

PHYTASE FROM *BACILLUS SP. STRAIN LA12*: ISOLATION, PURIFICATION AND CHARACTERIZATION

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ABSTRACT

Phytate take effect as an anti-nutrient element in food and feed materials. Thus, phytase, by catalyzing phytate, hydrolyzing the phosphomonoester bonds and releasing the inorganic phosphorous, decrease the phytate and enhance their nutritional value. Therefore, in this study, *Bacillus sp. strain LA12* was isolated from natural origins and the phytase production activity was evaluated. The novel extracellular phytase was produced and precipitated by saturated ammonium sulfate. The ion-exchange chromatography on DEAE-sepharose and the size-exclusion chromatography on Sephadex G-100 were used to purify the enzyme. The results showed that the purification yield and concentration of final enzyme were 5.9% and 18.4%, respectively. Based on SDS-PAGE results the molecular weight of the phytase was determined about 73 kDa. Optimal activity of the enzyme was obtained at pH of 5.5 and 60 °C. Kinetic parameters K_m and V_{max} were 0.197 mM and 1.174 $\mu\text{mol}/\text{min}$, respectively. Mg^{2+} , Co^{2+} and EDTA accelerated the effect on phytase activity; whilst adding other metal ions such as Ca^{2+} , Zn^{2+} and Fe^{2+} in both concentrations could decrease its activity. Moreover, Mn^{2+} ion didn't show indicative effect on its activity. The purified phytase exhibits good thermal stability after incubation at 50-70°C for 30 min, whereas the phytase activity drastically decreased up to 61% at 80°C. This study indicated that the purified phytase has the desired characteristics and can promisingly be used for hydrolyzing of phytate in food and feed.

Keywords: *Bacillus sp. strain LA12*, Phytase, Catalytic activity, Thermo-stability

INTRODUCTION

Phytase are group of enzymes able to hydrolyze phytate to other component such as inositol phosphate and inorganic phosphate. The food/feed from plant origin contain phosphorus at notable level in which the main part of phosphorus is in form of phytate as the salt form of Phytic acid. Phytic acid (Myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) is a predominant phosphorus storage in plants (>70–80% of the total phosphorus). The amount of phytic acid, based on total weight count, comprises 3% to 5% of cereal, oilseed and legumes (Reddy *et al.*, 1982a). As a matter of fact the application of phytase is one of the proper resources to overcome the phytic acid in food/feed. Phytic acid is recognized as anti-nutrient factor for monogastric animals which able to chelate divalent and trivalent cations such as calcium, magnesium, iron, manganese, copper, molybdenum and zinc therefore it has negative effect on their absorption and digestion (Singh and Satyanarayana, 2006, Vohra and Satyanarayana, 2003). Phytate may adversely affect mineral bioavailability (Luo *et al.*, 2007) along with it is not digest by monogastric animals, including human, due to lack of phytase performance. Thus, notable amount of ingested phytate phosphorus in these animals is excreted and is led to the environmental problems, e.g., water pollution (Bohn *et al.*, 2008, Vats *et al.*, 2005). Also being of phytate in foods is one of an important index in mineral related nutritional deficiency in human. In particular for individual who has tendency to plant food or follow vegetarian diet. In this regard as key role of phytase in human nutrition it could not only reduce phytate content but also prevent the mineral deficiency (Reddy *et al.*, 1982b).

From the past decade, attempt has been focused on the phytase enzyme due to the increasing concerns to wards phosphorus pollution in environment and also nutritional benefits. Phytases have a great advantage in raising the nutritional value of phytate in foods/ feed products. The extraction of Phytases from microorganisms (bacteria, yeast, fungi) or from the plants origin have been evaluated in numerous studies (De Angelis *et al.*, 2003, Greiner and Carlsson, 2006, Kaur *et al.*, 2007). Based on literature review, many isolated microbial strains containing efficient phytases have been introduced for industrial applications.

In this study, the enzyme of phytase was purified from *Bacillus sp. strain LA12* and after characterization the optimum condition of activity regarding pH and temperature also thermo stability properties were identified.

MATERIAL AND METHODS

Chemicals

The salt of Phytic acid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemical agent and used media were purchased from Merck (Darmstadt, Germany) also the all reagents were analytical grade.

Bacterial Sample Collection

There are various reports on phytase producer strains of bacteria in poultry farm area soil samples (Sreedevi *et al.*, 2013; Vihnudas *et al.*, 2012 & Tungala *et al.*, 2012). Therefore, in this research more than fifty soil samples from the surface layer of different chicken farm zones in Iran (Fars, 15-50 km north of Shiraz) were used for study.

Isolation of phytase hydrolyzing bacteria

After thorough dilution of 10 g of each soil sample in 90 ml citrate sodium solution (2% w/v) using stomacher (Seaward Medical stomacher, London, UK). The Phytase Screening Medium (PSM) containing calcium phytate was used to select the phytase-producing bacteria and the inoculated plates were incubated at 37 °C for 24-72 h. Each of isolates bearing clear zones on PSM were selected for next examination (Bala *et al.*, 2014).

Molecular identification

DNA isolation

One of the best phytase producer isolates was selected for molecular characterization and identification. A loop of mature colony was transfer to nutrient broth and incubated for 24 h at 37°C. After centrifugation of culture (4,000 g for 10 min) the pellet was harvested and utilized for DNA extraction by the DNA kit (Thermo Fisher Scientific, Germany) based on defined protocol.

16S rRNA sequencing

The extracted DNA was used as a template for Polymerase chain reactions (PCR) amplification of 16S rDNA using primer pair Bacillus Primer-VivantisF (5'-TCACCAAGGCACGATGCG-3') and Bacillus Primer-VivantisR (5'-CGTATTCACCGCGGCATG-3'). The PCR program was done using a thermocycler (Eppendorf, Hamburg, Germany) at 94°C for initial denaturation (3 min), 94°C for denaturation by 35 cycles of 30 sec, 53°C for annealing and 72°C for 2 min extension, also 72°C for final extension (10 min). Then the electrophoresis of PCR products were carry out using a agarose gel (1% w/v) in Tris Boric acid EDTA buffer (100 V for 45 min). The determination of the size of DNA fragments was done using a 100 bp DNA Ladder (Fermentase, Canada). The amplified PCR product was fractionated on agarose gel (0.7 %). The obtained fragments on the gel were cut and purified by gel extraction kit (Axygen Biosciences, Union City, CA, US). Then the purified product was sequence using a sequencer. 16S rDNA sequence of this strain was submitted to a Basic Local Alignment Search Tool (BLAST) search of the NCBI (National Center for Biotechnology Information) GenBank database (www.blast.ncbi.nlm.nih.gov) to identify the organism.

Enzyme production

The phytase enzyme was produced according to Singh and Satyanarayana (2008)(Singh and Satyanarayana, 2008) study with some modification. Briefly, the isolate was cultivated in Luria broth (LB) media for 24 h at 37°C and used as the seed culture. Phytase production media (in a final volume of 1 L) contained: 10 g D-glucose, 4 g Naphytate, 2 g NH₄NO₃, 0.5 g KCl, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O and 0.01 g MnSO₄·H₂O. The prepared liquid fermentation inoculated with 1% of seed culture and incubated in a Benmarin shaker (250 rpm) set at 37°C. After 72 h, the culture was centrifuged at 10,000 g for 20 min. The obtained supernatant was used for the further enzyme purification stages (Gulati et al., 2007b).

Enzyme purification

Step 1: At first, ammonium sulfate was added to collected supernatant. Then the sample was centrifuged at 10,000 g for 30 min at 4°C. The pellet was removed and re-suspended with phosphate buffer 0.1 M (pH 7.0). The obtained sample was desalted using a dialysis bag (diameter: 0.45 μm).

Step 2: Then, the dialyzed fraction was subject to DEAE-sepharose anion-exchange column chromatography (pH 7.0). The fractions with phytase activity were collected and concentrated using PEG 20,000.

Step 3: In the last step of purification, the concentrated sample was loaded onto a Sephadex G-100 chromatography column (2.5 × 35 cm) which was equilibrated with a phosphate buffer (pH7.0). Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished for determining the protein purity (Laemmli, 1970).

Measurement of protein content

After each steps of enzyme purification the total protein concentration of samples were calculated according to the method, of Lowry et al. (1951), using bovine serum albumin and reported as milligrams of protein per mL of sample.

Measurement of phytase activity

Phytase activity of the samples at each step of the purification was analyzed according to the standard method described by Engelen and Van Der Heeft (1994)(Engelen et al., 1994). Briefly, 100 μl of enzyme samples were suspended in 900 μl of 0.1 M acetate buffer (pH 6) and 500 μl of 0.1 M sodium phytate solution which was prepared in acetate buffer. The samples were incubated for 30 min at 37°C. After that, 500 μl of color reagent was added to the sample to stop the reaction. The absorbance was measured at 415 nm by a spectrophotometer (UNICO, UV-2100, China). The blank sample was the substrate inoculated with deionized water. The phytase activity was calculated using absorbance data. One phytase unit (U) is defined as the amount of enzyme per milliliter of culture medium, which releases 1 μg of inorganic phosphorus per minute (Vohra and Satyanarayana, 2003).

Phytase molecular weight estimation

The molecular weight of the purified phytase was determined by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method as described by Laemmli (1970) (Laemmli, 1970).

Characterize of the purified phytase.

The favor temperature for phytase activity was optimized after incubation of the enzyme at different temperatures (30, 37, 40, 50, 60, 70, 80 and 90°C) for 30

min. The percent of residual activity was measured and then the optimum temperature was reported.

The optimum pH for the phytase activity was measured using different buffers with pH ranging from 2.0 to 9.0 at 37 °C. The used buffers in the study were a citrate buffer for pH 3.0-5.8, a Tris-maleate buffer for pH 6.2-7.4 and a Tris-HCl buffer for pH 7.6-9.0 (Bogar et al., 2003).

Kinetic properties of phytase

The kinetic parameters of purified phytase were evaluated by measuring the rate of sodium phytate hydrolysis at the optimum pH and temperature conditions based on method of Lineweaver–Burk plot (Lineweaver and Burk 1934). Sodium phytate was used in concentration ranged from 0.2 to 10 mM. Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) were calculated from the slope and intercept of the regression line of Line weaver–Burk plots using Microsoft Excel 2010.

Effect of metal ions and EDTA on phytase activity

Effect of various cations (Ca²⁺, Zn²⁺, Mn²⁺ and Fe²⁺) and EDTA on phytase activity was evaluated by the method described by Escobin-Mopera (2012) (Escobin-Mopera et al., 2012). For this experiment, a 100 μl of the phytase solution was mixed with 100 μl of metal ions and EDTA solutions which was prepared in 0.05 M sodium acetate buffer with concentrations of 1 mM and 5 mM. After incubation of the solutions at 30°C, the activity of the phytase was assayed.

Statistical analyses

Experiments were done in triplicates based on two different occasions (n=6) so the mean value was calculated. All obtained data was analyzed using One-way Analysis of Variance (ANOVA) and PostHoc Multiple comparison test (LSD) using SPSS version 19.0 (USA).

RESULTS AND DISCUSSION

Microbiological analysis

Thirty one phytase producing bacteria were isolated from the soils samples according the formed clear zones on PSM medium. After further screening on liquid medium, a gram positive bacteria was choose as the best phytase producer strain. The result of nucleotide sequence of 16S rRNA revealed species of isolate as *Bacillus*. The accession number for the sequence was MG322293. Some bacteria belonging to both Gram-positive and Gram-negative group which isolated from soil e.g. *Klebsiella sp.* (Sajidan et al., 2004) *Bacillus laevolacticus* (Gulati et al., 2007a), *Staphylococcus lentus* (Hussin et al., 2007), *Acromobacter sp.*, *Tetrathiobacter sp.* and *Bacillus sp.* (Kumar et al., 2013) have been reported able to produce phytase.

Phytase purification

As presented in Table 1, the crude enzyme had the highest total protein and phytase activity and lowest specific phytase activity. After successive (NH₄)₂SO₄ precipitation, the phytase was purified 1.4 fold and yield of 57.7%. The purification degree of phytase by ammonium sulfate precipitation in *Bacillus sp.* strain LA12 was 100-fold less compared to *Amycolatopsis vancoresmycina* (Bandyopadhyay et al., 2017), but it was more than *Bacillus megaterium EBD 9-1 strain* (Demirkan et al., 2017). The enzyme purification followed by DEAE-sepharose and Sephadex G-100 and the purification increased to 9 and 18.4 fold, respectively. The results showed that the specific activity of the enzyme increased after each purification step, while the yield of enzyme purification significantly decreased. DEAE cellulose column was used for phytase purification from *Amycolatopsis vancoresmycina* with a 3.53 fold purification (Bandyopadhyay et al., 2017).

Figure 1 shows the SDS-PAGE analysis of the resulted proteins from purification steps. The protein band with determined molecular weight of 73 kDa was observed in the last step of protein purification (G-100 size-exclusion chromatography). The obtained molecular weight in our study was higher than *Amycolatopsis vancoresmycina S-12*, *Klebsiella pneumonia 9-3B*, *Bacillus megaterium* and *Enterobacter sp.* ACSS which achieved in Bandyopadhyay et al. (2017), Escobin-Mopera et al. (2012), Demirkan et al. (2017) and the studies conducted by Chanderman et al. (2016) (Bandyopadhyay et al., 2017, Chanderman et al., 2016, Demirkan et al., 2017, Escobin-Mopera et al., 2012), respectively.

Effect of pH and temperature on the phytase activity

Generally, the phytase derived from different sources has a wide range of features, while the industrial applications of enzymes strongly affected by its properties (Yao et al., 2012). Since the thermo stability of enzymes has a major

role in their application, the thermal dependence of phytase activity has shown in Figure 2. Our results indicated that the activity of phytase was increased by increasing the temperature from 30 °C to 60°C, however more increase in thermal treatment resulted in slow decrease in phytase activity. This may be due to heat denaturation of enzyme with increasing temperature. Phytase displayed the highest activity at 60°C, so the optimum temperature of the enzyme activity was at 60°C. In the present study, the optimum temperature was closely similar to those observed for *Enterobacter* and *Serratia* species (Kalsi et al., 2016), *Bacillus megaterium* (Demirkan et al., 2017), *Bacillus subtilis* B.S.46 (Rocky-Salimi et al., 2016), *Geobacillus* (Jorquera et al., 2017), *Penicillium simplicissimum* (Boyce and Walsh, 2007) and *Aspergillus niger* (Vats and Banerjee, 2005), while it lower than the values reported for *Rhizomucor pusillus* (Chadha et al., 2004) and *Thermomyces lanuginosus* (Gulati et al., 2007b).

Our results indicated that a negligible change is observed in the phytase activity by incubation of the phytase solution in the temperature range of 50-70°C for 30 min. Furthermore, the activity after incubation of phytase solution at 80°C for 10, 20 and 30 min were 68, 51 and 39%, respectively. Therefore, the thermal stability was higher than the enzyme obtained from those *Bacillus* species that reported by (Jorquera et al., 2017).

The purified phytase activity was significantly ($p < 0.05$) affected by the pH value of the medium (Fig.3). The phytase activity has increased by increasing the pH values from 2 to 5.5. By increasing the pH above 5.5, the phytase activity suddenly decreased. The enzyme activity was completely lost at pH values of 7.5 and higher. This may be related to denaturation of the enzyme in the alkaline condition. Phytase showed optimum activity at pH 5.5. Under high alkaline and acidic conditions, the phytase lost its activity which it may be due to structural change in the phytase proteins. Overall, the enzyme showed good stability at board pH spectra, since its activity preserved in board pH spectra. The optimum pH of phytase activity was comparable to other species presented in Table 3. Generally, the pH of the different part of digestive tract including salivary glands, stomach and small intestine are 5, 2-4 and 4-6, respectively (Simon and Igbasan, 2002). Therefore, obtained results demonstrated that the enzyme has a good activity in the digestive tract.

Kinetic properties of phytase

After purification of enzyme, the Kinetic parameters of phytase based on sodium phytate as substrate were estimated by Lineweaver–Burk plot. The Michaelis–Menten constant (K_m) was found to be 0.197 mM and the Lineweaver–Burk plot demonstrated the maximum velocity (V_{max}) was found 1.174 $\mu\text{mol}/\text{min}$. A lower K_m indicates higher affinity of enzyme towards the substrate and V_{max} is dependent on the affinity of the enzyme for the substrate and catalytic power.

The difficulty of comparing the kinetic values of phytase is due to the use of different substrate concentration also different experimental conditions by researchers. K_m estimated in this work was close to ones obtained from *Pichia anomala* (0.2 mM, Vohra et al., 2011), *Geobacillus stearothermophilus* strain DM12 (0.177 mM, Parhamfar et al., 2015), *Yersinia enterocolitica* (0.19 mM, Niu et al., 2017) and *Candida melibiosica* 2491 (0.21 mM, Georgiev et al., 2018), regarding affinity to substrate.

The phytase from *Bacillus sp. strain LA12* showed lower K_m and V_{max} values upon comparison with other reported phytases. *Aspergillus niger* NCIM 563 phytase demonstrated a high K_m value of 2.01 mM and V_{max} 5.02 $\mu\text{mol}/\text{min}$ (soni et al., 2010), *Thermomyces lanuginosus* IMI 096218 showed K_m 0.285 mM and V_{max} 0.126 $\mu\text{M}/\text{min}$ (Bujna et al., 2016), *Bacillus subtilis* displayed K_m 0.38 mM and V_{max} 769 U/mg (Jian et al., 2018) and *Aspergillus fumigatus* showed K_m 7.2 mM and V_{max} 35.7 $\mu\text{mol}/\text{min}$ (sanni et al., 2019).

According to these results, it can be concluded that *Bacillus sp. strain LA12* phytase had high affinity with low K_m and V_{max} towards sodium phytate. Furthermore, the evaluation of the kinetic constants indicated that the efficacy of enzyme could be proper for industrial applications.

Effect of metal ions on phytase activity

Different metal ions and modulator such as EDTA had significant effects on purified phytase activity (Table 2). The different phytase activities were reported after incubating metal ions and EDTA. Comparing to the control sample, EDTA is led to stimulate the phytase activity at 5 mM. The results showed that Ca^{2+} , Zn^{2+} , Mn^{2+} and Fe^{2+} at both concentrations decreased the phytase activity. The variation of phytase activity in presence of Ca^{2+} was comparable to phytases of other fungi and yeast species (Table 3). Zn^{2+} had the highest inhibition effect on the phytase activity (reduced phytase activity up to 63% at the concentration of 5 mM). It may be related to –SH groups in the active site of the phytase. Mg^{2+} and Co^{2+} slightly increased the phytase activity at the concentration of 1mM, while at concentration of 5 mM, these metal ions significantly inhibit the activity. These results were in agreement with those achieved by (Wang et al., 2004) that Co^{2+} increased the phytase activity of *K. pneumoniae subsp. pneumoniae* XY-5. They concluded that the decrease in phytase activity could be due to the changing the enzyme conformation. Their finding was inconsistent with that observed in *Bacillus* which the phytase activity was stimulated by calcium ions (Greiner and Carlsson, 2006). Also, EDTA even lightly promoted the phytase activity at

the concentration of 5mM. Kerovuo et al. (2000) (Kerovuo et al., 2000) concluded that the EDTA stimulation of phytase activity may be related to metal ions chelation in which form the accessibility of substrate be grater for enzyme. This means that chelating agent, e.g., EDTA can be used as an additive to enhance the efficacy of phytase, The screening of the best bacteria species which has high phytase production efficacy from soil was the major limitations of present study.

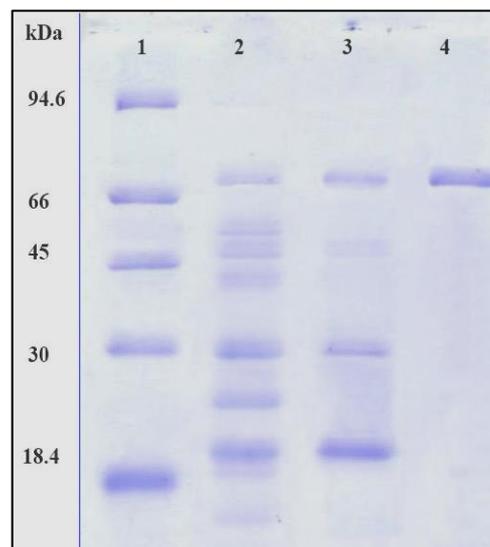


Figure 1 The SDS-PAGE of phytase. lane 1: Ladder 2: the sample after $(\text{NH}_4)_2\text{SO}_4$ precipitation; lane 3: Purified protein (DEAE-sepharose anion-exchange column chromatography); lane 4: Purified protein (G-100 size-exclusion chromatography).

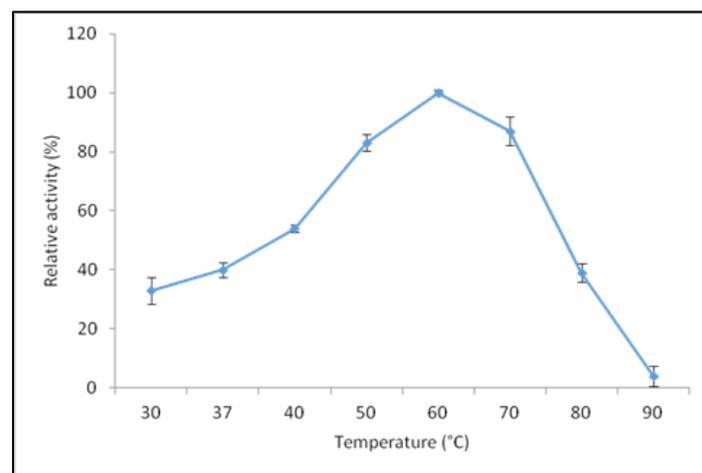


Figure 2 Effect of temperature on phytase activity.

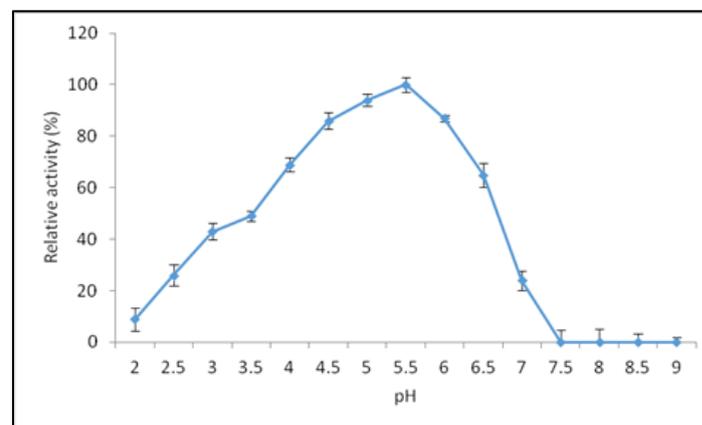


Figure 3 Effect of pH on phytase activity.

Table 1 Purification of the phytase from *Bacillus sp. strain LA12*.

Purification Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude Extract	6082.2	1962	3.1	100	1
(NH ₄) ₂ SO ₄ precipitation	3510	780	4.5	57.7	1.4
DEAE-Sephadex	2388.5	85	28.1	39.2	9
Sephadex G-100	360.36	6.3	57.2	5.9	18.4

Table 2 Activity of the purified phytase from *Bacillus sp. strain LA12* at different concentration metal ions and EDTA

Metal ions	Ion concentration	
	1(mM)	5(mM)
Ca ²⁺	97	80
Zn ²⁺	84	37
Mg ²⁺	103	99
Mn ²⁺	99	90
Fe ²⁺	95	75
Co ²⁺	106	100
EDTA	100	102
Control	100	100

Table 3 Phytase properties from different microorganisms.

Phytase source	Optimal temperature (°C)	pH optimum	Molecular weight (kDa)	Ca ²⁺ demand (mM)	Reference
Fungi					
<i>Buttiauxella sp. GC21</i>	55	4.5	45	No effect	(Shi et al., 2008)
<i>Aspergillus ficuum NTG-23</i>	67	1.3	65.5	No effect	(Zhang et al., 2010)
<i>Penicilliumoxalicum EUFR-3</i>	40	7	-	Inhibited	(Kaur et al., 2017)
<i>Sporotrichum thermophile BJTLR50</i>	60	5	-	-	(Singh and Satyanarayana, 2012)
Yeast					
<i>Hansenulafabianii J640</i>	50	4.5	49	No effect	(Watanabe et al., 2009)
<i>Kodamaeaohmeri BG3</i>	65	5	51	No effect	(Li et al., 2009)
<i>Saccharomyces cerevisiae CY</i>	40	3.6	55	Inhibited	(In et al., 2008)
<i>Debaryomycescastellii CBS 2923</i>	60	4-4.5	51.2	-	(Ragon et al., 2008)
<i>Schizosaccharomycesoctosporus</i>	50	4	-	Inhibited	(Pable et al., 2014)
Bacteria					
<i>Escherichia coli</i>	60	4.5	46.2	No effect	(Miksch et al., 2002)
<i>Enterobacter sp.</i>	50	7-7.5	-	Inhibited	(Yoon et al., 1996)
<i>Bacillus laevolacticus</i>	70	7-8	-	Inhibited	(Gulati et al., 2007b)
<i>Bacillus sp. 9B</i>	60	5	-	Inhibited	(Jorquera et al., 2017)
<i>Geobacillus sp</i>	50	5	-	No effect	(Jorquera et al., 2017)

CONCLUSION

Phytase is an enzyme is able to catalyze the phosphorus release from phytate. The availability of phosphorous and minerals is enhanced in monogastric animals by adding phytase to their feeds. Considering that some of microorganisms are good phytase producer, the *Bacillus sp. strain LA12* was assayed to produce a novel extracellular phytase. The purified phytase was thermostable and its stability was greater at acidic pH compared to alkaline pH. SDS-PAGE test revealed that molecular weight of the phytase was 73 kDa. The optimum temperature and pH for phytase activity was 55°C and 5.5, respectively. Comparing the biochemical properties of phytase obtained from *Bacillus sp. strain LA12* with others sources demonstrated that it has potential for applying in various industries. Therefore, further research is needed to optimize the culture conditions in order to enhance the production yeild and also activity of phytase.

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Ethics Approval: The study project had approved by the research ethics committee of Semnan University of Medical Sciences (approval ID: IR.SEMUMS.REC.1399.148).

Conflict of interest: It is declared that there is no conflict of interest in publication of this work.

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