Volume 13, Issue 1, Page 123-127, 2024 https://doi.org/10.46810/tdfd.1440314 Research Article

BİNGÖL UNIVERSITY

Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

Partially Purification and Biochemical Characterization of Phytase Enzyme from *Lactobacillus brevis* Isolated from Fresh Kashar Cheese

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(Received: 21.02.2024, Accepted: 15.03.2024, Online Publication: 26.03.2024)

Keywords
Lactobacillus brevis, Phytase, Enzyme purification

Abstract: In the last 20 years, phytase enzyme has attracted the attention of scientists in the fields of environmental protection, nutrition and biotechnology. Myo-inositol hexaphosphate phosphohydrolase (phytase), which is a type of phosphatase enzyme, catalyzes the hydrolysis of phytate into less phosphorylated inorganic phosphates and phytate. Phytases of microbial origin are widely used in biotechnological applications (paper industry, feed industry, food industry and soil improvement). In the present study, phytase enzyme was partially purified from *Lactobacillus brevis* NM-34 strain isolated from fresh kashar cheese and the pH and temperature values at which the enzyme showed optimum activity were determined. *L. brevis* NM-34 exhibited a phytase activity of 243.80 U/mL as a result of ammonium sulphate precipitation. In the ammonium sulfate range (40-60%), where the highest phytase activity was observed, the protein concentration was measured as 0.989 mg/mL. K_m and V_{max} values of phytase enzyme were determined as 0.0146 mM and 1.6 µmol/min, respectively. The pH and temperature values at which the partially purified phytase showed optimum activity were found to be pH 5 and 50 °C, respectively. Based on the findings from our research, the enzyme purified from this bacterium was found to have unique properties that make it suitable for use in industrial applications.

Taze Kaşar Peynirinden İzole Edilen *Lactobacillus brevis*'ten Fitaz Enziminin Kısmi Saflaştırılması ve Biyokimyasal Karakterizasyonu

Anahtar Kelimeler
Lactobacillus brevis, Fitaz, Enzim saflaştırması

Öz: Son 20 yılda fitaz enzimi çevre koruma, beslenme ve biyoteknoloji alanlarında bilim insanlarının dikkatini çekmiştir. Fosfataz enzim grubunda yer alan miyo-inositol heksafosfat fosfohidrolaz (fitaz), fitatın daha az fosforile edilmiş inorganik fosfatlara ve fitata hidrolizini katalizler. Mikrobiyal kaynaklı fitazlar biyoteknolojik uygulamalarda (kâğıt endüstrisinde, yem sanayinde, gıda endüstrisinde ve toprak iyileştirmede) yaygın bir şekilde kullanılmaktadır. Mevcut çalışmada, taze kaşar peynirinden izole edilen *Lactobacillus brevis* NM-34 suşundan fitaz enzimi kısmi olarak saflaştırıldı ve enzimin optimum aktivite gösterdiği pH ve sıcaklık değerleri belirlendi. *L. brevis* NM-34 amonyum sülfat çöktürmesi sonucunda 243.80 U/mL'lik bir fitaz aktivitesi sergiledi. En yüksek fitaz aktivitesi görülen amonyum sülfat aralığında (%40-60) protein konsantrasyonu 0.989 mg/mL olarak ölçüldü. Fitaz enziminin K_m ve V_{max} değerleri sırasıyla 0.0146 mM ve 1.6 µmol/min olarak belirlendi. Kısmi olarak saflaştırılan fitazın optimum

aktivite gösterdiği pH ve sıcaklık değerleri sırasıyla pH 5.0 ve 50 °C olarak bulundu. Araştırmamızdan elde edilen bulgulara dayanarak, bu bakteriden saflaştırılan enzimin endüstriyel uygulamalarda kullanılmasını uygun kılan kendine özgü niteliklere sahip olduğu tespit edilmiştir.

1. INTRODUCTION

Recently, there has been increasing interest in lactic acid bacteria (LAB) that can produce lactic acid in the fermentation of carbohydrates and are now widely used in many different industrial fields [1,2]. LAB are catalase negative, non-sporulating, gram positive, acid tolerant, cocci or rod-shaped microorganisms living on carbohydrate-rich substrates [1,3,4,5].

LAB is a large group of bacteria, including genera such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus* and *Streptococcus*, which are widespread in a variety of environments and have many ecological roles, especially playing an important role in the fermentation processes of food products [6,7]. LAB on the 'Generally Recognized as Safe' list are very useful in many industries as they produce organic acids and other metabolites that prevent spoilage in food, increase flavour development, as a result of fermentation [6,8].

Phytic acid (PA) is an organic form of phosphorus that is present in nature and plays a non-nutritive role, accounting for 60-90% of the total phosphorus content in many seeds and grains during the ripening period [8,9,10]. Phytase enzyme is an important phosphatase that mainly catalyzes phytate into myo-inositol and inorganic phosphate [11,12].

Phytases are isolated from many organisms such as plants, bacteria and fungi [10,13,14]. However, it seems that plants are more preferred than other phytase sources in commercial industrial applications due to the catalytic properties of microbial phytases, their abundance, versatility, easier production, economic stability and environmental friendliness [13,15,16]. The application of microorganisms or enzymes isolated from them in sectors vital to human health is a subject of debate concerning their safety. Phytase enzymes produced by LAB can be used in various industries as they produce safe, cost-effective enzymes with high purity and stability [10,17].

In this study, it was aimed to partially purify phytase from *L. brevis* NM-34 strain isolated from fresh kashar, to determine the optimum pH and temperature values at which the enzyme showed the highest activity, and to determine the protein concentration, K_m and V_{max} values of the enzyme after purification.

2. MATERIAL AND METHOD

2.1. Bacterial Strain

L.brevis NM-34 strain was isolated from fresh kashar cheese and identified.The strain is preserved in the culture collection of N. Dikbas in the Department of Agricultural Biotechnology, Faculty of Agriculture, Atatürk University.

2.2. Phytase Enzyme Production

The strain was inoculated from M17 Agar to M17 Broth and incubated at 35°C for 2 days. The precipitate was then centrifuged at 9000 rpm at 4 °C for 10 min. The supernatant was used to measure enzyme activity [8].

2.3. Partial Purification of Phytase

Partial purification of phytase enzyme was carried out according to Demir et al. [18] *L. brevis* NM-34 strain grown in M17 Broth under appropriate conditions (48 h at 35°C) was centrifuged (10 min at 9000 rpm at 4°C). Phytase was partially purified using ammonium sulfate at a concentration of 0–80%. The partially purified enzyme was dissolved in sodium acetate buffer (0.1 M, pH 6.0) and stored at +4 °C for further studies.

2.4. Measurement of Enzyme Activity

Phytase activity measurement was determined according to Dikbas et al. [8]. Samples containing 0.1 mL of phytase were incubated at 50 °C for 10 min after adding sodium phytate (0.25 mL of 2 mM). After hydrolysis, the reaction was terminated by adding 10% (w/v) trichloroacetic acid. The sample was measured against a blind sample in a spectrophotometer (700 nm).

2.5. Protein Determination

Protein concentration was performed according to Bradford [19] using bovine serum albumin as standard during purification and measured spectrophotometrically at 595 nm.

2.6. Determination of Optimum pH Value of Enzyme

Substrate solutions of the enzyme were prepared by using buffer solutions with different pH values (sodium acetate (pH 2.0-3.0), sodium citrate (pH 4.0-5.0-6.0), Tris-HCl (pH 7.0-8.0-9.0), sodium carbonate (pH 10.0-11.0)) to determine the pH value at which phytase showed optimum activity. The pH value at which the enzyme showed the highest activity was determined by measuring the absorbance values in the spectrophotometer (700 nm) [20].

2.7. Determination of Optimum Temperature of Enzyme

In order to determine the temperature value at which phytase showed the highest activity, the reactions were carried out in the range of 10-90°C with a temperature increase of 10°C. A water bath was used for the determination of temperature measurements. The optimum temperature of the enzyme was determined by measuring absorbance values in a spectrophotometer (700 nm) [21].

2.8. Determination of K_m and V_{max} Values

The activity of phytase enzyme was measured under optimum conditions at different substrate concentrations and K_m and V_{max} values were determined by drawing Lineweaver-Burk graph [22].

3. RESULTS

3.1. Partial Purification Results of Phytase Enzyme

Phytase enzyme from *L.* brevis NM-34 strain was partially purified by ammonium sulfate precipitation in the range of 0-80%, and phytase activity of each range was measured. The purification range with the highest activity was determined to be 40-60% with an activity of 243.80 U/mL (Figure 1). The protein concentration in the range of 40-60% was measured as 0.989 mg/mL.

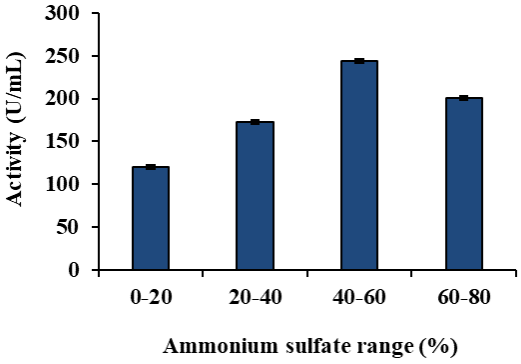

Figure 1. Phytase enzyme activity of ammonium sulfate ranges

3.2. Optimum pH and Temperature Results of Phytase Enzyme

The pH and temperature values at which the phytase enzyme purified from *L. brevis* NM-34 strain showed optimum activity were determined as pH 5.0 and 50 °C, respectively. It was determined that phytase showed an activity of 67.1 U/mL at pH 5.0 and 115.87 U/mL at 50 °C (Figure 2,3).

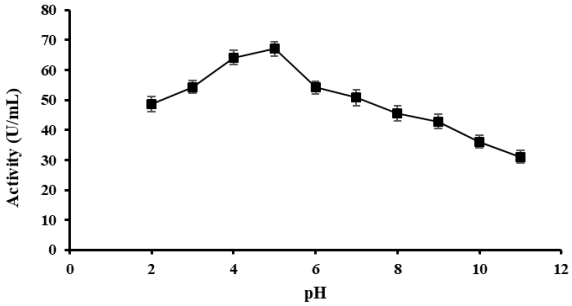

Figure 2. Effect of pH on activity of phytase enzyme

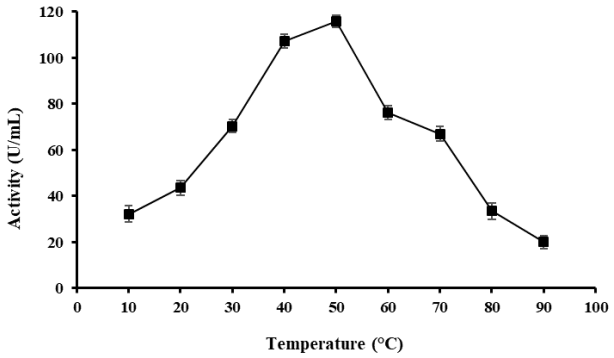

Figure 3. Effect of temperature on activity of phytase enzyme

3.3. K_m and V_{max} Values

As a result of testing the phytase enzyme for *L. brevis* NM-34 strain against sodium phytate, the enzyme showed a K_m value of 0.0146 mM and a V_{max} value of 1.6 µmol/min (Figure 4).

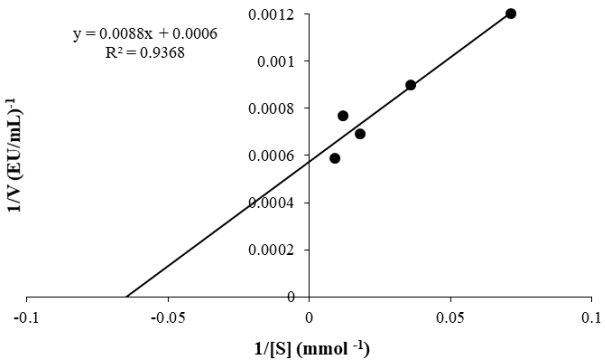

Figure 4. Graph for determining K_m and V_{max} values of phytase enzyme for phytate

4. DISCUSSION AND CONCLUSION

Enzymes are routinely used in many industrial fields. Phytase, one of the industrial enzymes, plays an important role in the field of biotechnology due to its worldwide adoption and being the subject of various studies [9]. Phytases exhibit exceptional versatility in a variety of fields, including human dietary enrichment, animal feed development and industrial processes, driving interest in the discovery of new microorganisms capable of producing phytases [15].

In the present study, the phytase activity of *L. brevis* NM-34 strain was found to be 243.80 U/mL after partial purification. Demir et al. [22] reported the specific activity of phytase of *L. coryniformis* as 202.25 (EU/mg protein). Sharma et al. [23] cloned the phytase gene identified as PhyLf isolated from *L. fermentum* NKN51 and determined the specific activity of phytase as 174.5 U/mg. Bhagat et al. [24] reported that the phytase of *L. paracasei* isolated from Kalarei showed a specific activity of 278 U/mg. Karagöz et al. [11] and Ahire et al. [25]

determined the specific activity of phytase purified from *L. plantarum* as 278.82 EU/mg and 48.59 U/mg, respectively. Dikbaş et al. [8] determined the phytase activity of *L. brevis* isolated from kashar cheese as 212.97 U/mL. Dikbaş et al. [15] reported the specific activity of phytase enzyme purified and isolated from *L. coryniformis* as 188.31 (EU mg $^{-1}$ protein).The data obtained in our study are consistent with the literature.

In this study, the concentration of the protein was measured as 0.989 mg/mL after partial purification. Dikbaş et al. [8] from *L. brevis* and Dikbaş et al. [15] from *L. coryniformis* determined the protein value of the phytase enzyme they purified as 0.504 mg/mL and 0.16 mg/mL, respectively. Karagöz et al. [11] reported the protein concentration of phytase purified from *L. plantarum* as 0.11 mg/mL. Sandez Penidez et al. [26] reported the protein value of phytase enzyme partially purified from *L. plantarum* as 0.08 mg/mL. Demir et al. [22] determined the protein amount of phytase purified from *L. coryniformis* as 0.12 mg/mL in their study. These findings in the literature support our results.

After partial purification of the enzyme, the phytase enzyme for *L. brevis* NM-34 strain was tested against sodium phytate as substrate and the kinetic parameters of the enzyme were calculated by Lineweaver-Burk plot. K_m and V_{max} values of the enzyme were determined as 0.0146 mM and 1.6 µmol/min, respectively. It can be concluded that the partially purified phytase has a high affinity for sodium phytate with low K_m and V_{max} values compared to phytases purified from different sources. Furthermore, the evaluation of the kinetic parameters of phytase showed that the enzyme could be used in industrial applications. Our results are similar to other studies in the literature [8,22,27,28].

In order to determine the optimum temperature and pH values of the phytase enzyme, activity measurements were made in the range of pH 2.0-11.0 and in the temperature range of 10-90°C. The optimum temperature at which phytase showed optimum activity was 50 °C and the pH value was determined as pH 5.0. In contrast to the results obtained, Sumengen et al. [29] determined the temperature and pH values at which the phytase of *L. plantarum* isolated from turnip showed optimum activity as 120 °C and pH 3.4, respectively. Arif et al. [30] reported that *L. gallinarum* (PDP10), *L. reutri* (PDP24) and *L. fermentum* (FYP38) isolated from fermented milk products showed the highest phytase activity at pH 5 at 35°C. Bhagat et al. [24] reported the optimum temperature and pH of the enzyme purified from *L. paracasei*, a species with the highest phytase activity, as 37 °C and 5.5, respectively. Similar to our results, Demir et al. [22] found that phytase purified from *L. coryniformis* isolated from curd cheese and Sharma et al. [23] found that recombinant PhyLf phytase isolated from *L. fermentum* NKN51 showed optimum activity at pH 5.0 at 60 °C. Dikbaş et al. [8] determined the pH and temperature at which the phytase enzyme partially purified from *L. brevis* showed optimum activity as pH 3.0 and 60 °C, respectively. Sandez Penidez et al. [26] found that *L. plantarum* CRL1964 (PhyLP) phytase exhibited optimum activity at pH 4.5 and 55°C. Based on the literature studies and the findings obtained, it is seen that the phytase enzyme purified from *Lactobacillus* spp. is generally acidic and shows optimum activity in the range of 35-70 °C.

The results obtained in the present study show that *L. brevis* NM-34 strain can produce phytase with high activity. According to the results obtained, our study confirmed that the phytase enzyme produced by *L. brevis* is an important biotechnological product that can be used in many different industrial fields due to its characteristic properties. Lactic acid bacteria are microorganisms that are important for food safety and preservation and have received limited research. Therefore, further studies are needed to isolate phytase-producing LABs and determine the biochemical properties of the enzymes to further increase their commercial applicability.

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