CHARACTERIZATION OF THERMOSTABLE β-GALACTOSIDASE FROM *Anoxybacillus ayderensis* AND OPTIMAL DESIGN FOR ENZYME INHIBITION USING SEMIPARAMETRIC EIV MODELS

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Abstract: The thermostable β-galactosidase from *Anoxybacillus ayderensis* was partially purified by Sephadex G-75 with a fold purification of 14.4 and a yield of 13.4%, respectively. The optimal activity and stability of β-galactosidase was pH 9.0 and a temperature of 60 °C. The molecular weight of the subunits was estimated as 90 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The effect of different concentrations of chelating agents (ethylenediaminetetraacetic acid, 1,10 phenantroline), some chemicals (dithiothreitol, phenylmethlysulfonyl fluoride, 4-Chloromercuribenzoic acid, N-ethylmaleimide, iodoacedemide and β-mercaptoethanol) and divalent metal ions (Mg^{2+},Ca^{2+}, Cu^{2+} and Zn^{2+}) on enzyme activity was assayed. According to the results, the thermostable β-galactosidase was significantly activated by Ca^{2+} while inhibited Zn^{2+} and Cu^{2+} ions. In addition, the enzyme activity was inhibited by EDTA, PCMB, N-ethylmaleimide and 1,10 phenantroline. We used a new semiparametric errors in variables model in order to explain the combined effect of the chemicals on enzyme activity, considering that there is a measurement error in nonparametric variable and there is no information at all about the error distribution and compared the no measurement error case and errors in variables case.

Keywords: β-galactosidase, characterization, inhibition, EIV model

Introduction

Enzymes are used in a number of new applications such as food, feed, agriculture, paper, leather and textile industries, which is costly. At the same time fast technological developments; The chemical and pharmaceutical industry, which includes enzyme technology, which is a stronger trend in health, energy, raw materials and environmental issues (Beilen and Lie 2002).

With the advancement of biotechnology and the purification of enzymes, the number of enzyme applications has increased a lot and many possibilities for industrial processes have emerged with the availability of thermostable enzymes. Thermostable enzymes, which are mainly isolated from thermophilic organisms, find many commercial applications due to their general internal stability (Haki and Rakshit 2003).

β-Galactosidase is found in various microorganisms, plant and animal tissues in nature. The commercial importance of microbial β-galactosidases is greater when compared to other sources because of their easy control of their production and their high productivity. (Juajun et al. 2010).

In this study, we purified thermostable β-galactosidase from thermophilic *Anoxybacillus ayderensis* and also we tried to explained the combined effect of the some chemicals on b- galactosidase activity using semi-parametric errors in variables model.

Materials and Methods

Moderately thermophilic *Anoxybacillus ayderensis* (NCBI GenBank database accession number is KP992869) was obtained from Dicle University and it was grown in 100 mL Luria-Bertani medium (LB) incubated at 50 °C for 24 h. After incubation, cells were harvested by centrifugation (8200 g, 30 min, 10 °C) and were lyzed by ultrasonicator (for 3 times 25 sec.) and centrifuged. Supernatants (crude extracts) containing the β-galactosidase were used for purification steps. β-galactosidase purification and anzyme activity determined with *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG, Sigma) method according to Matpan Bekler et al. (2017). The protein content was determined by the Lowry method (1951) using bovine serum albumin (BSA) as a standard. The molecular weight of β-galactosidase was estimated by SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electro-phoresis) according to Laemmli (1970) and zymography analysis was performed as described by Gül-Güven et al. (2007).

After purification steps, the purified enzyme was characterized. Optimum pH was determined under standard assay conditions using *o*-NPG (pH 4.0–10.0). For the pH stability, the purified enzyme was incubated at 60 °C for 1 h in different buffers (4.0 to 11.0). Optimum temperature was determined under standard assay conditions using *o*-NPG at temperatures ranging from 20 to 90 oC. The thermostability was incubated at 60 °C and 70 °C for different time (0-120 min). The unheated purified enzyme was taken as 100%. The remaining β-galactosidase activity was measured under standard assay conditions.

The effect of chelating agent (1,10- phenanthroline: phen), some chemicals dithio-threitol (DTT), phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), iodoacetamide (Iod A) and *β*-Mercaptoethanol (*β*-Mer) and divalent metal ions (Mg^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}) on *β*- galactosidase activity was assayed using 1.5 mM *o*-NPG solution in 0.1 M sodium phosphate buffer (pH 9.0) at 60 °C. The partially purified enzyme was pre-incubated with all agents and then enzyme activity was measured. The combined effects of some chemicals on *β*- galactosidase activity by *Anoxybacillus ayderensis* were studied using semiparametric errors in variables methodology as described by Matpan Bekler et al (2017).

Results and Discussion

The β-galactosidase was purified as described in materials and methods. The purification steps resulted in 14.4 fold purification and a yield of 13.4% (Table 1).

Table 1: Purification steps of β-galactosidase

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	201,6	34943,8	173,3715	1	100
Ammonium sulphate precipitation and dialysis	16,2	16215,2	1003,118	8,0	46,4
Sephadex G-75	2,7	4683,9	1721,989	14,4	13,4

The purified β-galactosidase was subjected to SDS-PAGE analysis and BNG staining (Figure 1a, b). Data of the SDS-PAGE (Figure 1a, lane 2) showed that the purified β-galactosidase had a molecular weight of 90 kDa.

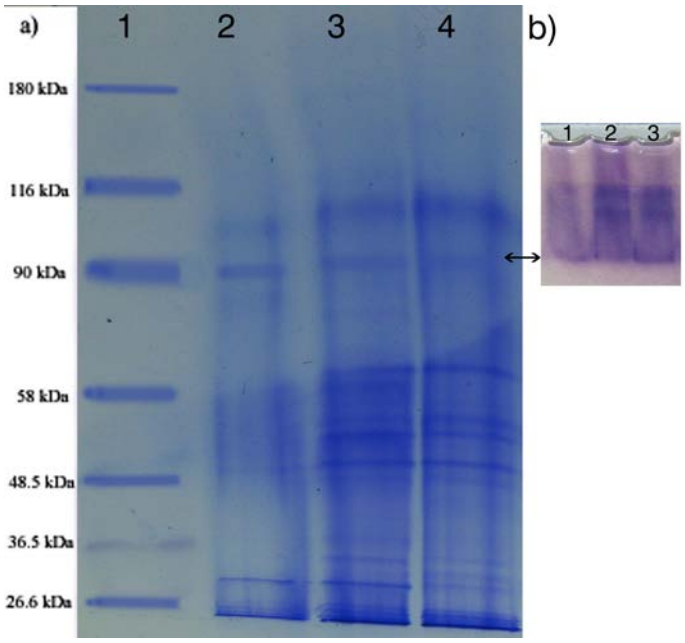

Figure 1. SDS-PAGE CBB-staining (a) BNG-staining (b) analysis of β-galactosidase from *Anoxybacillus ayderensis*. a: Lane 1, molecular mass markers [Sigma SDS7B2: a2-macroglobulin (180 kDa), β- galactosidase (116 kDa), lactoferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephospate isomerase (26.6 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.

Matpan Bekler et al. (2017) reported that the molecular weight of purified β-galactosidases from *Anoxbacillus* were 68.5 kDa. According to Uniport Protein sequence databases (http://www.uniprot.org/), molecular weight of β-galactosidases belonging to *Anoxybacillus* are between 49.1-116.7 kDa.

The optimum pH for β-galactosidase activity was found to be 9.0 (Figure 2). The enzyme was also stable at pH 9.0 (Figure 3).

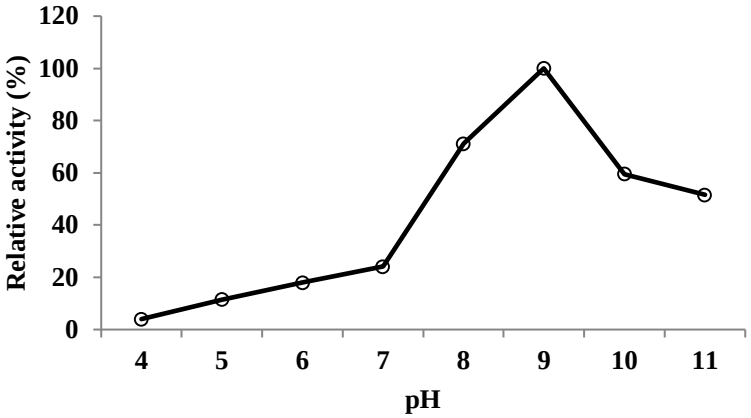

Figure 2. Effect of pH on β-galactosidase.

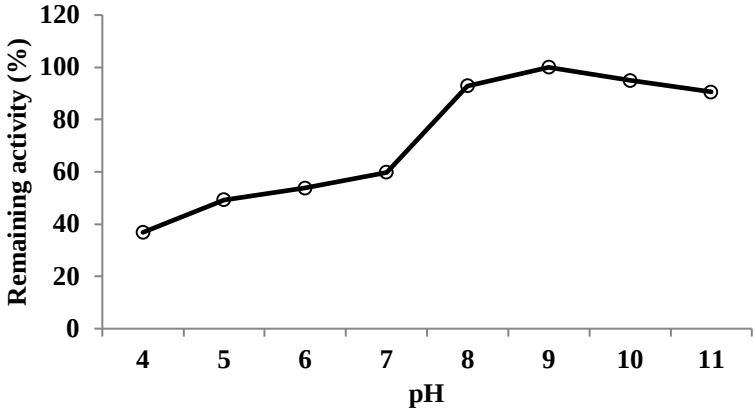

Figure 3. Effect of pH on stability of β-galactosidase.

The purified β-galactosidase activity increased with temperature up to 60 °C, after 70 °C the enzyme activity decreased (Figure 4).

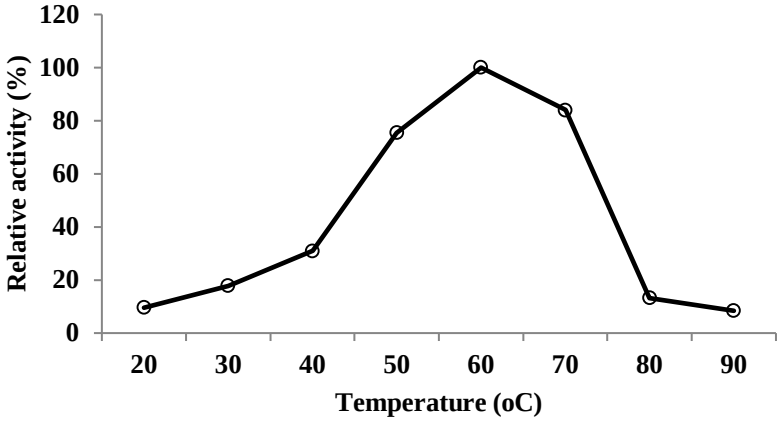

Figure 4. Effect of temperature on β-galactosidase.

Vetere and Paoletti (1998), Chakraborti et al. (2000) and Di Lauro et al. (2008) reported optimum temperature of β-galactosidase activity was 60 °C for *B.circulans*, *Bacillus sp*. and *A. acidocaldarius*, respectively. The enzyme is highly stable at 60 °C for 120 min (Figure 5). Thermostable β-galactosidases are tolerant to immobilization and pasteurization have an economic advantage (Ohtsu et al. 1998).

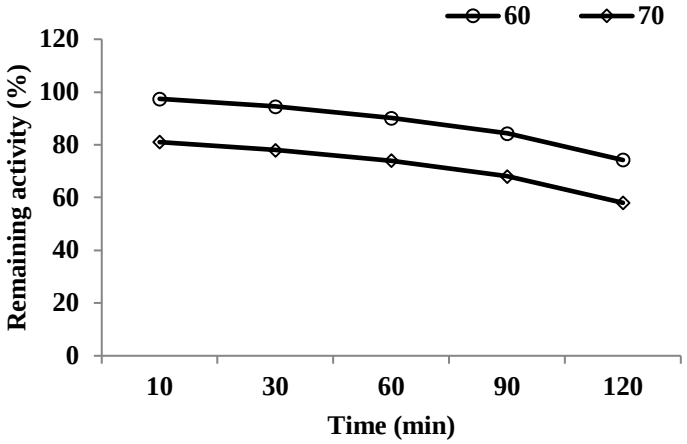

Figure 5. Effect of temperature on stability of β-galactosidase.

the effect of some metal ions on enzyme activity In Table 2 we can see that. The enzyme activity was significantly inhibited by divalent metal ions Cu^{2+} and Zn^{2+} in the total activity result. In previous studies,Ladero et al. (2002) and Shipkowski et al. (2006) stated that Cu^{2+} ions inhibited the enzyme activity. In addition, Ca^{2+} (at 1 mM to 20 mM) activated the enzyme activity while addition of EDTA to assay mixute leads to enzyme inactivation. It is known that enzyme activity in most β-galactosidases increases with Ca^{2+} (Berger et al. 1997) β-galactosidase is considered a metalloenzyme since treatment of the unfolded protein with chelating agents and subsequent refolding experiments ought to be carried out in order to leave out the structural and catalytic metal ions on the enzyme.

Table 2: Effect of some metal ions on β-galactosidase

	Percentage activity retained (%)				
Concentrations / Metal	**1 mM**	**2 mM**	**5 mM**	**10 mM**	**20 mM**
EDTA	68	64	63	60	58
CaCl2	108	111	114	132	178
MgCl2	94	94	94	94	95
ZnCl2	48	46	46	46	39
CuCl2	29	24	23	11	6

Optimal combinations of β-Mer and Iod A for maximum β-galactosidase activity was determined as 8 mM β-Mer and 2 mM Iod A while for minimum β-galactosidase activity was determined as 4 mM β-Mer and 10 mM Iod A. (Fig. 6) The optimal combinations of DTT and Iod A for maximum β-galactosidase activity was determined as 8 mM DTT and 8 mM Iod A and for minimum β-galactosidase activity was determined as 1 mM DTT and 4 mM Iod A (Fig. 7). The combined effect of DTT and β-Mer for maximum β-galactosidase activity was determined as 8 mM DTT and 8 mM β-Mer and minimum β-galactosidase activity was determined as 4 mM DTT and 4 mM β-Mer (Fig. 8). According to our experimental design, in Fig. 9, the combined effect of PMSF and NEM on maximum activity was obtained at 2 mM PMSF and 8 mM NEM. Therefore, it was concluded that no thiol or carboxylic residue are essential for its catalytic activity. It can be seen that there is also a decrease in enzyme activity at 4 mM PMSF and 1 mM NEM because of the measurement error.

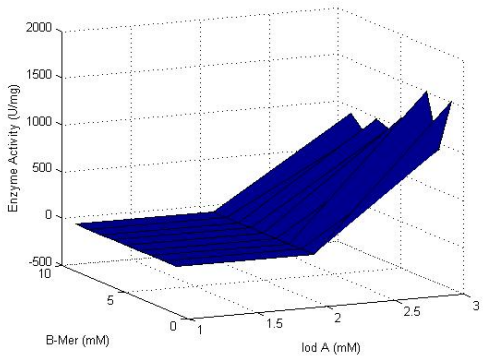

Figure 7: Response surface plot showing the effect on β-mer, Iod A and their mutual effect on the β-galactosidase activity.

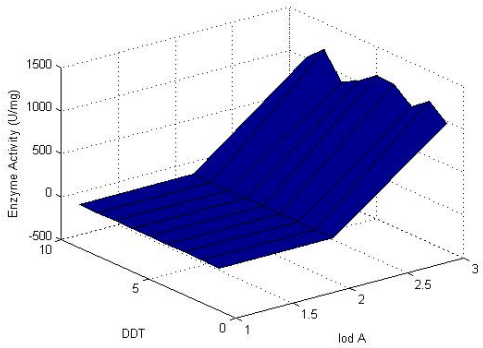

Figure 8: Response surface plot showing the effect on DTT, Iod A and their mutual effect on the β-galactosidase activity.

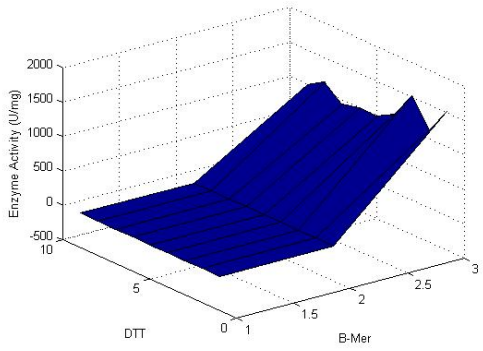

Figure 6: Response surface plot showing the effect on DTT, β-mer and their mutual effect on the β-galactosidase activity.

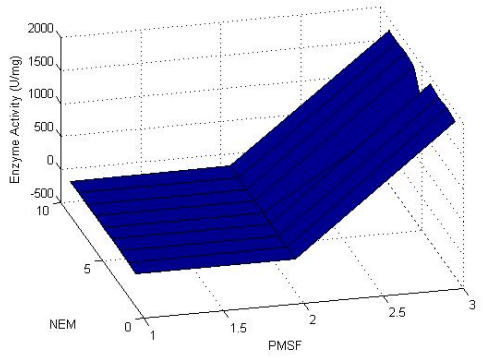

Figure 6: Response surface plot showing the effect on NEM, PMSF and their mutual effect on the β-galactosidase activity.

Conclusion

The main goal of this study was to purify, characterize and reveal the effect of concentrations of some chemicals on β-galactosidase activity and for that purpose experimental design in semiparametric regression method was used as variable of nonparametric part had unknown measurement error. The significant achievement of the present work was to enable us to have no information about a measurement error in nonparametric variable and the combined effect of the six chemicals on the enzyme activity. The method that was selected for the optimization of chemical concentration was found to be quite simple, adequate, and taking less time and material.

References

Beilen, J.B., Li, Z. (2002) Enzyme technology: an overview. Current Opinion in Biotechnology, 13:338–344.

Berger J-L, Lee BH, Lacroix C. (1997) Purification, properties and characterization of a high-molecular-mass β-galactosidase isoenzyme from Thermus aquaticus YT-I. Biotechnology and Applied Biochemistry. 25(1):29–41.

Chakraborti S, Sani RK, Banerjee UC, Sobti RC. (2000) Purification and characterization of a novel β-galactosidase from Bacillus sp MTCC 3088. Journal of Industrial Microbiology and Biotechnology. Jan 24(1):58–63.

Di Lauro B, Strazzulli A, Perugino G, Cara FL, Bedini E, Corsaro MM, Rossi M, Moracci M. (2008) Isolation and characterization of a new family 42 β-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*: Identification of the active site residues. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1784(2):292–301.

Gul-Guven R, Guven K, Poli A, Nicolaus B. (2007) Purification and some properties of a β- galactosidase from the thermoacidophilic Alicyclobacillus acidocaldarius subsp. rittmannii isolated from Antarctica. Enzyme and Microbial Technology 40: 1570-1577

Haki, G.D., Rakshit, S.K., (2003) Developments in industrially important thermostable enzymes a review. Bioresource Technology, 89: 17-34.

Juajun O, Nguyen T-H, Maischberger T, Iqbal S, Haltrich D, Yamabhai M. (2010) Cloning, purification, and characterization of β- galactosidase from Bacillus licheniformis DSM 13. Applied Microbiology and Biotechnology 89(3):645–54.

Ladero M, Perez M, Santos A, Garcia-Ochoa F. (2002) Hydrolysis of lactose by free and immobilized β-galactosidase from Thermus sp. strain T2. Biotechnology and Bioengineering. 81(2):241–52.

Laemmli U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriphage T4. Nature 277:680-685

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

MATLAB:https://www.mathworks.com

Matpan Bekler F, Yalaz S, Acer O, Guven K. (2017) Purification of thermostable β-galactosidase from *Anoxybacillus* sp. KP1 and estimation of combined effect of some chemicals on enzyme activity using semiparametric errors in variables model. Fresenius Environmental Bulletin 26(3): 2251-2259.

Ohtsu N, Motoshima H, Goto K, Tsukasaki F, Matsuzawa H. (1998) Thermostable β-galacto-sidase from an extreme thermophile, *Thermus* sp. A4: Enzyme purification and character-ization, and gene cloning and sequencing. Bioscience, Biotechnology, and Bio-chemistry 62(8):1539–45.

Shipkowski S, Brenchley JE. (2006) Bioinformatic, genetic, and biochemical evidence that some glycoside hydrolase family 42 β-galactosidases are arabinogalactan type I oligomer hydrolases. Applied and Environmental Microbiology 72(12):7730–8.

Vetere A. (1998) Separation and character-ization of three β-galactosidases from *Bacillus circulans*. Biochimica et Biophysica Acta (BBA)-General Subjects. Oct 1380(2):223–31.

