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Full Length Research Paper

Purification, characterization of phytase enzyme from *Lactobacillus plantarum* bacteria and determination of its kinetic properties

Elif Sarıbuga[1], Hayrunnisa Nadaroglu[2]*, Neslihan Dikbas[1*], Merve Senol[1] and Bülent Cetin[3]

[1]Department of Biotechnology, Faculty of Agriculture, Ataturk University, 25240, Erzurum, Turkey.
[2]Department of Food Technology, Erzurum Vocational Training School, Ataturk University, 25240, Erzurum, Turkey.
[3]Department of Food Engineering, Faculty of Agriculture, Ataturk University, 25240, Erzurum, Turkey.

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Phytases (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyze the release of phosphate from phytates. Many of the cereal grains, legumes and oilseeds store phosphorus in phytate form. Phytases can be produced by plants, animals and microorganisms. However, the ones with microbial origin are the most promising for commercial uses and biotechnological applications. In this study, phytase enzyme isolation from *Lactobacillus* spp. ATCC strain and its characterization was carried out. Phytase production from bacterial strains was determined by zone production formed around colonies after 48 h of incubation at 30°C in MRS medium. Optimum pH and optimum temperature values of the phytase enzyme that was partially purified by precipitation of ammonium sulphate from *Lactobacillus plantarum*, extracellularly from bacteria put into liquid culture medium, were measured. Optimum activity of the enzyme derived from *L. plantarum* bacterium was at 30°C and pH 6.0. It was observed that *L. plantarum*'s extracellular enzyme maintains its 90% of activity at 10-100°C for 120 min. Effects of certain metal ions on activity of phytase enzyme derived from *L. plantarum* were also investigated. Of these, $CuCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$ and $ZnCl_2$ decreased enzyme activity significantly. $FeCl_2$ increased enzyme activity by 121%. Based on these results, the phytase enzyme of *L. plantarum* is considered suitable for use in many industrial areas, in feed and food industries in particular, due to its thermal stability and resistance to metal ions.

Key words: Purification, characterization, phytase, *Lactobacillus plantarum*.

INTRODUCTION

Phytases (myo-inositol hexakisphosphate phosphohydrolas, EC 3.1.3.8) are enzymes that catalyze phytic acid's (myo-inositol hexaphosphate) hydrolysis reaction to myo-inositol phosphate, inorganic monophosphate and free myo-inositol (Kerovuo, 2000). Phytase enzyme was first found in rice bran (Suzuki et al., 1907) and in the blood of calves (McCollum and Hart, 1908). Then, the presence of phytase enzyme in plants, yeast, fungi and

*Corresponding author. E-mail: hnisa25@atauni.edu.tr; neslidikbas@atauni.edu.tr. Tel: 0-90-442-2311818. Fax: 0-90-442-2360982.

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microorganisms was identified (Li et al., 2013). Today, bacterial phytases have more alternative uses than other phytase sources, due to their features such as commercial substrate specificity, resistance against metal ions, thermal stability, proteolysis-resistance and high catalytic activity (Konietzyn and Greiner, 2004; El-Toukhy et al., 2013).

Phytases increase the bioavailability of phosphorus contained in the structure of raw feed and cannot be utilized normally by facilitating its digestion. Phytase enzyme breaks down phytic acid in producing fertilizer and paper pulp and hence inhibits the formation of carcinogenic or toxic substances, and helps to decrease environmental pollution caused by phosphorus. In addition, it was also found that plant growth is stimulated when phytase enzyme is added to soil, depending on the increase in the decomposition rate of phytin (Dalal, 1978; Findenegg and Nelemans, 1993).

Phytase enzyme was purified and characterized from several microbial sources, such as *Pseudomanas* spp. (Irving and Cosgrove, 1971), *Bacillus subtilis* (Powar and Jagannathan, 1982), *Bacillus subtattoilis* (Shimizu 1993), *Bacillus amyloliquefaciens* (Kim et al., 1998), *Eschericia coli* (Greiner et al., 1993), *Klebsiella aerogenes* (Tambe et al., 1994), *Klebsiella terrigena* (Greiner et al., 1997), *Klebsiella oxytoca* (Jareonkitmongol et al., 1997), and *Enterobacter* spp. (Yoon et al., 1996).

Lactic acid bacteria (LAB) are Gram-positive, facultative anaerobic, catalase-negative, immobile (with one or two exceptions), cytochrome-free bacteria that do not produce spores except *Sporolactobacillus inulinus*, and produce lactic acid as an end product during carbohydrate fermentation. These bacteria are almost never present in water and soil, however, their various genus and species can be seen in milk, dairy products, dairy farms, plants, plant waste, and intestinal systems of humans, animals and other living organisms; and these bacteria are non-toxic (Hofvendahl et al., 2000; Tungala et al., 2013). *Lactobacillus plantarum* used in our study is a LAB and is an important bacteria in terms of biosecurity.

The aim of this study was to produce phytase enzyme extracellularly from *L. plantarum* to purify partially, and study its certain kinetic properties.

MATERIALS AND METHODS

Microorganism strains and medium used

The lactic acid bacteria *L. plantarum* used in the study were provided by Laboratory of Microbiology, Department of Food Engineering, University of Ataturk. Bacteria seeded into MRS agar first, and activated by incubating for 48 h at 26 to 30°C. Later, a sample taken from a single colony was added to MRS Broth and allowed to incubate for 48 h again. Then, extracellularly obtained crude extract was used in the enzyme studies. During this time, liquid cultures were allowed to stand in the refrigerator (+4°C).

Ammonium sulphate precipitation for phytase enzyme

After centrifugation of bacteria broth at +4°C at 9000 x *g* for 10 min, supernatant and the precipitate were separated. The precipitate was discarded after centrifugation and the supernatant fraction was subjected to ammonium sulfate precipitation. In order to determine the precipitation range of phytase enzyme, ammonium sulphate precipitation was performed at 0-20, 20-40, 40-60 and 60-80% saturations. Precipitates were dissolved in small amount of 0.1 M Tris-HCl buffer (pH 5.5), and phytase activity was determined both in the precipitate and supernatant (Nadaroglu and Tasgin, 2013). Active fractions were dialyzed against the same buffer. Active fractions were pooled and allowed to stand at 4°C.

Measuring enzyme activity

Enzyme activity was determined by using Na-phytate. In short, 0.1 mL enzyme solution and 250 µL Na-fitate were mixed in a vortex and incubated for 10 min at 37°C. Then, the reaction was stopped by adding 500 µL 10% TCA to the medium, and after incubating for 5 min at 90°C, a 500 µL coloring solution was added and allowed to stand for 15 min, then it was centrifuged for 5 min at 3000 x *g*. After centrifugation, absorbant change in the samples was measured spectrophotometrically (PG Instrument T80 Spectrophotometer) at 700 nm against blind sample.

Determining optimum pH value of enzyme

The enzyme activity was measured in the range of pH 2.0-11.0, in order to determine optimum pH of the phytase enzyme purified from *L. plantarum*. 10 mM acetate buffer was used for the pH 2.0-6.0 range, Tris/HCl buffer was used for the pH 6.0-9.0 range, and carbonate buffer was used for the pH 9.0-11.0 range to measure enzyme activity spectrophotometrically for NA-phytate substrate (Figure 1).

To determine the optimum pH of phytase enzyme in the range of pH 2.0-11, Sodium Acetate (pH 11-6.0), Tris (pH 7.0-9.0), and Carbonate (pH 10-2.0) buffers were used, and optimum pH value for Na-phytate substrate was determined spectrophotometrically by using the above-mentioned standard method (Figure 1).

Determining stable pH value of enzyme

Activity measurements were performed in the range of pH 2-11 in order to determine pH stability of the phytase enzyme produced extracellularly and purified from *L. plantarum*. For this purpose, the buffer solutions mentioned above and enzyme solutions that were adjusted for each pH values were put into the reaction medium, and enzyme activity was determined at certain intervals for 10 h and the pH stability of the enzyme was determined (Figure 2).

Determining optimum temperature value of enzyme

For determination of optimum temperature of enzyme activity, the above-mentioned activity assay was used for activity measurements at 10°C intervals ranging from 10 to 100°C (Figure 3).

Determining stable temperature value of enzyme

In order to determine temperature stability of the phytase enzyme purified from *Lactobacillus plantarum* extracellularly, the enzyme solution was allowed to stand at temperature values ranging from 10 to 100°C, and activity measurements were performed at every 15 min for 2 h (Figure 4).

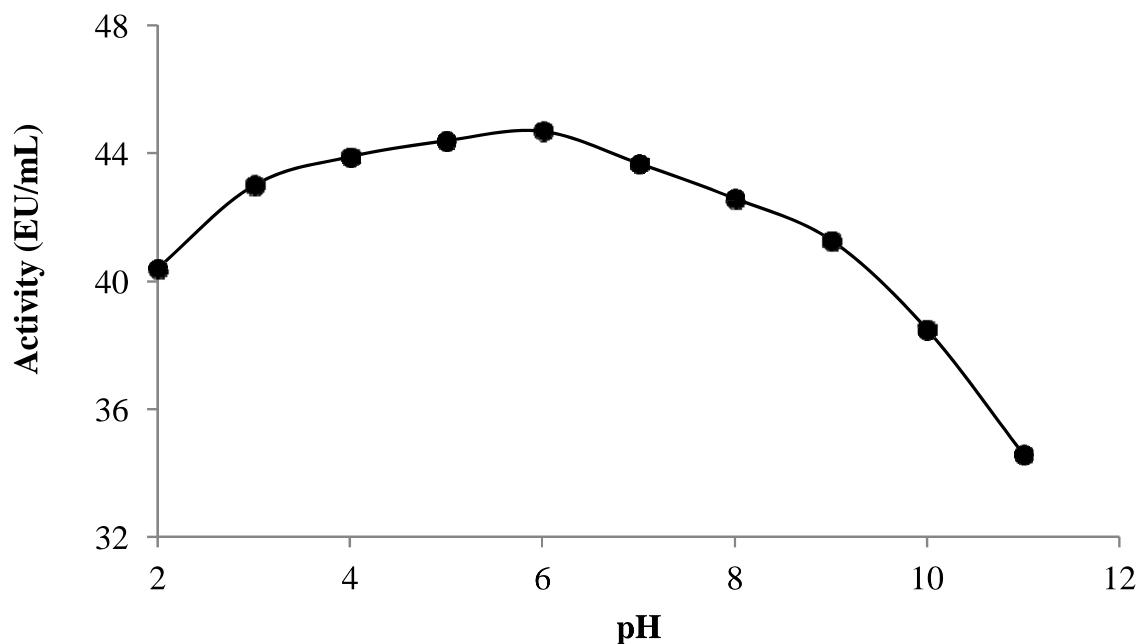

Figure 1. The effect of pH on the activity of purified phytase from *L. plantarum.*

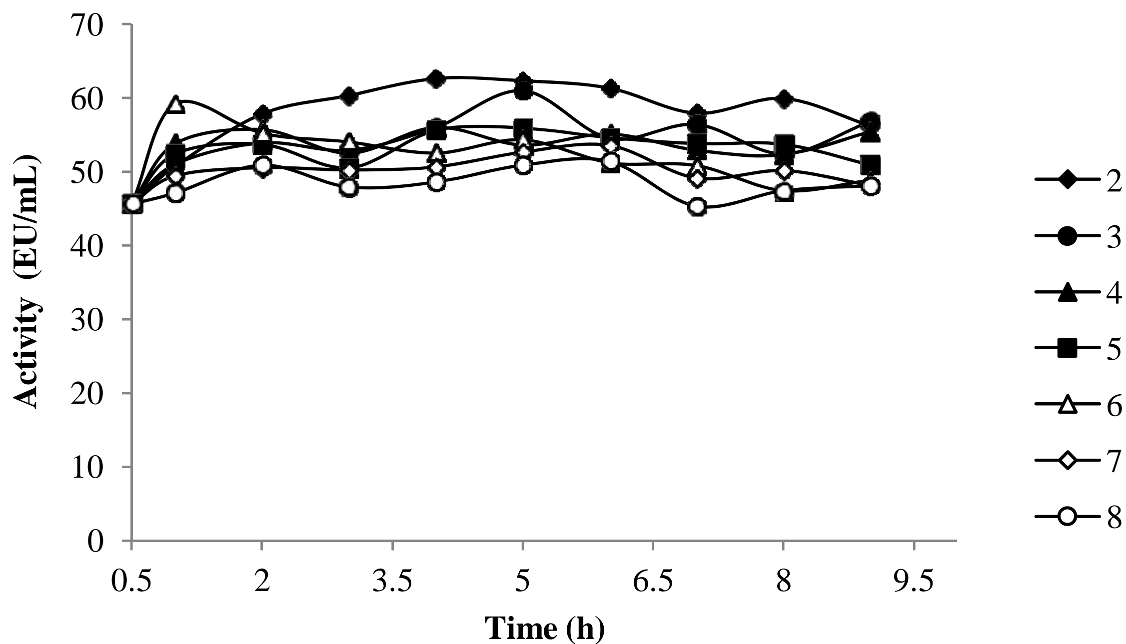

Figure 2. Effect of pH on the activity of the purifed phytase from *L. plantarum*. Enzymes and substrate were dissolved either in 10 mM buffers of various pH. Other conditions were as given for the standard assay method

Determining protein content

Bradford method was used to determine the amount of protein in the enzyme solution quantitatively. In this method, negatively charged Coomassie Brilliant Blue G-250, that binds positive charge in the proteins in acidic medium, is used as a dye. The sensitivity of this method is 1 to 100 µg (Bradford 1976).

Determining the effects of certain metal ions on enzyme activity

Effects of certain metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} on enzyme activity were also investigated. For this purpose, effects at 0.5 and 1 mM concentrations were investigated for each metal ion (Nadaroglu and Tasgin, 2013).

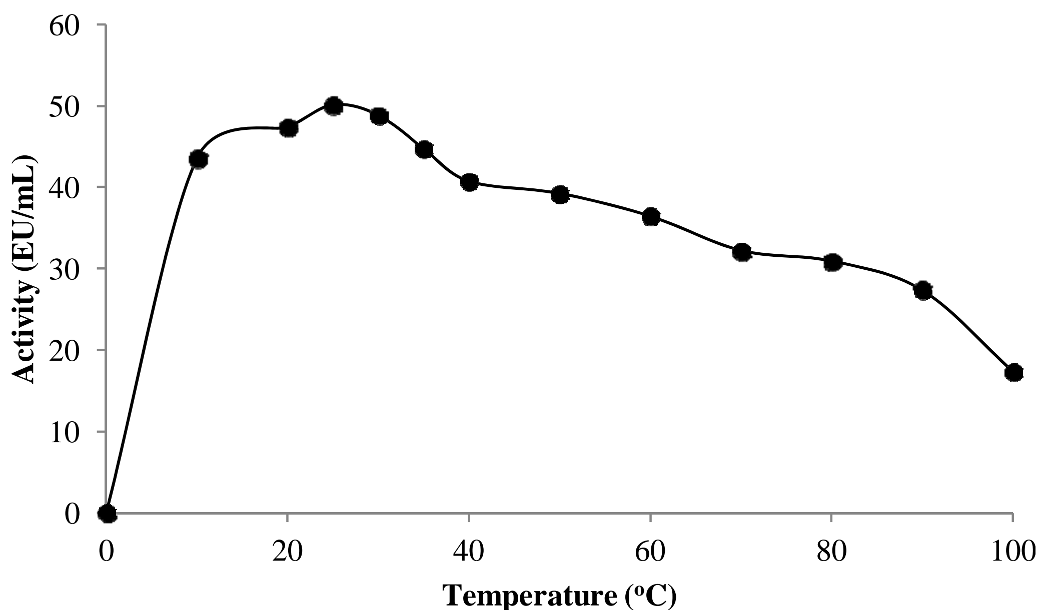

Figure 3. Effect of temperatures on the activity of purifed phytase from *L. plantarum.*

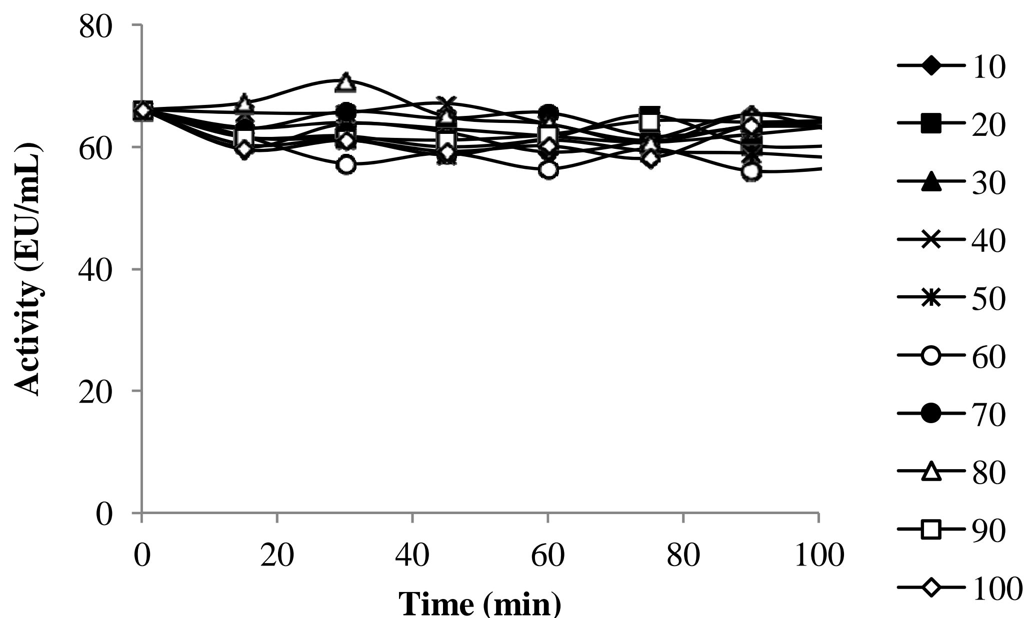

Figure 4. Effect of temperatures on the stability of the purifed phytase from *Lactobacillus plantarum*. Enzymes were incubated at different temperatures for various periods, then directly put into an ice water bath and the residual phytase activity was measured as described in the standard assay method.

RESULTS AND DISCUSSION

In this study, phytase enzyme activity and some kinetic properties of *L. plantarum*, which is a LAB used in many areas, were studied.

Extracellular enzyme activity of *L. plantarum* was determined as 167.3 EU/mL as a result of standard activity assay measurements (Table 1). Looking at the studies in the literature, Songre-Ouattara et al. (2008) have determined intracellular enzyme activity of *L. plantarum* 4.4 as 348.7±17.4 U/mL, intracellular enzyme activity of *L. plantarum* 6.1 as 276.3±51.4 U/mL, and

Table 1. The purification process of phytase from *Lactobacillus plantarum*.

Parameter	Volume (mL)	Activity (EU/mL)	Total activity EU	Total activity %	Protein amount (mg/mL)	Specific activity (EU/mg)	Purification fold
Crude extract	50	167.3	8365	100	6.59	25.39	-
Amonium sulphate precipitation (60-80%)	50	45.6	2280	27.3	0.29	157.75	6.21

Table 2. The effect of some chemical compounds on phytase activity

Chemical compound	Concentration (mM)	Relative activity (%)	Concentration (mM)	Relative activity (%)
None	-	100 ± 0.0	-	100 ± 0.0
Ca^{2+}	0.5	82.9	1	62.0
Fe^{2+}	0.5	153.8	1	235.6
Zn^{2+}	0.5	65.6	1	28.2
Cu^{2+}	0.5	65.6	1	8.16
Mn^{2+}	0.5	80.7	1	56.3

intracellular enzyme activity of *L. fermentum* 7.4 as 276.3±13.2U/mL. Our results comply with literature.

Phytase enzyme was partially purified by ammonium sulfate precipitation of the crude extract. For this purpose, ammonium sulphate precipitation was performed at 0-20, 20-40, 40-60 and 60-80% saturations in order to determine the precipitation range of phytase enzyme. It was determined that enzyme is precipitated in 80-100% range. The enzyme was 6.21-fold purified by this precipitation from *L. plantarum* a by 27.3 yield.

The optimum pH of the phytase enzyme purified from *L. plantarum* was 6 (Figure 1). It was observed that purified phytase enzyme is stable at pH 2.0-11.0 values for 10 h and the enzyme activity was not affected by change in pH much (Figure 2).

Haros et al. (2008) reported that the optimum activity of phytase isolated from *L. plantarum* W42 and *L. plantarum* JBRS is at pH 6.0-6.5, and the optimum activity of phytase isolated from *L. plantarum* 110 is at pH 7.5; and these are in line with our findings.

The activity was measured in the range of 10-100°C, in order to determine optimum temperature of the phytase enzyme obtained from *L. plantarum* (Figure 3). According to the results, the optimum temperature of the phytase enzyme from *L. plantarum* was 30°C. Pure phytase enzyme was incubated for 2 h at 10-100°C, and its temperature stability was found by activity measurements at certain intervals. For phytase enzyme, it was observed after 2 h that the enzyme was stable at the applied temperature values and maintains its 90% of activity (Figure 4).

Effects of certain metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} on the activity of phytase enzyme purified from *L. plantarum* were also studied. It was determined that Cu^{2+}, Mn^{2+} and Ca^{2+} inhibited pure phytase enzyme activity, whereas Fe^{2+} activates the enzyme (Table 2).

Shimizu (1992) found that the activity of extracellular phytase enzyme purified from *B. subitilis* (natto) N-77 strains is inhibited by addition of EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+}. Yoon et al. (1996) have found that *Enterobacter* spp., which is an extracelluar phytase producer, was inhibited by the addition of 4.1 mM Zn^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+} and ethylenediaminetetraacetic acid (EDTA). Yanke et al. (1999) have determined that 5 mM concentration of Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} inhibits phytase enzyme of *Selenomonas ruminantum*, whereas Pb^{2+} activates the enzyme.

In a study conducted by Choi et al. (2001), it was found that the activity of extracellular phytase enzyme obtained from *Bacillus* spp. KHU-10 is inhibited by EDTA and metal ions such as Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, $Hg^{2+}$and Mn^{2+}. These literature findings support the findings in our study.

Conclusion

Based on the results of our study, it was revealed that the phytase enzyme purified partially from *L. plantarum* (ATTC) bacteria is extremely resistant against pH and temperature changes and metal ions that can be found in certain media; and maintains its activity for a long time. It was concluded that it would be appropriate to use the resulting phytase enzyme especially in food, feed and paper industries, in environmental treatment and in soil improvement by enabling its conversion to myo inositol derivatives through hydrolysis of phytic acid found in

grains and legumes.

Conflict of Interests

The author(s) have not declared any conflict of interests

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