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

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**Keywords**

Enzyme activity inhibition, Thermostable β-galactosidase, Enzyme purification, Anoxybacillus sp. AH1

**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. Ca²⁺, Zn²⁺, and Mg²⁺ significantly activated β-galactosidase activity, whereas enzyme activity was inhibited significantly by Cu²⁺ as well as by the metal ion chelators1,10-phenanthroline (phen) and ethylenediaminetetraacetic acid (EDTA). The Purified β-galactosidase activity was increased by PMSF (phenylmethanesulfonyl 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.

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

**Anahtar Kelimeler**

Enzim aktivitesi inhibisyonu, Termobazıl β-galaktosizaz, Enzim saflaştırma, Anoxybacillus sp. AH1

**Öz:** Termofoilik bakterilerden elde edilen termobazıl β-galaktosizazlar, endüstriyel ve biyoteknolojik uygulamalarda çeşitli avantajlara sahip oldukları için ilgi çekmektedir. Bu çalışmada, *Anoxybacillus* sp. AH1'den üretilen, oldukça termobazıl olan β-galaktosizaz, saflaştırıldı ve karakterize edildi. En yüksek enzim üretimi, bakterinin 24 saat inkübe edilmesinden sonra elde edildi. Enzim, anonyum sülfat köktürümesi, diyaliz ve jel filtrasyon kromatografisi (Sephadex G-75) kullanarak saflaştırıldı. Saflaştırma aşamalarından sonra, β-galaktosizazın % 13,9 verimle 10,2 katda saflaştırıldığı tespit edildi. β-galaktosidazın moleküler kütesi, 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-galaktosizazın *Km* ve *Vmax* değerleri sırasıyla 1.249 mM ve 0,5 μmol dakika⁻¹ olarak hesaplandı. Ca²⁺, Zn²⁺ ve Mg²⁺ β-galaktosidaz aktivitesini önemli ölçüde aktive ederken, Cu²⁺ 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 (phenylmethanesulfonyl fluoride), PCMB (p-chloromercuribenzoic acid), DTT (dithiothreitol), ve β-ME (β-mercaptoethanol) ile artar iken, 1 mM NEM (N-ethylmaleimide) ile tamamen inhibe edildiği belirlenirdi.
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 napthophilia [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), diithiothreitol (DTT), iodoacetamide (IAA), N-ethylmaleimide (NEM), sodium dodecyl sulphate (SDS), phenylmethylsulfonyl 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

1 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 °C for 24 hours. Then cells were centrifuged at 10,000 rpm at 4 °C for 10 minutes. 0.1 M Sodium phosphate buffer (NaH2PO4 / Na2HPO4) 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 °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 determine 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 °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 α-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 Na2CO3. 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 °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 α-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 (Cu2+, Zn2+, Ca2+, and Mg2+) 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](image)

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 | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|-----------------------------------------------|-------------------|-------------------|--------------------------|-------------------|----------|
| Crude extract                                 | 123.3             | 27225.4           | 212.2                    | 1                 | 100      |
| Ammonium sulphate and precipitation/takeoff   | 16.1              | 13900.5           | 731                      | 3.5               | 44.4     |
| Sophares 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, Osiriphan and Jaturapiree [21] also found the molecular weight of β-galactosidase purified from Anoxybacillus 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.
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].

To perform Kinetic studies of the purified enzyme, various concentrations of o-NPG were used as substrate. As shown in Figure 5, $K_m$ and $V_{max}$ values were calculated as 1,249 mM and 0.5 μmol minutes$^{-1}$, respectively, using the Lineweaver–Burk plot.
The affinity of the enzyme to its substrate is reflected by $K_m$. The worth of $K_m$ is comparatively smaller, displaying a higher enzyme affinity for its substrate. As substrate concentrations become very high, $V_{max}$ is the limiting velocity. $V_{max}$ is expressed in product units that are generated per unit of time. High affinity is expressed by low $K_m$ value. The enzyme's $K_m$ 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 $\beta$-galactosidase. In the studies published recently, $K_m$ and $V_{max}$ 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 $\mu$mol mg$^{-1}$ for K. oxytoca ZIUH1705 [24].

3.3. Investigation of the Effect of Various Chemicals Reagents and Metal Ions on the Purified $\beta$-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 $\beta$-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. $\beta$-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 $\beta$-galactosidase of Anoxybacillus sp. AH1 is a metal-dependent enzyme.

In the previous studies, Rani et al. [1] also reported that $\beta$-galactosidase of A. flaviithermus 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 $\beta$-galactosidase activity of Anoxybacillus sp. KP1, whereas Cu$^{2+}$ inhibited $\beta$-galactosidase activity as well. Calcium is well known as an important component of milk. For this reason, evaluation of $\beta$-galactosidase stability is necessary with different amounts of CaCl$_2$ (calcium chloride) [4]. As shown in Table 2, the activity of $\beta$-galactosidase was enhanced significantly in the presence of Ca$^{2+}$. In most $\beta$-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 $\beta$-galactosidase activity as well.

As can be seen in Table 2, DTT and $\beta$-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) is an alkylating agent 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 $\beta$-galactosidase activity in Alicyclobacillus acidocaldarius subsp. rittmannii. Actually, to our knowledge, there are not many studies on the inhibition of $\beta$-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 $\beta$-galactosidase

| Divalent metal ions | $\beta$-Me reagents | $\beta$-ME reagents | percentage activity retained (%) |
|--------------------|---------------------|--------------------|--------------------------------|
| Ca$^{2+}$          | NT                  | NT                 | 0                              |
| Mg$^{2+}$          | NT                  | NT                 | 0                              |
| Zn$^{2+}$          | NT                  | NT                 | 0                              |
| Cu$^{2+}$          | 100                 | 100                | 0                              |

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 $\beta$-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 $\beta$-galactosidase is needed to be evaluated. Mg$^{2+}$ is known to be required for enzyme activity in most $\beta$-galactosidases as well. We evaluated the effect of various inhibitors and chemicals on the $\beta$-galactosidase activity, which may further clarify the nature of the purified enzyme. This $\beta$-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 thermoactive 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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