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Characterization of a Thermally Stable β-galactosidase Produced by Thermophilic *Anoxybacillus* sp. AH1

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Abstract: Thermostable β-galactosidases from thermophilic bacteria have attracted increasing interest to have various advantages in industrial and biotechnological applications. In this study, a highly thermally stable β-galactosidase produced by *Anoxybacillus* sp. AH1 was purified and characterized. The highest enzyme production was achieved after the bacterium was incubated for 24 hours. The enzyme was purified by precipitation with ammonium sulphate dialysis, gel filtration chromatography using Sephadex G-75. After the purification steps, β-galactosidase was found to be purified 10.2-fold and a yield of 13.9%. The molecular mass of the β-galactosidase was estimated to be 75 kDa by SDS-PAGE. The purified enzyme was highly stable and retained at 71% of the original activity at 60 °C and 53% at 70 °C within 120 minutes. The *Km* and *Vmax* values of purified β-galactosidase were calculated as 1.249 mM and 0.5 μmol minutes^{-1}, respectively. Ca^{2+}, Zn^{2+}, and Mg^{2+} significantly activated β-galactosidase activity, whereas enzyme activity was inhibited significantly by Cu^{+2} as well as by the metal ion chelators1,10-phenanthroline (phen) and ethylenediaminetetraacetic acid (EDTA). The Purified β-galactosidase activity was increased by PMSF (phenylmethylsulfonyl fluoride), PCMB (p-chloromercuribenzoic acid), DTT (dithiothreitol), and β-ME (β-mercaptoethanol) at 2 mM, but inhibited completely by NEM (N-ethylmaleimide) at 1 mM.

Thermofilik *Anoxybacillus* sp. AH1'den Üretilen Termostabil β-galaktosidazın Karakterizasyonu

Anahtar Kelimeler
Enzim aktivitesi inhibisyonu,
Termostabil β-galaktosidaz,
Enzim saflaştırma,
Anoxybacillus sp. AH1

Öz: Termofilik bakterilerden elde edilen termostabil β-galaktosidazlar, endüstriyel ve biyoteknolojik uygulamalarda çeşitli avantajlara sahip oldukları için ilgi çekmektedir**.** Bu çalışmada, *Anoxybacillus* sp. AH1'den üretilen, oldukça termostabil olan β-galaktosidaz, saflaştırıldı ve karakterize edildi. En yüksek enzim üretimi, bakterinin 24 saat inkübe edilmesinden sonra elde edildi. Enzim, amonyum sülfat çöktürmesi, diyaliz ve jel filtrasyon kromatografisi (Sephadex G-75) kullanılırak saflaştırıldı. Saflaştırma aşamalarından sonra, β-galaktosidazın % 13,9 verimle 10,2 kata kadar saflaştırıldığı tespit edildi. β-galaktosidazın moleküler kütlesi, SDS-PAGE ile 75 kDa olarak tahmin edildi. Saflaştırılmış enzimin oldukça stabil olduğu ve 120 dakika sonunda 60 °C'de orijinal aktivitenin % 71'ini, 70 °C'de ise % 53'ünü koruduğu tespit edildi. Saflaştırılmış β-galaktosidazın *Km* ve *Vmax* değerleri sırasıyla 1,249 mM ve 0,5 μmol dakika^{-1} olarak hesaplandı. $Ca^{2+,}$ Zn^{2+} ve Mg^{2+} β-galaktosidaz aktivitesini önemli ölçüde aktive ederken, Cu^{2+} ve metal iyon şelatörleri, 1,10-phenanthroline (phen) ve ethylenediaminetetraacetic acid (EDTA) enzim aktivitesini önemli ölçüde inhibe etmiştir. Saflaştırılmış β-galaktosidaz aktivitesi 2 mM PMSF (phenylmethylsulfonyl fluoride), PCMB (p-chloromercuribenzoic acid), DTT (dithiothreitol), ve β-ME (β-mercaptoethanol) ile artar iken, 1 mM NEM (N-ethylmaleimide) ile tamamen inhibe edildiği belirlendi.

1. INTRODUCTION

β-Galactosidase (beta-D-galactohydrolase, EC3.2.1.23) is a hydrolase enzyme that hydrolyses the complex lactose into simple sugars, glucose, and galactose [1-2]. β-galactosidase is known as a commercially significant enzyme widely used especially in the food and pharmaceutical sectors [2-3]. The main biotechnological uses of β-galactosidase in the dairy industries are the production of galacto-oligosaccharides (GOSs) to use in probiotic foodstuffs and remove lactose from milk for lactose-intolerant people. GOSs are known as largely indigestible oligosaccharides and can support the growth of useful gut bacteria [4-5]. β-Galactosidase is largely used in the dairy industry to prevent crystallization of lactose in concentrated frozen dairy products such as condensed milk and ice cream, and also to solve the problem of whey disposal by converting whey into lactic acid. [6]. Besides, the hydrolysis of whey turns lactose into a very beneficial product, such as sweet syrup; this can be used in the various dairy, confectionery, bakery, and beverage industries. For this reason, lactose hydrolysis not only allows the non-lactose intolerant population to consume milk but also helps solve the environmental problem of whey destruction [1]. In addition, there are many studies on the use of whey in the production of many precious products. In this regard, thermostable β-galactosidases obtained from thermophilic bacteria have attracted increasing attention for use in such industrial processes [4].

Many studies on thermostable galactosidase which have been isolated from different microorganisms have been done so far, such as *Alicyclobacillus acidocaldarius* subsp. *rittmannii* [7], *Bacillus stearothermophilus* [8], *Streptococcus thermophilus* [9], *Thermotoga naphthophila* [10]. There are many studies on various enzymes from *Anoxybacillus*, for instance amylase [11], protease [12], glucose isomerase [13], carboxylesterase [14], esterase [15], and lipase [16]. However, to our knowledge, there have been a few studies so far on the characterization of thermostable β-galactosidase in *Anoxybacillus species* [1, 17-18].

In the present study, we aimed to purify and characterize a biotechnologically important thermally stable β-galactosidase from thermophilic *Anoxybacillus* sp. AH1.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals used in this study were of analytical grade. Bovine serum albumin (BSA), Sephadex G-75, 3,5- p-chlorobenzoic acid (PCMB), 1,10-phenanthroline (phen), dithiothreitol (DTT), iodoacetamide (IAA), N-ethylmaleimide (NEM), sodium dodecyl sulphate (SDS), phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma (Sigma–Aldrich, St Louis, MO, USA). All culture media (Luria-Bertani medium), β-mercaptoethanol (β-ME), and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Germany).

2.2. Bacterial strain, medium, and β-galactosidase activity assay

In this study, thermophilic *Anoxybacillus* sp. AH1 isolated by Acer et al. [11] was used. To determine enzyme activity, *Anoxybacillus* sp. AH1 was grown on the solid Luria-Bertani medium (LB) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and then incubated at 65 °C for 2 days. After incubation of 48 hours, observed blue colonies transferred into LB liquid medium and incubated 60 °C for 48 h in a shaker. To follow bacterial growth, absorbance was measured at 600 nm. The activity of β-galactosidase was detected by the release of *o*-NPG (*o*-nitrophenyl-β-D-galactopyranoside, Sigma) according to Gül-Güven et al. [7].

2.3. Investigation of the Effect of Incubation time on β-Galactosidase Production and Bacterial Growth

I mL of fresh culture was transferred into 100 mL of the flasks containing 25 mL of LB and 3, 6, 9, 12, 24, 36, 42, 48, 60, 72 hours period the samples were taken to measure enzyme activity. In order to determine growth, the increase in absorbance at 600 nm was measured. The enzyme activity is measured by using a supernatant.

2.4. β-Galactosidase Purification

For β-galactosidase purification procedures, *Anoxybacillus* sp. AH1 cells were grown in 100 mL of LB medium at 60 ^{o}C for 24 hours. Then cells were centrifuged at 10.000 rpm at 4 ^{o}C for 10 minutes. 0.1 M Sodium phosphate buffer (Na_2HPO_4 / NaH_2PO_4) at pH 7.0 was used to re-suspend the pellet. For breaking cells, a Sonicator Ultrasonic Processor was used. After sonication, cells were centrifuged at 10.000 rpm at 4 ^{o}C for 10 minutes. The supernatant represented the crude extract.

Ammonium sulphate to 70% saturation was used to precipitate the supernatant. Sodium phosphate buffer (pH 7.0, 0.1 M) was used to dissolve the centrifuged precipitate and after which the sample was dialyzed overnight against the sodium phosphate buffer (pH 7.0, 0.1 M). After the dialyzed, enzyme was concentrated by stirred ultrafiltration cell (PBGC membrane, Millipore), and applied to gel filtration chromatography using Sephadex G-75 (1.5 cm ×30 cm), pre-equilibrated with the same buffer. To elute purified enzyme, sodium phosphate buffer (0.1 M, pH 7.0) with the flow rate of 3 mL minutes^{-1} was used. Ultrafiltration was used for collecting the enzyme-containing fractions. All purification steps were fulfilled at 4 °C. After each step was completed, enzyme activity was measured. Lowry method [19] was used to determine the protein content.

2.5. Molecular Weight Estimation by Electrophoresis and Gel Filtration Chromatogram

To estimate the molecular weight of the subunits, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a vertical gel electrophoresis system was used. SDS-PAGE was carried out according to Laemmli [20]. Zymography (6-bromo-2-naphthyl-galactopyranoside-BNG staining) analysis of the enzyme activity was carried out according to Gül-Güven et al. [7].

2.6. Effect of pH and Temperature on the Purified Enzyme Activity

To determent the optimum pH of the purified enzyme activity, the enzyme activity was studied at pH values ranging from 4.0 to 11.0 at 60 ^{o}C. The pH stability of the purified enzyme was determined by incubating purified enzyme at 60 °C for 2 hours in different buffers (sodium citrate buffer (pH 4.0-6.0); sodium phosphate buffer (pH 7.0–9.0); and glycine-NaOH buffer (pH 11.0).

The optimal temperature for enzyme activity was measured by incubating purified enzyme with *o*-NPG at different temperatures between 20 and 90 °C for 15 minutes at pH 8.0. The reactions were stopped with 500 mL of 1 M Na_2CO_3. The residual enzyme activity was measured after the purified enzyme incubated at 60 and 70 °C for 30, 60, 90, and 120 minutes to determine the thermal stability of the enzyme. For the control, non-subjected to heating enzyme activity was determined and considered as 100%.

For determination of the optimum pH of the purified enzyme activity, the enzyme activity was studied at pH values ranging from 4.0 to 11.0 at 60 ^{o}C. The pH stability of the purified enzyme was determined by incubating purified enzyme at 60 °C for 2 hours in different buffers (sodium citrate buffer (pH 4.0-6.0); sodium phosphate buffer (pH 7.0–9.0); and glycine-NaOH buffer (pH 11.0).

2.7. Kinetic Properties of Purified Enzyme

Lineweaver–Burk plot was used to calculate the *Km* and *Vmax* values. The enzyme was assayed at various *o*-NPG substrate concentrations ranging from 0.5 to 15 mM in 0.1 M phosphate buffer at pH 8.0 at 60 °C for 10 minutes.

2.8. The Effects of Inhibitors

The effects of various chemicals (IAA, β-ME, PMSF, PCMB, DTT, and NEM) and different chelating agents (EDTA and phen), and metal ions (Cu^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+}) on β- galactosidase activity were determined by preincubating enzyme with all agents for 15 minutes. To calculate the remaining enzyme activity, the enzyme activity was determined under standard assay conditions. The activity was used as a control and taken as 100% in the absence of any additives. Chloride forms were used for all the metal. For dissolving divalent metals, chelating agents, chemicals, Tris-HCl buffer (0.1 M, pH 7.0) was used, while ethanol used for dissolving PMSF and NEM, and methanol used for phen.

3. RESULTS AND DISCUSSION

3.1. β-Galactosidase Production and Purification

In this study, the time-dependent β-galactosidase production (129.092 U/mg) was maximum obtained at 24 hours (Fig. 1).

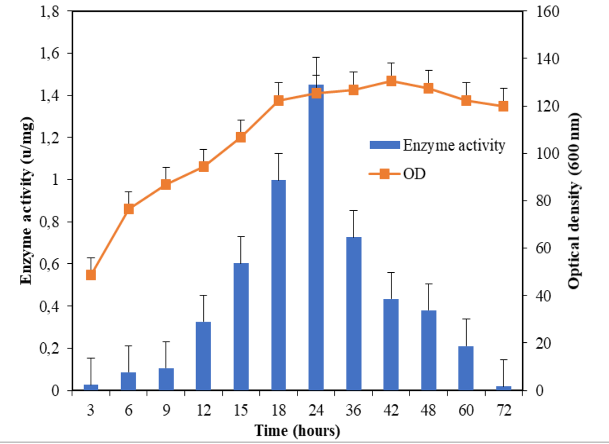

Figure 1. Time-dependent bacterial growth and enzyme activity

Table 1 shows the purification steps used for the purification of the enzyme. β-galactosidase was found to be purified up to 10.2-fold with a 13.9% yield of the pure enzyme**.**

Table 1. Purifications steps of β-galactosidase

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	128.3	27225.4	212.2	1	100
Ammonium sulphate and precipitation/dialysis	16.3	12080.5	743	3.5	44.4
Sephadex G-75	1.7	3781.3	2162.5	10.2	13.9

The partially purified enzyme was subjected to SDS-PAGE analysis and BNG staining (Fig. 2a, b) to estimate the molecular weight of the enzyme and show β-galactosidase activity. According to data of the SDS-PAGE (Figure 2a, lanes 2,3, and 4), the molecular weight of the purified enzyme was found as 75 kDa. In the previous studies, Osiriphun and Jaturapiree [21] also found the molecular weight of β-galactosidase purified from *Anoxbacillus* sp. B1.2 as 75 kDa. Besides, the molecular weight of β-galactosidase was reported as 68 kDa, 42 kDa, and 113 kDa from *Anoxybacillus* sp. KP1 [17], *Aspergillus terreus* [22], and *Bacillus velezensis* [23]. The molecular weight of β-galactosidases belonging to *Anoxybacillus* was recorded by Uniport Protein sequence databases (http://www.uniprot.org/) in the range of 49.1-116.7 kDa.

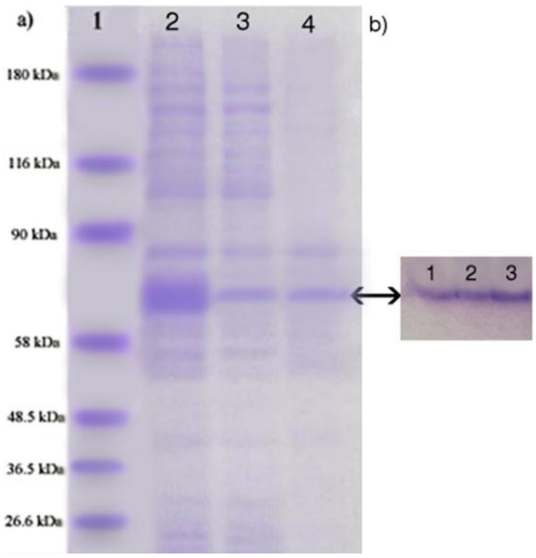

Figure 2. SDS-PAGE CBB-staining (a) BNG-staining (b) analysis of β-galactosidase from *Anoxybacillus* AH1. a: Lane 1, molecular mass markers [Sigma SDS7B2: a2- triosephosphate isomerase (26.6 kDa), lactic dehydrogenase (36.5 kDa), fumarase (48.5 kDa), pyruvate kinase (58 kDa), lactoferrin (90 kDa), β- galactosidase (116 kDa), macroglobulin (180 kDa)]; lanes 2 CBB-staining of partially purified β- galactosidase (Sephadex G- 75), 3b: BNG-staining lane 1, crude extract; lane 2, ammonium sulphate precipitation and dialysis; lane 3, Sephadex G-75.

3.2. Effect of Thermal, pH, and Kinetic Features on β-Galactosidase

As shown in figure 3a, purified β-galactosidase exhibits the highest activity at pH 8 and the enzyme retained galactosidase activity of 60%, 80%, 69%, and 58% at pH 7.0, 9.0, 10.0, and 11.0, respectively. In the recent studies, maximum enzyme activity was reported at pH 7.0, 9.0, 7.2, 7.0 and 6.0 from *Anoxybacillus falvithermus* [1], *Anoxybacillus ayderensis* FMB1[18], *Streptococcus thermophilus* [9], *Aspergillus terreus* [22], and *Klebsiella oxytoca* ZJUH1705 [24], respectively. We also determined that purified β-galactosidase displayed 100%, 95%, 90%, 64%, 61% stability at pH 8.0, 7.0, 9.0, 10.0 and 11.0 for 2 hours, respectively (Fig. 3b).

In the present study, the purified β-galactosidase exhibited maximum activity at 60 °C and displayed 87% of its peak activity at 70 °C (Fig. 4a). In recent studies, Matpan-Bekler et al. [17], Rani et al. [1] and Di Lauro et al. [25] reported optimum β-galactosidase activities from *Anoxybacillus* sp. KP1, *A. flavithermus*, and *Alicyclobacillus acidocaldarius* at 60 °C, respectively. On the other hand, Murphy et al. [26] reported optimum β-galactosidase activity from *Alicyclobacillus vulcanalis* as 70 °C. As seen in Figure 4b, the purified β-galactosidase was highly stable up to 2 hours. It was found that the purified enzyme retained 71% of the original activity at 60 °C and 53% at 70 °C. The tolerance of thermostable β-galactosidases to pasteurization and immobilization is known to have an economic advantage [27] and is of great interest for possible use in the industrial processing of lactose-containing fluids [7].

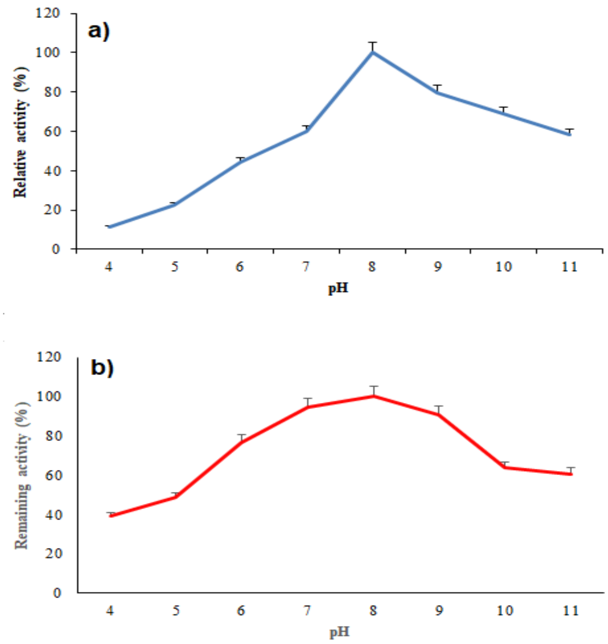

Figure 3. Effect of a) pH on the β-galactosidase activity b) pH on the stability of β-galactosidase activity

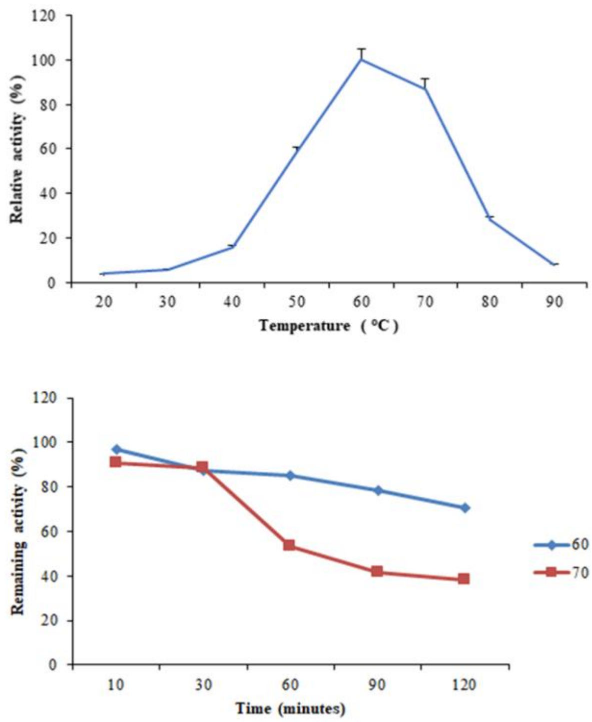

Figure 4. Eff ect of: a) temperature on the *Anoxybacillus* sp. AH1 β-galactosidase activity. b) temperature on the stability of *Anoxybacillus* sp. AH1 β-galactosidase activity.

To perform Kinetic studies of the purified enzyme, various concentrations of *o*-NPG were used as substrate. As shown in Figure 5, *Km* and *Vmax* values were calculated as 1,249 mM and 0.5 µmol minutes^{-1}, respectively, using the Lineweaver–Burk plot.

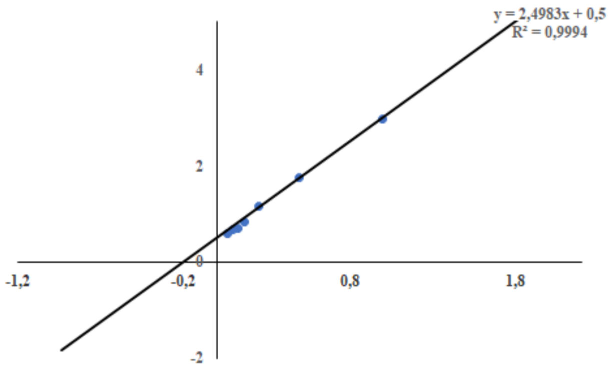

Figure 5. Lineweaver–Burk plot for *Km* and *Vmax* values of the β-galactosidase in the presence of different concentrations of *o*-NPG

The affinity of the enzyme to its substrate is reflected by *Km*. The worth of *Km* is comparatively smaller, displaying a higher enzyme affinity for its substrate. As substrate concentrations become very high, *Vmax* is the limiting velocity. Vmax is expressed in product units that are generated per unit of time. High affinity is expressed by low *Km* value. The enzyme's Km values range is in a wide range especially for most enzymes of industrial significance. Due to the high catalytic efficiency and specificity of the enzyme, *o*NPG is by far the best substrate to determine the activity of β-galactosidase. In the studies published recently, *Km* and *Vmax* values for *o*NPG substrate were reported as 1.3 mM and 3.23 U/mg/min for *A. flavithermus* PW10 [23], 0,48 mM, and 0.96 for *A. terreus* (KUBCF1306) [22], 5.62 mM and 167.1 μmol mg^{-1} for *K. oxytoca* ZJUH1705 [24].

3.3. Investigation of the Effect of Various Chemicals Reagents and Metal Ions on the Purified β-Galactosidase

Understanding the interaction between enzymes and metal ions is important for enzyme activity because proteins are known as essential biological molecules that are required for the appropriate functioning of cells and organisms. As shown in Table 2, purified β-galactosidase activity was enhanced significantly by Ca^{2+} (60% at 10 mM), Mg^{2+} (77% at 20 mM) and Zn^{2+} (43% at 10 mM) whereas Cu^{2+} (100% at 1mM) and metal ion chelators, phen (100%) and EDTA (70%) at 10 mM, significantly inhibited enzyme activity. β-galactosidase inhibition in the presence of metal ions present in milk and dairy products is an important aspect. According to the data obtained from our study, we can suggest that β-galactosidase of *Anoxybacillus* sp. AH1 is a metal-dependent enzyme.

In the previous studies, Rani et al. [1] also reported that β-galactosidase of *A. flavithermus* activity was enhanced in the presence of Ca^{2+}, Mg^{2+} and Zn^{2+} while decreased in the presence of EDTA. Matpan Bekler et al. [17] reported that Ca^{2+} and Mg^{2+} enhanced the β-galactosidase activity of *Anoxybacillus* sp. KP1, whereas Cu^{2+} inhibited β-galactosidase activity as well. Calcium is well known as an important component of milk. For this reason, evaluation of β-galactosidase stability is necessary with different amounts of $CaCl_2$ (calcium chloride) [4]. As shown in Table 2, the activity of β-galactosidase was enhanced significantly in the presence of Ca^{2+}. In most β-galactosidases, Mg^{2+} is known to be required for enzyme activity as well. In recent studies, Ustok et al. [28], Matpan Bekler et al. [17], and Rani et al. [1] reported that Mg^{2+} increased β-galactosidase activity as well.

As can be seen in Table 2, DTT and β-ME reagents containing SH groups increased the purified-galactosidase activity by 122 and 18%, respectively, at 2 mM. Nevertheless, NEM completely inhibited the enzyme activity at 1 mM. We can conclude that there is at least one essential cysteine residue that is modified by chemicals in the active site of the enzyme due to inhibition by NEM. On the other hand, it is interesting to note that the IAA (Iodoacetamide) which is an alkylating reagent through SH group had little effect on the enzyme. Enzyme activity was also enhanced by PMSF for 70% at 8 mM. In recent studies, Gül-Güven et al. (2011) also reported reagents containing SH groups such as 2-mercaptoethanol and DTT enhanced β-galactosidase activity in *Alicyclobacillus acidocaldarius* subsp. rittmannii. Actually, to our knowledge, there are not many studies on the inhibition of β-galactosidase purified from the species *Anoxybacillus* genus. Therefore, this study may be a guide for future studies.

Table 2 Effect of metal ion chelators, divalent metal ions, and chemicals on the activity of purified β-galactosidase

Divalent metals, ion chelators, and chemicals	c mM^{-1} Percentage activity retained (%)								
	0.2 mM	0.4 mM	1 mM	2 mM	4 mM	5 mM	8 mM	10 mM	20 mM
Ca^{2+}	NT	NT	117	123	NT	127	NT	160	239
Mg^{2+}	NT	NT	108	144	NT	148	NT	177	211
Cu^{2+}	NT	NT	0	0	NT	0	NT	0	0
Zn^{2+}	NT	NT	102	116	NT	120	NT	143	222
EDTA	NT	NT	62	58	53	NT	43	30	NT
PHE	NT	NT	NT	27	NT	NT	0	0	NT
DTT	NT	NT	194	222	233	NT	208	180	NT
β-ME	NT	NT	129	118	75	NT	92	8	NT
PMSF	NT	NT	99	160	165	NT	170	117	NT
PCMB	283	263	203	217	NT	NT	NT	NT	NT
NEM	NT	NT	0	0	0	NT	0	0	NT
IAA	NT	NT	113	111	91	NT	106	67	NT

NT=not tested, 0=activity not determined.

4. CONCLUSION

In the present study, we found that metal ions such as magnesium and calcium increased the activity of the purified β-galactosidase. Calcium is well known as an important component of milk. For this reason, In the presence of different amounts of $CaCl_2$, the stability of β-galactosidase is needed to be evaluated. Mg^{2+} is known to be required for enzyme activity in most β-galactosidases as well. We evaluated the effect of various inhibitors and chemicals on the β-galactosidase activity, which may further clarify the nature of the purified enzyme. This β-galactosidase from *Anoxybacillus* sp. AH1 was found to be highly temperature resistant and maintains high relative activity levels at temperatures up to 70 ° C. This property would allow this enzyme to be used for various industrial processes at high temperatures that require a thermo-active β-galactosidase, such as in the manufacture of synthetic disaccharide lactulose, or the manufacture of milk and milk products free of lactose. To our knowledge, there are not many studies on the inhibition of β-galactosidase purified from the species of *Anoxybacillus* genus. Therefore, this study may be a guide for future studies.

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REFERENCES

[1] Rani V, Sharma P, Dev K. Characterization of thermally stable β-galactosidase from *Anoxybacillus flavithermus* and *Bacillus licheniformis* isolated from Tattapani Hotspring of North-Western Himalayas, India. Int. J. Curr. Microbiol. 2019; 8 (1): 2517-2542.Carlson BM. Human embryology and developmental biology. 4th ed. St. Louis: Mosby; 2009.

[2] Banerjee G, Ray A, Das SK, Kumar R, Ray AK. Chemical extraction and optimization of intracellular β-galactosidase production from the bacterium *Arthrobacter oxydans* using Box-Behnken design of response surface methodology. Acta Alimentaria. 2016;45 (1):93-103.

[3] Natarajan J, Christobell C, Kumar DM, Balakumaran MD, Kumar MR, Kalaichelvan, PT. Isolation and characterization of β-galactosidase Producing *Bacillus* sp. from dairy effluent. World Appl. Sci. J. 2012;17 (11):1466-1474.

[4] Gül-Güven R, Kaplan A, Guven K, Matpan-Bekler F, Dogru M. Effects of various inhibitors on β-galactosidase purified from the thermoacidophilic *Alicyclobacillus acidocaldarius* subsp. rittmannii isolated from Antarctica. Biotechnol. Bioprocess Eng. 2011;16 (1):114-119.

[5] Liu Z, Zhao C, Deng Y, Huang Y, Liu B, 2015. Characterization of a thermostable recombinant β-galactosidase from a thermophilic anaerobic bacterial consortium YTY-70. Biotechnol. Biotechnol. Equip. 2015;29 (3):547-554.

[6] Chanalia P, Gandhi D, Attri P, Dhanda S. Purification and characterization of β-galactosidase from probiotic *Pediococcus acidilactici* and its use in milk lactose hydrolysis and galactooligosaccharide synthesis. Bioorg. Chem.2018; 77:176-189.

[7] Gul-Guven R, Guven K, Poli A, Nicolaus B. Purification and some properties of a β-galactosidase from the thermoacidophilic *Alicyclobacillus acidocaldarius* subsp. rittmannii isolated from Antarctica. Enzyme Microb. Technol. 2007; 40 (6): 1570-1577.

[8] Chen W, Chen H, Xia Y, Yang J, Zhao J, Tian F, et al. Immobilization of recombinant thermostable β-galactosidase from *Bacillus stearothermophilus* for lactose hydrolysis in milk. J. Dairy Sci. 2009; 92 (2): 491–498.

[9] Princely S, Basha NS, Kirubakaran JJ, Dhanaraju, MD. Biochemical characterization, partial purification, and production of an intracellular beta-galactosidase from *Streptococcus thermophilus* grown in whey. Eur. J. Exp. Biol. 2013; 3 (2): 242-251.

[10] Kong F, Wang Y, Cao S, Gao R, Xie G Cloning, purification and characterization of a thermostable β-galactosidase from *Thermotoga naphthophila* RUK-10. Process Bioche. 204; 49 (5): 775–82.

[11] Acer Ö, Pirinççioğlu H, Bekler FM, Gül-Güven R, Güven K. 2015. *Anoxybacillus* sp. AH1, an α-amylase-producing thermophilic bacterium isolated from Dargeçit Hot Spring. Biologia. 2015; 70 (7): 853-862.

[12] Matpan-Bekler F, Acer Ö, Güven, K. Production and purification of novel thermostable alkaline protease from *Anoxybacillus* sp. KP1. Cell. Mol. Biol. 2015; 61 (4): 113-120.

[13] Karaoglu H, Yanmis D, Sal FA, Celik A, Canakci S, Belduz AO. 2013. Biochemical characterization of a novel glucose isomerase from *Anoxybacillus gonensis* G2T displays a high level of activity and thermal stability. J. Mol. Catal. 2013;97: 215–24.

[14] Ay F, Karaoglu H, Inan K, Canakci S, Belduz AO, 2011. Cloning, purification, and characterization of a thermostable carboxy- lesterase from *Anoxybacillus* sp. PDF1. Protein Expr. Purif. 2011;80 (1): 74–9.

[15] Chiş L, Hriscu M, Bica A, Toşa M, Nagy G, Róna G, et al. 2013. Molecular cloning and characterization of a thermostable esterase/lipase produced by a novel *Anoxybacillus flavithermus* strain. J. Gen. Appl. Microbiol. 2013;59 (2): 119-134.

[16] Bakir ZB, Metin K. Purification and characterization of an alkali-thermostable lipase from thermophilic *Anoxybacillus flavithermus* HBB 134. J Microbiol Biotechnol, 2016;26 (6): 1087-97.

[17] Matpan Bekler F, Yalaz S, Acer O, Guven K. Purification of thermostable β-galactosidase from *Anoxybacillus* sp. KP1 and estimation of the combined effect of some chemicals on enzyme activity using semiparametric errors in variables model. Fresenius Environ Bull. 2017;26: 2251-2259.

[18] Matpan-Bekler F, Yalaz S, Güven RG, Acer O, Güven K 2018. Characterization of thermostable β-galactosidase from *Anoxybacillus ayderensis* and optimal design for enzyme inhibition using semiparametric EIV models. TOJSAT. 2018;8 (2): 32-37.

[19] Lowry OH, Rosebrough NJ, Farr AL. Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry. 1951;193 (1): 265–275.

[20] Laemmli U. Cleavage of structural proteins during the assembly of the head of Bacteriphage T4. Nature. 1970;277: 680-685.

[21] Osiriphun S, Jaturapiree P. Isolation and Characterization of β-galactosidase from the Thermophile B1.2. AJOFAI. 2009;04: 135-143.

[22] Vidya B, Palaniswamy M, Angayarkanni J, Nawaz KA, Thandeeswaran M, Chaithanya, KK, et al. Purification and characterization of β-galactosidase from newly isolated *Aspergillus terreus* (KUBCF1306) and evaluating its efficacy on breast cancer cell line (MCF-7). Bioorg. Chem. 2020; 94:103442.

[23] Liu Y, Wu Z, Zeng X, Weng P, Zhang X., Wang, C. A novel cold-adapted phospho-beta-galactosidase from *Bacillus velezensis* and its potential application for lactose hydrolysis in milk. Int. J. Biol. Macromol. 2021;166: 760-770.

[24] Huang J, Zhu S, Zhao L, Chen L, Du M, Zhang C, et al. A novel β- galactosidase from *Klebsiella oxytoca* ZJUH1705 for efficient production of galacto-oligosaccharides from lactose. Appl. Microbiol. Biotechnol. 2020;104: 6161-6172.

[25] Di Lauro B, Strazzulli A, Perugino G, Cara FL, Bedini E, Corsaro MM, et al. Isolation and characterization of a new family 42 β-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*: Identification of the active site residues. Biochim Biophys Acta Proteins Proteom. 2008;1784 (2): 292–301.

[26] Murphy J, Ryan MP, Walsh G. Purification and characterization of a novel β galactosidase from the thermoacidophile *Alicyclobacillus vulcanalis*. Appl. Biochem. Biotechnol. 2020;191(3):1190-1206.

[27] Ohtsu N, Motoshima H, Goto K, Tsukasaki F, Matsuzawa H. Thermostable β-galactosidase from an extreme thermophile, *Thermus* sp. A4: Enzyme purification and characterization, and gene cloning and sequencing. Biosci. Biotechnol. Biochem. 1998;62 (8): 1539–45.

[28] Ustok FI, Tari C, Harsa S. Biochemical and thermal properties of β-galactosidase enzymes produced by artisanal yoghurt cultures. Food Chem. 2010; 119 (3): 1114–20.