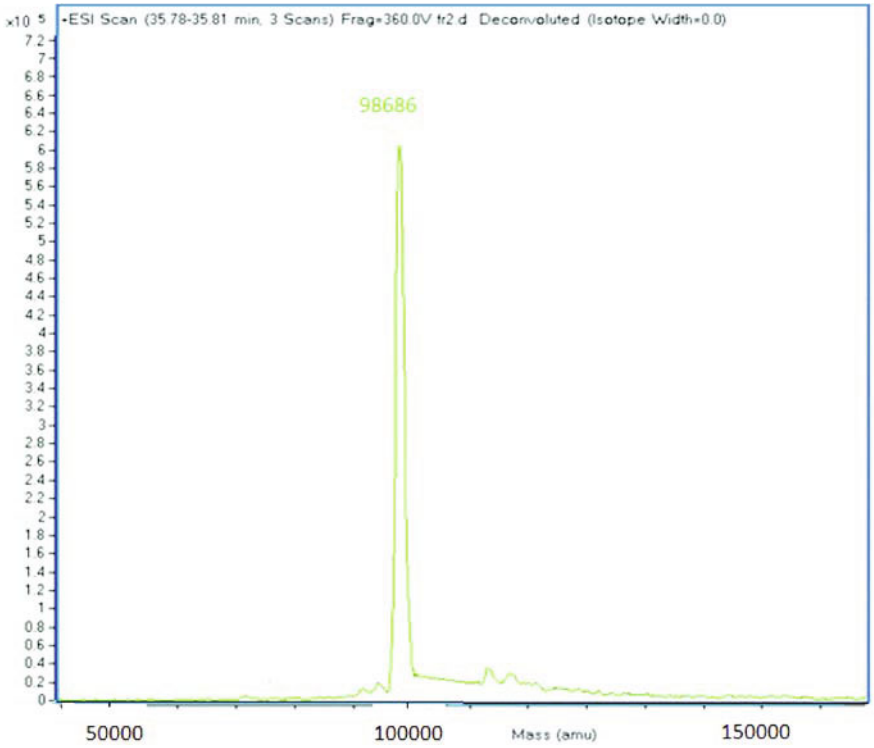


Fig. 2. Deconvoluted mass spectrum of the phytase of *Bacillus lehensis* MLB2.

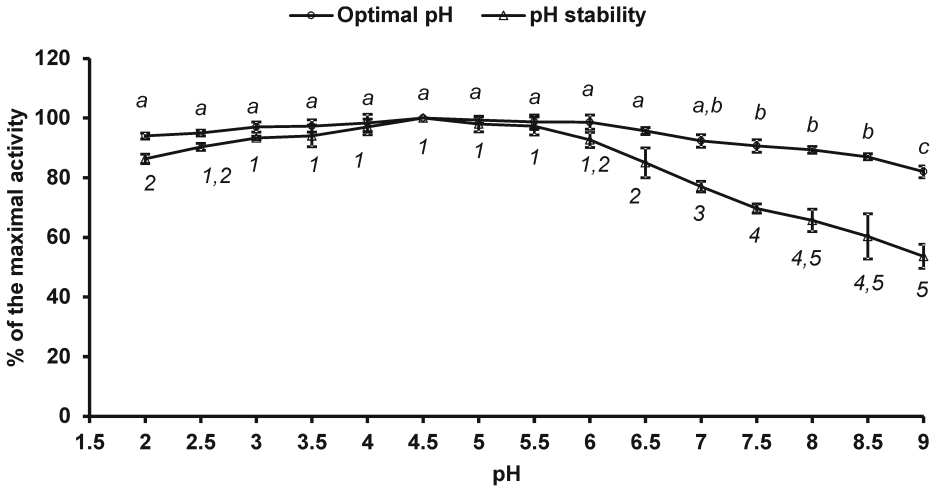


Fig. 3. Effect of pH on *Bacillus lehensis* phytase activity and stability. For optimal pH and pH stability studies, the pre-incubation was carried out at 37 ℃ for 30 min and 24 h, respectively, in different buffers of 0.1 M concentration. The phytase activities are expressed as the percentage of the maximal activities. The values with different letters or numbers on error bars for each pH treatment are significantly different at 5% significance level.

*Characterization of phytase of B. lehensis MLB2*

In addition to molecular weight, optimum pH and stability, optimum temperature and stability, catalytic residues in the active site, substrate specificity, and kinetic constants are used to characterize the bacterial or fungal phytases. The release of inorganic phosphate from feed substrates by the phytase of *B. lehensis* MLB2 was also investigated.

*Effect of pH on phytase activity and stability*

The effect of pH on the activity of the phytase was evaluated in the 2.0–9.0 range. The phytase was 100% active over an acidic pH range with maximum activity at pH 4.5 (Fig. 3). Similarly, pH 4.0 was optimum for the phytase purified from *K. pneumonia* 9-3B (Escobin-Mopera et al. 2012) and *L. sanfranciscensis* CB1 (De Angelis et al. 2003); whereas 5.0 was optimum for phytases of *P. syringae* MOK1 (Cho et al. 2003), and *Shigella* sp. CD2 (Roy et al. 2012). The optimum pH in the 5.0–6.0 range was also reported for *Pseudomonas* sp. JPK1 (Park & Cho 2011) and *B. subtilis* MJA (El-Toukhy et al. 2013). The neutral pH was also optimum for the phytases of *Bacillus* species (Kerovuo et al. 1998; Hong et al. 2011). Likewise, a significant phytase activity was also observed for the $\beta$-propeller phytases, which are active at neutral or slightly alkaline pH (El-Toukhy et al. 2013). The variation in phytase activities at different pH optima was ascribed to phytase molecular conformation or sterospecificity (Nampoothiri et al. 2004).

The stability of the phytase was evaluated at pH 2.0–9.0. The phytase of *B. lehensis* MLB2 showed a remarkable stability and retained 100% of its activity at pH 2.5–6.0 after 24 h. However, a loss of about 30–45% was observed at alkaline pH (7.5–9.0) (Fig. 3). Good stability of $>$ 80% was also observed for the phytase of *B. subtilis* MJA in the range of pH 2.0–8.0 for only 4 h at 25 ℃ (El-Toukhy et al. 2013). The comparable stability of 83% in the range of pH 4.0–8.0 for only 6 h was recorded for the phytase obtained from *B. subtilis* CF92 (Hong et al. 2011). Likewise, Bakthavatchalu et al. (2013) reported a phytase that was $>$ 80.0% stable at pH 5.0–8.0 for only 2 h. Low pH stability of 40% at pH 4.0–4.5 was reported for the phytase purified from *Pseudomonas* sp. JPK1 (Park & Cho 2011). The phytase purified from the present study appeared to have a higher stability in the acidic region than others originating from bacterial species, since none is retaining a significant activity after 24 h, suggesting the phytase to be applied as feed additive in order to release inorganic phosphate from phytate feed in the stomach. Therefore, the phytase active at acidic pH is preferred as feed additive, since, for instance, the pH in poultry gut ranges from 2.5 to 6.0 (Radcliffe et al. 1998), pH 5.0 is in salivary glands (Casey & Walsh 2003), pH 4.0–6.0 in the upper part of small intestine (Haefner et al. 2005), and pH 1.5–3.5 in the gastrointestinal tract of monogastric animals (Zhang et al. 2010).

*Effect of temperature on phytase activity and stability*

The effect of temperature on the *B. lehensis* phytase activity was studied in the 0–90 ℃ range. The phytase exhibited a significant activity for all the temperatures investigated with an optimum at 37 ℃, although statistically at par with 0, 20, 30, 50 and 60 ℃. A loss of about 10% phytase activity was seen in the 70–90 ℃ range (Fig. 4). Similarly, various phytases with optimum activity at 37 (El-Toukhy et al. 2013), 40 (Cho et al. 2003), 45 (De Angelis et al. 2003), 50 (Park & Cho 2011; Escobin-Mopera et al. 2012; Bakthavatchalu et al. 2013), 55 (Jareonkitmongkol et al. 1997; Kerovuo et al. 1998), and 60 ℃ (Hong et al. 2011; Roy et al. 2012), were obtained from various bacterial species. Most of the optimum temperatures reported for bacterial phytases are therefore in the 37–60 ℃ range. Likewise, an optimum temperature in the 45–55 ℃ range was also observed for various commercial phytases (Boyce & Walsh 2006). The gastrointestinal temperature of poul-

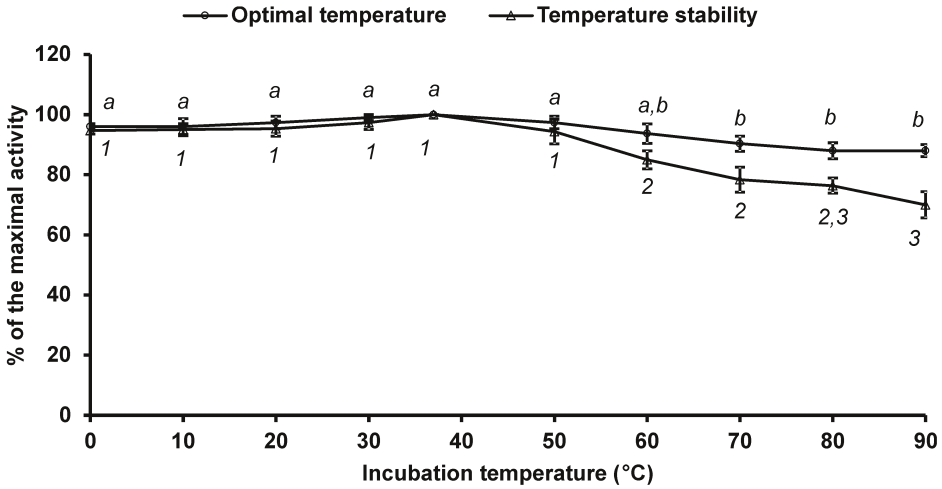


Fig. 4. Effect of temperature on *Bacillus lehensis* phytase activity and stability. For optimal temperature and temperature stability profiles, the pre-incubation was done at different temperatures for 30 min and 24 h, respectively, in 0.1 M acetate buffer (pH 4.5). The phytase activities are expressed as the percentage of the maximal activities. The values with different letters or numbers on error bars for each temperature treatment are significantly different at 5% significance level.

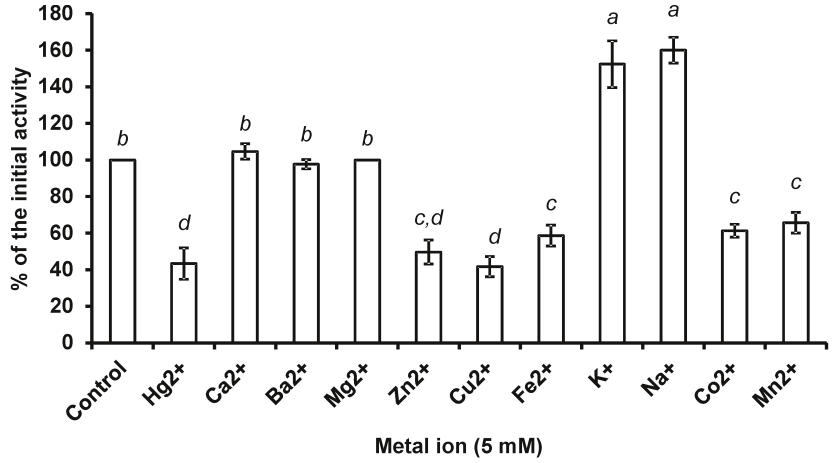


Fig. 5. Effect of mono- and di-valent metal ions on *Bacillus lehensis* phytase activity. The phytase was pre-incubated in 5 mM metal ion at 37 ℃ for 30 min. The residual phytase activity was expressed as percentage of the initial activity without metal ion (control), taken as 100%. The values with different letters on error bars for each ion treatment are significantly different at 5% significance level.

try or pig is in the 37–40 ℃ range (Lei & Porres 2003). The phytase of *B. lehensis* MLB2 may thus be an ideal choice as a feed supplement since 100% enzyme activity was retained in this range.

When the thermostability profile of the phytase of *B. lehensis* MLB2 was studied, the retention of full activity was observed over the 0–50 ℃ range after an overnight incubation. However, a loss in relative phytase activity in the range of 15% (at 60 ℃) to 30% (at 90 ℃) was recorded after 24 h (Fig. 4). The phytases obtained from *L. sanfranciscensis* CB1 (De Angelis et al. 2003) and *Shigella* sp. CD2 (Roy et al. 2012) retained full activity at 70 ℃ but only for 30 min. The phytase produced by *K. oxytoca* MO-3 was 100% stable at 60 ℃ for 20 min (Jareonkitmongkol et al. 1997). The phytase of *P. aeruginosa* F6 showed a significant stability at 50 ℃ for 4 h (Bakthavatchalu et al. 2013). A retention of 40% phytase activity for 30min at 80 ℃ was observed for the enzyme from *B. subtilis* CF92 (Hong et al. 2011). The phytase of *B. lehensis* MLB2 had a broad temperature adaptability (0–90 ℃) in comparison with other bacterial phytases reported, since none retained a significant activity after an overnight incubation. The significant thermostability of the present phytase can be ascribed to the presence of the carbohydrate moiety. This is advantageous as the present phytase will resist the pelletization process taking place at elevated temperature for a brief period during animal feed preparation. Indeed, the pelleting of animal feeds may occur up to 95 ℃ (Bakthavatchalu et al. 2013; El-Toukhy et al. 2013).

*Effect of metal ions on phytase of B. lehensis MLB2*

The effect of various metal ions on phytase activity was studied. $K^+$ and $Na^+$ enhanced the phytase activity, whereas $Ca^{2+}$, $Mg^{2+}$, and $Ba^{2+}$ did not have any effect on the enzyme (Fig. 5). Similar to the phytase of the present study, $Ca^{2+}$ (Kerovuo et al. 1998; Escobin-Mopera et al. 2012; Roy et al. 2012), or $Ca^{2+}$ and $Mg^{2+}$ (Bakthavatchalu et al. 2013; El-Toukhy et

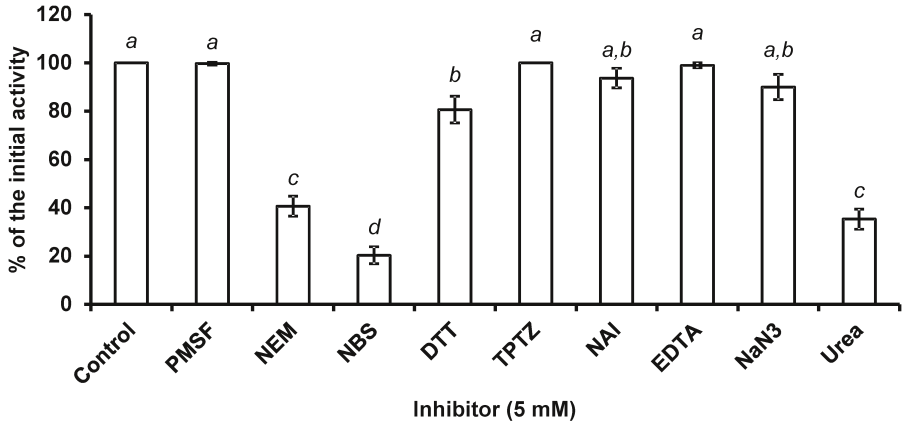


Fig. 6. Effect of specific inhibitors on *Bacillus lehensis* phytase activity. The phytase was pre-incubated in 5 mM specific inhibitor at 37 ℃ for 30 min. The residual phytase activity was expressed as percentage of the initial activity without inhibitor (control), taken as 100%. The values with different letters on error bars for each inhibitor treatment are significantly different at 5% significance level.

al. 2013) also activated the phytases purified from various bacterial species. The stimulation of phytase by mono- or di-valent metal ions suggested the important role of these ions in maintaining the phytase active site and improving the phytase thermostability. $Co^{2+}$, $Fe^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ partially inhibited the phytase of *B. lehensis* MLB2, while $Cu^{2+}$ and $Hg^{2+}$ inhibited the enzyme (Fig. 5). Similarly, the phytases of *K. oxytoca* MO-3 (Jareonkitmongkol et al. 1997), *L. sanfranciscensis* CB1 (De Angelis et al. 2003); *Pseudomonas* sp. JPK1 (Park & Cho 2011), *K. pneumonia* 9-3B (Escobin-Mopera et al. 2012), *Shigella* sp. CD2 (Roy et al. 2012), and *B. subtilis* MJA (El-Toukhy et al. 2013) were significantly inhibited by $Zn^{2+}$, $Fe^{2+}$, and/or $Cu^{2+}$. Likewise, the phytase purified from *P. syringae* MOK1 was strongly inhibited in the presence of $Mn^{2+}$, $Cu^{2+}$ and $Cd^{2+}$ (Cho et al. 2003), which was attributed to the formation of poorly soluble complexes when combined to phytate, reducing phytate active concentration (Wyss et al. 1999). Most of the bacterial phytases are thus inhibited by $Cu^{2+}$, $Zn^{2+}$ and $Fe^{2+}$.

*Effect of specific inhibitors on phytase of B. lehensis MLB2*

The effect of different specific inhibitors on the phytase of *B. lehensis* MLB2 was analyzed (Fig. 6). The enzyme was not affected by TPTZ, PMSF, NAI, and $NaN_3$. NEM and NBS were the only reagents, which showed an inhibitory effect, apart urea indicating the probable presence of cysteine and tryptophan in the active site of the phytase (Fig. 6). Similarly, the tryptophan and arginine were in the active site of *B. subtilis* strain VTT E-68013 (Kerovuo et al. 1998). Contrastingly, NEM had no effect on the phytase from *K. oxytoca* MO-3 (Jareonkitmongkol et al. 1997), and PMSF inhibited both the phytases of *L. sanfranciscensis* CB1 (De Angelis et al. 2003) and *Pseudomonas* sp. JPK1 (Park & Cho 2011). The phytase of *B. lehensis* MLB2 was not inhibited by EDTA, confirming the non-metalloproteinic nature of the present phytase (Fig. 6). A similar observation was reported for the bacterial phytases from *K. oxytoca* MO-3 (Jareonkitmongkol et al. 1997), *B. subtilis* CF92 (Hong et al. 2011), and *K. pneumonia* 9-3B (Escobin-Mopera et al. 2012). In contrast, the phytases of *B. subtilis* strain VTT E-68013 (Kerovuo et al. 1998), *P. syringae* MOK1 (Cho et al. 2003), and *Pseudomonas* sp. JPK1 (Park et al. 2012) were inhibited by the EDTA addition.

Table 3. The activity of *Bacillus lehensis* MLB2 phytase against phytate and various phosphorylated substrates.[a]

| Substrate (0.5%, w/v) | % of the maximal activity |
|---|---|
| Sodium phytate | $100.0 \pm 0.0^a$ |
| Glucose-6-phosphate | $0.0 \pm 0.0^b$ |
| Fructose-6-phosphate | $0.0 \pm 0.0^b$ |
| $\alpha$-Glycerophosphate | $8.2 \pm 0.0^b$ |
| $\beta$-Glycerophosphate | $5.4 \pm 0.0^b$ |
| 4-Nitrophenylphosphate | $0.0 \pm 0.0^b$ |

[a] The sodium phytate hydrolysis was considered as 100% for comparison. The values with different letters are significantly different at 5% significance level.

*Substrate specificity of phytase of B. lehensis MLB2*

The substrate specificity of the phytase of *B. lehensis* MLB2 was assessed. The enzyme only hydrolyzed its substrate and had no or negligible activity in the presence of other substrates tested (Table 3). Likewise, the phytases of *P. syringae* MOK1 (Cho et al. 2003), *Pseudomonas* sp. JPK1 (Park & Cho 2011), *Shigella* sp. CD2 (Roy et al. 2012), and *P. aeruginosa* F6 (Bakthavatchalu et al. 2013) were highly active only towards their phytate substrates and possessed no or little activity with other phosphate substrates. In contrast, the CF92 phytase had a broad substrate specificity and was able to hydrolyze most of the tested phosphate substrates with the highest activity towards sodium phytate and $\alpha$-naphthyl phosphate, moderate with adenosine triphosphate, and the lowest against adenosine diphosphate, 4-nitrophenylphosphate, $\alpha$-glycerophosphate, and 4-nitrophenylphosphate (Hong et al. 2011). Similarly,

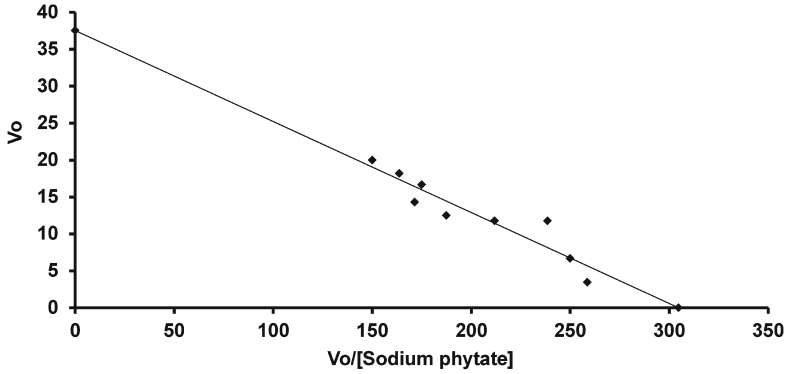


Fig. 7. Eadie-Hofstee plot for determination of $K_m$ and $V_{max}$ of phytase of *Bacillus lehensis* MLB2. The phytase was incubated with different concentrations of sodium phytate.

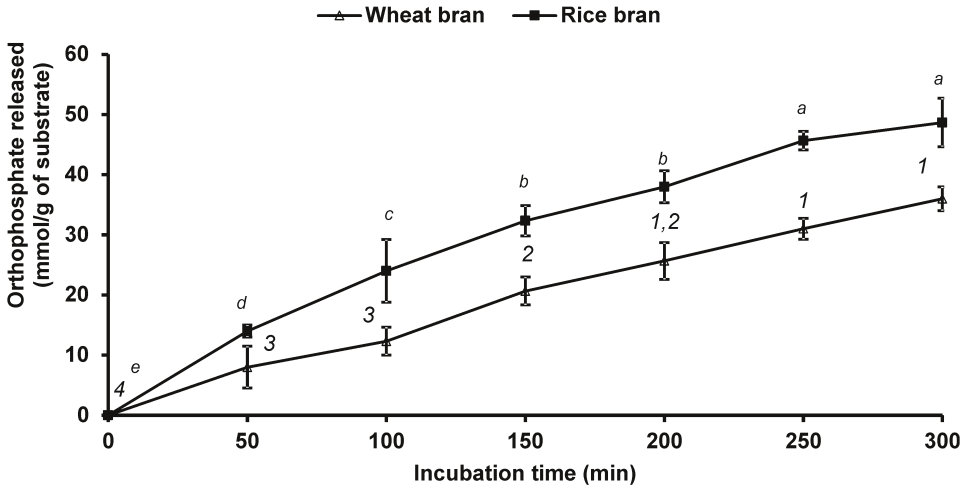


Fig. 8. Release of inorganic phosphate from feedstuffs by the phytase from *Bacillus lehensis* MLB2. The values with different letters or numbers on error bars for each treatment are significantly different at 5% significance level.

the phytase of *L. sanfranciscensis* CB1 possessed the broad substrate specificity and was active towards sodium phytate followed by ATP, AMP, $\alpha$-D-glucose-1-phosphate, D-fructose-1,6-phosphate, and D-fructose-6-phosphate (De Angelis et al. 2003). The phytase of *B. subtilis* strain VTT E-68013 also showed substrate specificity on phytate, ADP, and ATP (Kerovuo et al. 1998). The absolutely strict substrate specificity shown by the phytase of *B. lehensis* MLB2 suggested its animal feed applicability.

*Determination of kinetic constants of B. lehensis MLB2 phytase*

The $K_m$ and $V_{max}$ values were found to be 0.1232 mM and 37.54 U/mL, respectively, with sodium phytate as revealed by Eadie-Hofstee plot (Fig. 7). Comparable $K_m$ of 0.25 mM (Roy et al. 2012), 0.267 mM (and a $V_{max}$ of 0.2843 µmol/min) (Bakthavatchalu et al. 2013), 0.38 mM (and a $V_{max}$ of 769U/mg) (Cho et al. 2003), 0.42 mM (and a $V_{max}$ of 4.35 µmol/min) (Hong et al. 2011), and 0.485 mM (and a $V_{max}$ of 510 U/mg) (El-Toukhy et al. 2013) were reported for various bacterial phytases. However, a very low $K_m$ of 0.04 mM was recorded for the phytase of *K. pneumoniae* 9-3B (Escobin-Mopera et al. 2012). The lower $K_m$ observed for the phytase of *B. lehensis* MLB2 indicates the higher affinity of the enzyme for its substrate (phytate).

*Inorganic phosphate release from feed substrates by B. lehensis phytase treatment*

In the present study, wheat bran and rice bran served as feed ingredients. Haefner et al. (2005) proposed wheat bran as an example of the feed ingredient with higher phytate concentration. After the investigation, 36 and 49 mmol inorganic phosphate was released per g of wheat bran and rice bran, respectively, for 300 min at pH 4.5 after *B. lehensis* phytase application (Fig. 8). Similarly, a significant inorganic phosphate was liberated from wheat bran by the action of the phytase of *Pseudomonas* sp. JPK1 (Park & Cho 2011). Likewise, orthophosphate in the range of 1.72–4 mmol/g of grain was released from corn meal, soybean meal, and wheat bran, after application of *P. syringae* MOK1 (Cho et al. 2003). The ability of the phytase of *B. lehensis* MLB2 to digest synthetic substrate like phytate, and natural substances, such as rice bran and wheat bran, and liberate a significant amount of inorganic phosphate suggested its use as feed supplement, and phosphate pollution gets reduced.

*Conclusions*

In the present investigation, a positive phytase-producing bacterium was isolated from soil sample and identified as *B. lehensis* MLB2. The growth and optimization parameters indicate the organism to be a potential strain for the phytase production. The MLB2 phytase was purified, characterized and evaluated for the *in vitro* inorganic phosphate release from animal feed ingredients. It was found to be valuable for animal feed applicability owing to its stability at a broad range of temperature and acidic pH, absolute specificity towards phytate, and a significant liberation of orthophosphate from representative feedstuffs, reducing thus manure phosphorus pollution. However, mutation studies or strain improvement strategies could be further used to increase the phytase production. The whole-cell and enzyme immobilization can also be used to increase the phytase efficacy, especially in pelletization process.

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