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تولید و تعیین خصوصیت بیوشیمیایی فیتاز گرمادوست جدا شده از باکتری باسیلوس آمیلولیکوفاسینس LOR10

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چکیده. فیتاز ارزش تغذیه‌ای غذاهای گیاهی را با افزایش هضم‌پذیری پروتئین و در دسترس سازی مواد معدنی از طریق هیدرولیز فیتات در طی هضم در معده یا در طی پردازش غذا بهبود می‌بخشد. منابع میکروبی برای تولید تجاری فیتاز پتانسیل قابل‌توجهی دارند. بنابراین، هدف این تحقیق غربالگری و جداسازی باکتری-های مولد فیتاز از چشمه آب گرم با پتانسیل قابل توجه می‌باشد. شناسایی بهترین سویه با روش مولکولی 16S rDNA انجام شد. بهینه سازی تولید آنزیم در حضور منابع مختلف کربن، نیتروژن و فسفات انجام گردید. فعالیت و پایداری آنزیم در pH ها، دماها و یون‌های مختلف انجام شد. مقایسه توالی ژن 16S rDNA سویه LOR10 با سایر سویه‌ها در بانک ژنی با استفاده از Clustal omega میزان ۹۸ درصد همولوژی با باسیلوس آمیلولیکوفاسینس را نشان داد. نتایج بهینه‌سازی محیط نشان داد که گالاکتوز، عصاره مخمر و تری‌کلسیم فسفات به‌عنوان بهترین منبع کربن، نیتروژن و فسفات برای تولید فیتاز می‌باشند. دمای بهینه فعالیت در دمای ۷۰ درجه سانتی‌گراد به دست آمد. بهترین پایداری فیتاز نیز در pH حدود ۵ تا ۸ به دست آمد. فعالیت فیتازی در حضور کلرید-کلسیم، کلریدروی و سولفات‌منیزیم حدود ۱/۴، ۲/۳ و ۱/۶ برابر به ترتیب افزایش یافت. خاطر نشان می‌شود که فعالیت فیتازی در حضور EDTA و SDS حدود ۳۰ درصد کاهش یافت. این نتایج خاطر نشان می‌سازد که فیتاز LOR10 پتانسیل قابل‌توجهی برای استفاده‌های تجاری از جمله مکمل غذایی را داراست.

واژه های کلیدی. باکتری‌های تجزیه‌کننده فیتات، جداسازی، چشمه آب‌گرم، شناسایی باکتریایی

Production and biochemical characterization of a thermostable phytase from *Bacillus amyloliquefaciens* LOR10

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Abstract. Phytase can improve the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through phytate digestion in the stomach and the food processing industry. Microbial sources are more promising for the production of phytases on a commercial scale. The objectives of this exploration were to screening and isolation of phytase-producing bacteria from hot spring with commercial interest. Molecular identification of the best isolate was achieved by the *16S rDNA* gene. Optimization of phytase production was prepared in the presence of different phosphate, nitrogen, and carbon sources. Enzyme activity and stability were also explored in the presence of different pHs, temperatures, and ion compounds. Comparing the *16S rDNA* gene sequence of the isolate LOR10 with those in GenBank using Clustal omega shows 98% sequence homology with *Bacillus amyloliquefaciens.* Medium optimization studies showed that galactose, yeast extract, and tricalcium phosphate were the best sources of carbon, nitrogen, and phosphate for phytase production, respectively. The optimum temperature activity was also observed to be 70 ºC. Phytase stability was at its optimum in a pH range of 5.0–8.0. Phytase activity increased in the presence of $CaCl_2$, $ZnCl_2$, and $MnSO_4$ about 1.4, 2.3 and 1.6 folds, respectively. It could be mentioned that phytase activity decreased by about 30 % in the presence of EDTA and SDS. On the basis of the results, it could be concluded that LOR10 phytase has a great potential for commercial interest as an additive to animal plant-based foods.

Keywords. bacterial identification, hot spring, isolation, phytate-degrading bacteria

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INTRODUCTION

Phytase catalyzes the hydrolysis of phytate (myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate) through phytate digestion in the stomach and the food processing industry (In et al., 2004; Sapna & Singh, 2016). Most of the plant seeds and their co-products contain 1–2% phytic acid representing >60% of their total phosphorus (Lei & Porres, 2003; Puppala, et al., 2019). In monogastric or agastric animals, phytic acid is a polyanionic chelating agent that forms complexes with several divalent cations of primary nutritional importance, e.g., Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} (Gessler et al., 2018). Phytic acid can also form complexes with proteins and amino acids at both acidic and alkaline pHs (Saani et al., 2019). The resulting compounds are insoluble, difficult for humans to hydrolyze during digestion, and, thus are typically nutritionally less available for absorption (Afinah et al., 2010). Furthermore, the unutilized phytate from plant feeds becomes an environmental pollutant in the areas of intensive animal agriculture (Vasudevan et al., 2017). These problems can be overcome by supplementing the animal feed with phytases, which improve the nutritional status of the feed and further, declines environmental phosphorus pollution caused by the use of phosphorus in animal feed (Selle & Ravindran, 2008; Singha & Satyanarayana, 2011; Demir et al., 2018; Vasudevan et al., 2019).

Phosphate can be efficiently released from the phytate by phytase activity (Kumar et al., 2010). Phytase blended into the diets of poultry, swine, and fish to increase the availability of phosphorus, minerals, amino acids, and energy. The enzyme sequesters orthophosphate groups from the inositol ring of phytate to produce free inorganic phosphate (Baruah et al., 2007; Cao et al., 2007). Phytases are present in bacteria, fungi, animals, and plants. Commonly, the activity of an animal's phytase is insignificant compared to the phytase of microorganisms and plants (Lei et al., 2013). Phytases have been found in various microorganisms such as *Aspergillus fumigatus* (Vasudevan et al., 2017), *Escherichia coli* (Demir et al., 2018), *Enterobacter* sp.4 (Vasudevan et al., 2019), *B. amyloliquefaciens* (Selle & Ravindran, 2008), *Lactobacillus sanfranciscensis* (Raghavendra & Halami, 2009), *Bacillus* sp. (Choi et al., 2001), *Raoultella* sp. (Sajidan et al., 2004), *Citrobacter braakii* (Kim et al., 1998), and *Mitsuokella jalaludinii* (Lan et al., 2002). Owing to some properties such as catalytic productivity, substrate specificity, and resistance to proteolytic enzymes, bacterial phytases are a real alternative to the fungal enzymes (Konietzny & Greiner, 2004).

Because of their great industrial significance, there is an ongoing interest in isolating new microbial producing phytase and optimization of enzyme production. Hence the aim of this study is the isolation of the bacteria with phytase producing ability and characterization of this enzyme.

MATERIALS AND METHODS

Isolation of the phytate-degrading bacteria

Samples were gathered from the Loriya hot spring in Jiroft, which located in the southeast of Iran. Approximately 5ml of these samples was injected in 50 ml of the phytase-specific medium [1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate (Sigma)]. These flasks were maintained at 45 °C, with 180 rpm for three days. After that, 5ml of this culture was added to the same fresh medium and maintained in the same conditions. Finally, 0.1 ml of this culture was streaked onto the phytase-specific medium and incubated at 65 °C for 48h. Bacterial colonies capable of hydrolyzing sodium phytate, which can be recognized by their surrounding clear halo, were obtained by re-plating single colonies (Chunshan et al., 2001). To find the optimum time of phytase production, *Bacillus* sp. LOR10 was cultured in phytase specific medium, samples were picked up at 24 h intervals, and the phytase activity was measured as described below.

Measurement of phytase activity

The phytase activity was investigated using a modified ferrous sulfate molybdenum blue system (Greiner et al., 1997). Briefly, 50 µl of the enzyme solution was incubated with 450 µl of a substrate solution (1.5 mM sodium phytate in 0.25 M sodium acetate buffer, pH 5.0) at 45 ºC for 30 min. The reaction was then stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid. After that, the released inorganic phosphate was analyzed by adding 1ml of color reagent, containing 1% (w/v) ammonium molybdate, 3.2% (v/v) sulfuric acid, and 7.2% (w/v) ferrous sulfate, and the optical density measured at 700 nm. All the phytase activity determinations were accomplished in three independent experiments. One unit (U) of phytase activity was defined as the amount of enzyme that released 1 micromole of phosphate per minute at 45 ºC. The investigational error was not ever over 7%.

PCR amplification and *16S rDNA* sequencing

Genomic DNA of *Bacillus* sp. LOR10 was extracted according to Sambrook and Russell protocols (Sambrook & Russell, 2001), and its purity was checked by the A260/A280. Universal *16S rDNA* PCR forward primer 27F (5-

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AGTTTGATCCTGGCTCAG-3) and reverse primer 1492R (5-*GGC/TACCTTGTTACGACTT*-3) were used for the amplification of *16S rDNA* genes (Badoei-Dalfard & Karami, 2013; Ramezani-pour et al., 2015). PCR program was achieved as follows: (1) 94 ºC for 5 min as initial temperature, (2) a run of 30 cycles with each cycle consisting of 45 s at 94 ºC, 45 s at 48 ºC and 90 s at 72 ºC, and (3) 5 min at 72 ºC to permit for the extension of any incomplete products (Azadian et al., 2016; Farahmand et al., 2019). PCR products were electrophoresed on agarose gel (1.0 %) and subsequently amplified *16S rDNA* bands were purified by DNA extraction kit (Sinaclon, Iran). Then DNA sequencing was performed on both strands directly by SEQ-LAB (Germany).

The phylogenetic tree was prepared based on the comparison of *16S rDNA* sequences of *Bacillus* sp. LOR10 strain with *16S rDNA* sequences of strains of *Bacillus* species stored in the GenBank database (http://www.ncbi.nlm.nih.gov). All sequences were aligned with Clustal Omega (version 6.0) that was obtained from http://www.seqtool.sdsc.edu/CGI/Omega.cgi (Sievers et al., 2011), and the phylogenetic tree was prepared in MEGA5 (Tamura et al., 2007). The obtained *16S rDNA* sequence has been deposited in GenBank for isolate *Bacillus* sp. LOR10.

Examination of culture condition for phytase production

To determine the optimal condition for phytase production, *Bacillus* sp. LOR10 was inoculated into 50 ml of the liquid medium in a 250 ml Erlenmeyer flask and maintained on a shaker at 37 °C for three days. Carbon, nitrogen, and phosphate sources were explored on the phytase production (Parhamfar et al., 2015; Parhamfar et al., 2016). Carbon sources were 1.5% glucose, 1.5% galactose, and 0.75% glucose + 0.75% galactose. Nitrogen sources were 0.5% glycine, 0.5% yeast extract, and 0.5% 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 explored by using phytase specific medium with pH 5.0, 6.0, and 7.0. Samples were picked up each 24h, and phytase activity was determined as described above.

Partial purification of phytase

Bacillus sp. LOR10 was grown up in nutrient broth medium for 16 h with 168 rpm. The liquid medium used for the production of phytase was composed of [1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate]. The pH of the medium was adjusted to 6.8 before autoclave. The medium was inoculated at 10% (v/v) with a 16h old culture and incubated at 37 ºC with 190 rpm shaking for 48 h. After that, the culture medium was centrifuged at 10,000 × g for 10 min at 4 ºC. and subsequently, the supernatant was precipitated with 0–100% $(NH_4)_2SO_4$ to fraction the proteins at 4 ºC. After centrifuging at 12,000×g for 20 min at 4 ºC, the pellet was dissolved in a small amount of 10 mM sodium acetate buffer, pH 7.0, and dialyzed overnight against the same buffer. All the steps were carried out at 4 °C. The concentrated sample was applied on a Q-sepharose column (1.5×24 cm), which equilibrated with sodium acetate buffer (20 mM, pH 7.0). The proteins were eluted at a flow rate of 1.0 ml/min with the same buffer with NaCl gradient (0.0-1.0 M). All the steps were carried out at 4 °C. Fractions with high phytase activity were pooled and concentrated. SDS-PAGE was also performed on slab gel containing 10% (w/v) polyacrylamide.

Enzyme characterization

The temperature activity profile of LOR10 phytase was investigated from 30 to 90 ºC with 10 ºC intervals. The thermal stability profile was also considered by measuring the residual enzyme activity after incubating the LOR10 phytase in 0.25M sodium acetate (pH 6.5) at each temperature for 30 min, and then the enzyme samples placed on ice. Finally, the residual activity was measured as the standard condition.

The pH activity profile was determined by measuring the LOR10 phytase activity at 45 ºC, and pH 3.0-11.0 using the following buffers; sodium acetate (0.25M, pH 3.0-6.0), Tris-HCl (0.25M, pH 6.0-9.0), and glycine-NaOH (0.25M, pH 9.0-11). For the pH stability assays, LOR10 phytase was incubated in the same buffers over a range of pH from 3.0 to 11.0 at 45 ºC for 60 min. The residual phytase activity was measured under the standard conditions (pH 6.8, 45 ºC, 30 min).

LOR10 phytase activity in the presence of different ions was also investigated with the final concentration of 5 mM. For determining ion stability, the equal volumes of metal ions/SDS/EDTA (10 mM) were mixed with enzyme solution in 0.05 M sodium acetate buffer. The mixture was incubated for 1.0 h at room temperature, and then the residual activity was measured as the standard condition.

RESULTS

Isolation and identification of the phytase-producing bacteria

A total of 85 bacterial species were isolated from Loriya hot spring in Jiroft, located in the southeast of Iran. This hot spring has 68 ºC temperature and

pH 5.0. After phytase assay screening, the best isolate was selected based on a clear halo around the colony. This isolate was cultured in liquid media, and phytase production was considered. This strain was grown on the liquid medium containing sodium phytate as an inducer. The samples were picked up to measure the phytase activity at a 24h interval. Results showed that the maximum enzyme production occurred after 40 h of incubation (Fig. 1A). It also results in Figure 1B shows that pH decreased from 6.8 to 5.7 as enzyme production and phosphate solubilization increased.

PCR product of the *16S rDNA* gene was about 1500 bp, and its sequence was edited to a total length of 1260 bp. Comparing the *16S rDNA* gene sequence of the isolate LOR10 with those in GenBank using Clustal omega show 98% sequence homology with *B. amyloliquefaciens* and the phylogenetic tree was created by the neighbor-joining method by MEGA5 software (Fig. 2).

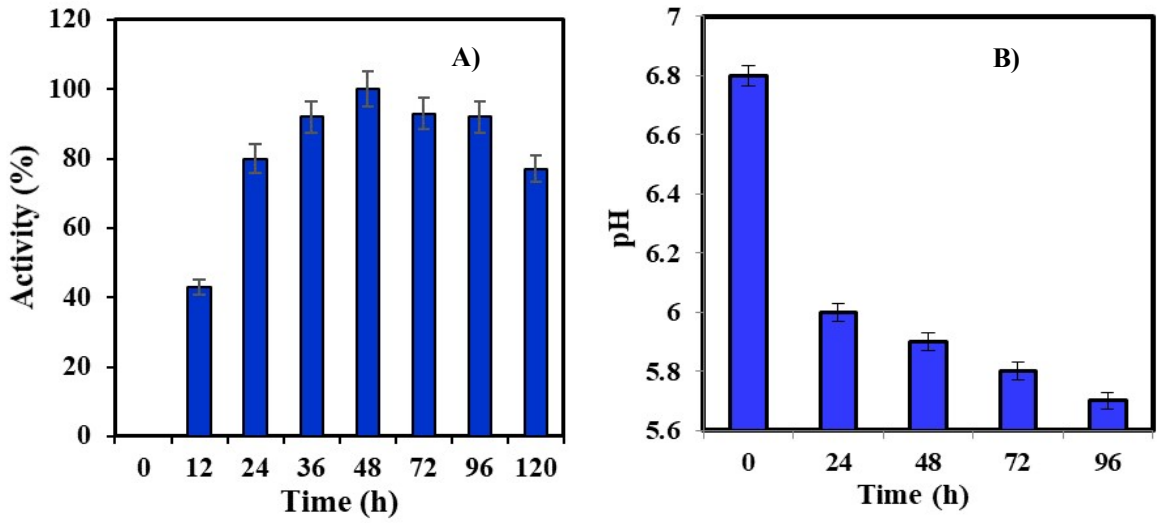

Figure 1. A. Time course of LOR10 phytase production. **B.** Time course of pH changes during incubation of *Bacillus amyloliquefaciens* LOR10.

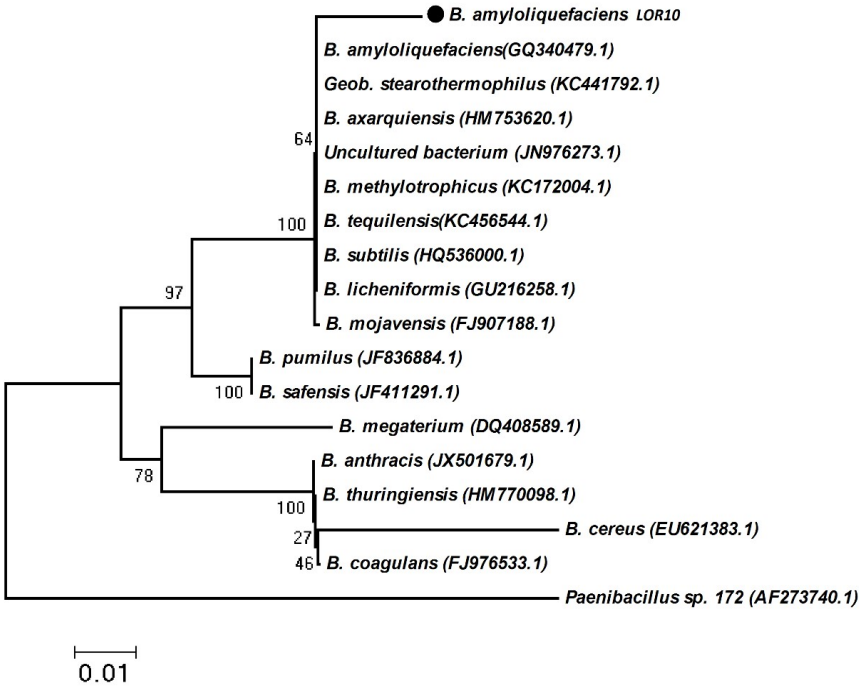

Figure 2. *16S rDNA* based phylogenetic analysis of *B. amyloliquefaciens* LOR10. Bootstrap values and scale bar depicting substitution rate per site are indicated. The phylogenetic tree constructed by the neighbor-joining method showing the position of isolate LOR10. *Paenibacillus* sp. 172 was used as an outgroup.

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Culture investigation for phytase production

To conclude the best conditions for phytase production, the LOR10 isolate was cultured into 50 ml of the liquid medium in 500 ml flask and maintained on a reciprocal at 37 ºC for three days. Figures 3 and 4 displayed diverse 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%) was added to the medium, and phytase activity was assayed at 24, 48, and 72 h of incubation. Results showed that all three carbon sources enhanced phytase production in 24 and 48 h of incubation and marginally decreased in 72h. High phytase activity was gotten in Gal (1.5%) medium after 48h of incubation (Fig. 3a). Results showed that glucose has less effect on phytase production.

The best nitrogen source for phytase production was yeast extract at 48h of incubation. It was also 40 % and 50% more efficient than glycine and ammonium sulfate, respectively (Fig. 3b). Results showed that glycine had less effect on phytase production among these compounds.

The effect of initial pH on the phytase production by LOR10 isolate was also investigated. Results showed that pH 7.0 was the optimum initial pH for phytase production after 24 h of incubation. But, after 48 and 72 h of incubation, pH 6.0 showed the best results for phytase activity. It is mentioned that after 72h incubation, pH 6 showed 10 and 20% more phytase activity than pH 5.0 and 7.0, respectively (Fig. 4A).

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 on Figure 4b. Results showed that tricalcium phosphate was the most suitable compound for phytase production. The nutrient broth has no significant effect on phytase production. A 30 % enhancement in the phytase production achieved after the addition of tricalcium phosphate to the liquid medium.

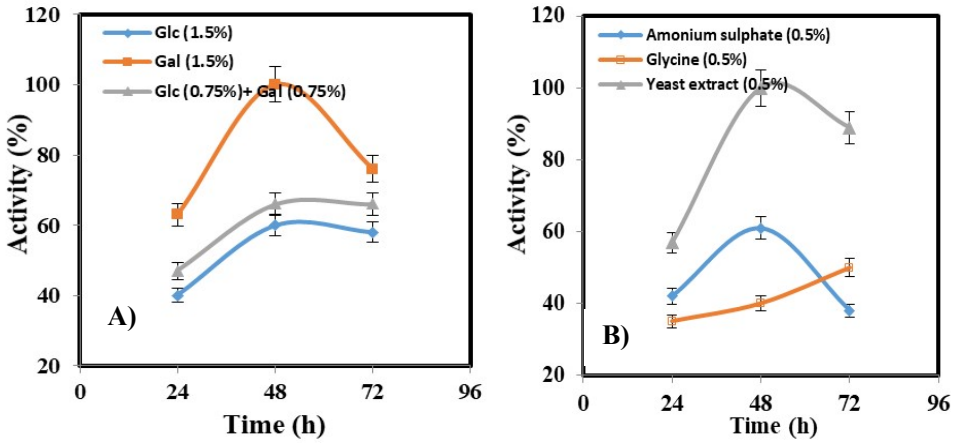

Figure 3. Effect of different carbon (**A**), nitrogen (**B**) on phytase production of *B. amyloliquefaciens* LOR10. Samples were picked up at 24h intervals and phosphate solubilizing determined as described in material and methods.

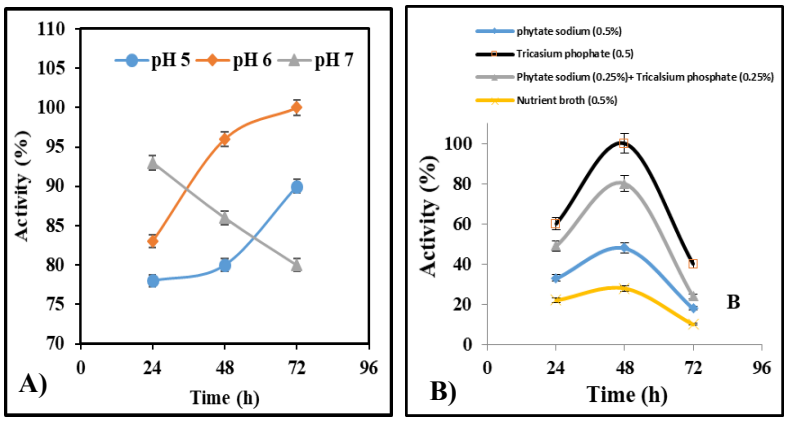

Figure 4. Effect of different initial pH (**A**) and phosphate sources (**B**) on phytase production of *B. amyloliquefaciens* LOR10. Samples were picked up at 24h intervals and phosphate solubilizing determined as described in material and methods.

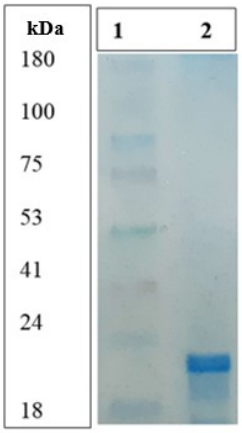

Figure 5. SDS-page of purified phytase from *B. amyloliquefaciens* LOR10 (2), protein markers (1).

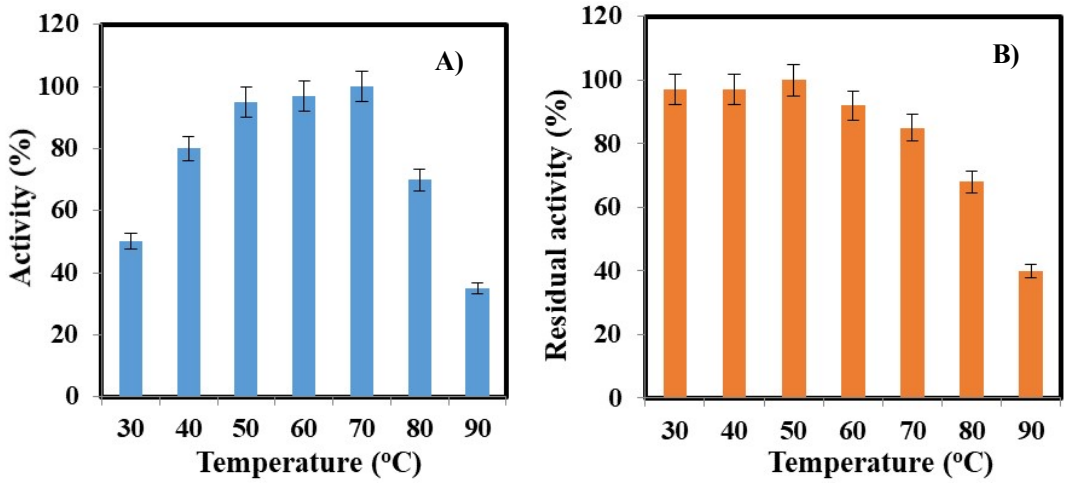

Figure 6. Temperature activity (**A**) and stability (**B**) profiles of LOR10 phytase. For temperature activity, the enzyme was assayed at different temperatures. For temperature stability, the enzyme was pre-incubated at different temperatures for 2h, and then the remaining activity was measured at the standard assay condition, which discussed in the materials and methods.

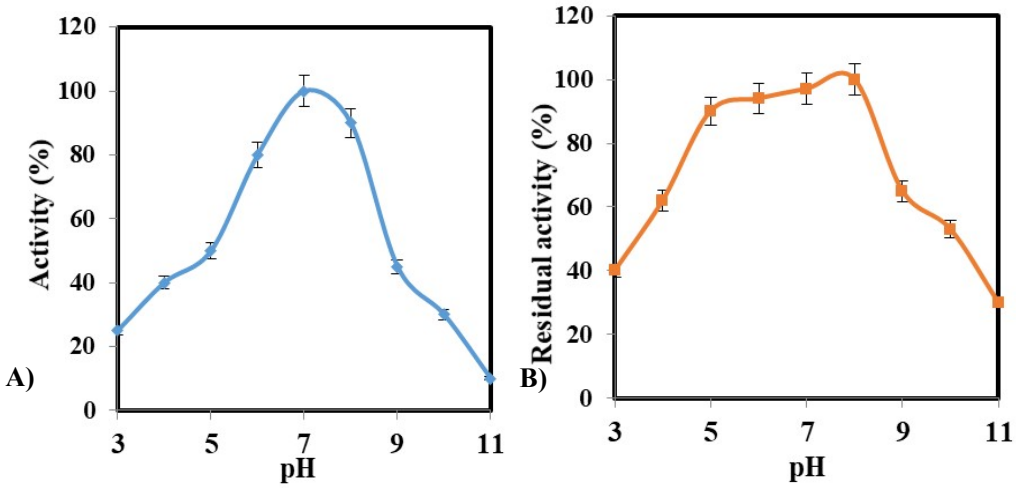

Figure 7. pH activity (**A**) and stability (**B**) profiles of LOR10 phytase. For pH activity, the enzyme was assayed at different pHs. For pH stability, the enzyme was pre-incubated at different pHs for 2h, and then the remaining activity was measured at the standard assay condition, which discussed in the materials and methods.

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Table 1. Relative activity and stability of LOR10 phytase in the presence of different ions and compounds

	Relative activity (%)	Relative stability (%)
No ion	100±0	100±0
CaCl2	142±1.2	137±1.3
MgCl2	105±1.03	112±1.3
ZnCl2	232±1.3	125±1.1
KCl	100±1.0	100±1.1
NaCl	100±0.9	152±1.16
MnSO4	163±1.4	192±1.5
FeSO4	70±0.2	75±0.3
EDTA	65±0.3	90±0.8
SDS	70±0.4	
	60±0.7	

Phytase characterization

SDS-Page results showed that the purified phytase from *B. amyloliquefaciens* LOR10 has a single band of about 22 kDa (Fig. 5). A variety of molecular weight for phytases from other *Bacillus* species had been described as 41 kDa from *Bacillus subtilis* (Farhat et al., 2008), 47 kDa from *Bacillus licheniformes* (Tye et al., 2002), and 28 kDa from *Bacillus tequilensis* Dm018 (Badoei-dalfard et al., 2019).

Phytase activity and stability at different temperatures

The temperature activity profile of LOR10 phytase was shown in Figure 6A. The optimal temperature activity was also detected in 70 ºC. LOR10 phytase showed about 35 % activity in 90 ºC. Results showed that the phytase activity was constant from 30 to 50 ºC, and then regularly decreased (Fig. 6B).

Phytase activity and stability at different pH

The pH activity profile of LOR10 phytase was shown in Figure 7. Results indicated that the LOR10 phytase showed maximum activity at pH 7.0 (Fig. 7A). Also, pH stability results were presented in Figure 7B. These results indicated that LOR10 phytase was stable from pH 5.0–8.0.

Effect of metal ion on phytase activity and stability

LOR10 phytase activity was also investigated at different ions. Results in Table 1 showed that the phytase activity had no change in the presence of $MgCl_2$, KCl, and NaCl. Phytase activity in the presence of $CaCl_2$, $ZnCl_2$, and $MnSO_4$ was increased about 1.4, 2.3, and 1.6 folds, respectively. It is mentioned that the phytase activity decreased by about 70 % in the presence of EDTA and SDS. Results of ion stability in Table 1 revealed that the phytase activity was improved by about 1.37, 1.52, and 1.92 folds in the presence of $CaCl_2$, NaCl, and $MnSO_4$, respectively.

DISCUSSION

A phytase-producing bacterium has been isolated from Loriya hot spring, that it is closely related to *Bacillus amyloliquefaciens*. The ideal enzyme creation was revealed after 40 h of cultivation in the liquid medium. Carbon sources investigation results showed that the high phytase activity (100 %) was obtained in galactose medium after two days of incubation, compared to phytase activity (60%) of the glucose medium.

The effect of different parameters on phytase production was also investigated based on previous reports (Parhamfar et al., 2016; Kim et al., 2002). However, phytase production by *Bacillus subtillus* using different carbon sources showed that glucose was the best carbon source (Kim et al., 2002). Nitrogen sources investigation showed that yeast extract had the best effect on the phytase production after 48 h of incubation. Ammonium sulfate and glycine have less effect about 60 and 40 % on phytase production in the same condition. It was also earlier described that the addition of yeast extract (0.01%) to culture improved phosphate solubilizing capacity of *Pseudomonas* sp.2 about 44%. The same results were also reported by Kim and coworkers. They indicated that yeast extract was the better nitrogen source for the phytase production by strain BPTK4 (Kim et al., 2002). The value of phytase production in the presence of different pHs showed that pH 6.0 was more effective than pH 5.0, 7.0 after 72 h of incubation. Interestingly, phytase production was gradually decreased in pH 7.0 but gradually increased in pH 5.0. It was also stated that the optimal pH by *Pseudomonas* sp. YH40 for phytase production was pH 6.0 (Kim et al., 2002). Phosphate sources exploration showed that tricalcium phosphate was the best phosphate source for phytase production. It was previously reported that none of the phosphate salts (calcium phytate, sodium phytate, K_2HPO_4, and Na_2HPO_4) had any effect on increasing phytase production by *Pseudomonas* sp. YH40 (Kim et al., 2002). The addition of KCl and $FeSO_4$ to the medium showed the same results as *Bacillus* sp.C43 phytase (Sreedevi & Reddy, 2012). LOR10 phytase

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has more than 90% of its stability in 40 to 60 ºC. This phytase displayed about 60% of its initial activity in 80 ºC. The thermal stability of phytase is deliberated to be an important and valuable principle for application as an animal additive. Power and Jagannathan described a phytase that displayed optimal activity at a temperature of 60 ºC and was stable up to 70 ºC (Powar & Jagannathan, 1982). *Bacillus* sp. (natto) phytase had an optimum temperature of 60 ºC (Shimizu, 1992). Although the maximum activity of LOR10 phytase was observed in pH 7.0, it showed more than 40% of its activity in acidic pH (4.0-7.0). LOR10 phytase showed about 90 % of its initial activity in the broad ranges of pH (5.0-8.0). The optimum pH activity of *B. subtillus* (natto) phytase was between 6.0-6.5 (Shimizu, 1992). pH stability of *Pseudomonas* sp. phytase was between pH 5.0-7.0, and the optimal pH of *Pseudomonas* sp. phytase was found pH 7.0 (Kim et al., 2002).

For the investigation of the effects of the ions on phytase activity and stability, several different metal ions were examined (Table 1). Results showed that LOR10 phytase was inhibited by EDTA and SDS about 30%, and stimulated by $CaCl_2$ (1.4 fold), $ZnCl_2$ (2.3 fold), and $MnSO_4$ (1.6 fold). LOR10 phytase stability showed that $CaCl_2$, $ZnCl_2$, NaCl, and $MnSO_4$ stimulated phytase stability from 1.25 to 1.95 folds. It was also reported that Ca^{2+} was required for the activity of *B. subtilis* phytase, and had no critical effect on *Bacillus* phytase (Sreedevi & Reddy, 2012), and had a slightly inhibitory effect on phytases from *E. coli* (Greiner & Konietzny, 1993) and *K. terrigena* (Greiner et al., 1997). It was previously reported that positive monovalent cations did not influence on the alkaline phosphatase activity, while positive bivalent cations had different effects on the enzyme; Mg^{2+}, Ca^{2+}, Co^{2+}, and Mn^{2+} activated the enzyme while Zn^{2+}, Cu^{2+}, and Cd^{2+} inhibited this enzyme (Kim et al., 2002).

Previous reports showed that SDS showed a strong inhibitory effect on other phytases, such as *A. niger* van Teighem phytase, *Eupenicillium parvum* BCC17694 phytase, and *S. thermophile* phytase (Vats et al., 2005; Singh et al., 2009; Fugthong et al., 2010). For example, *A. niger van Teighem* phytase lost 92% of its activity at 0.1% SDS.

Previous reports showed that metal ions could affect enzyme activity. Since metal ions act as Lewis acids, and coordination complexes of these can have different geometries, they may perform differently toward proteins as a ligand (Dokuzparmak et al., 2017). These differences may also result in metal binding to different sites, and, therefore, change the enzyme structure in different ways and affect the enzyme activity. Neira-Vielma and co-workers reported that the activity of the *A. niger* phytase increased in the presence of Zn^{+2} (Neira-Vielma et al., 2018), while phytase activity of *B. subtilis* CF92 (Hong et al., 2011) and *L. plantarum,* (Sumengen et al., 2012) was also inhibited in the presence of Zn^{+2}.

Phytases of *K. pneumoniae* 9-3B and *Bacillus subtilis* were stimulated by EDTA (Kerovuo et al., 2000; Choi et al., 2001). It might be owing to the chelating of metal ions from the phytase complex, which decreases the *K*m. Inhibition of alkaline phytase activity by EDTA has also been reported for the enzymes produced by *Bacillus* sp. DS11, *Bacillus subtilis* (Kim et al., 1998; Vohra and Satyanarayan, 2003), and *Lactobacillus brevis* phytase (Casey et al., 2003). The inhibition by EDTA indicates that some divalent ions are important for complete phytase activity. As mentioned above, EDTA removes divalent metal ions from proteins, thereby modifying their conformations (Ornela et al., 2019).

Finally, LOR10 phytase has engaging activity and stability under acidic pH, excellent thermal stability, and fine ion activity and stability. So, LOR10 phytase can be applied as feed additives, as it could release inorganic phosphate from feed during the digestion process of animals. Currently, phytase has emerged as the world's most extensively used food enzyme. It had been shown that the phytase enzyme has giant latent in food industrial zone for bread processing, isolation of plant proteins, grains wet milling, and cereal bran fractionation.

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