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A highly inducible *β*-galactosidase from *Enterobacter* sp.

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Abstract: *Enterobacter* sp. 3TP2A isolated from a petroleum station was found to produce a novel, highly inducible mesophilic intracellular *β*-galactosidase in the presence of lactose up to 76.5 U mg^{-1}. The enzyme was purified to 17.3-fold after gel permeation chromatography with a yield of approximately 11 %. The optimum pH and temperature values of the purified enzyme were found to be 8.0–9.0 and 35 °C, respectively. The molecular weight of the enzyme was approx. 60 kDa with a single band by both SDS-PAGE and native-PAGE, and estimated by gel filtration chromatography. The enzyme was inhibited by Zn^{2+} and EDTA, while Cu^{2+} had strong inhibitory effect even at low concentrations. Activation by Mg^{2+} and inhibition by EDTA show that the enzyme is metal-dependent or a metalloenzyme. The enzyme was slightly activated by 2-mercaptoethanol, while slightly inhibited by iodoacetamide. On the other hand, PCMB inhibited the enzymatic activity to a great extent, whereas it was completely inhibited by *N*-ethylmaleimide. The V_{max} and K_m values were calculated as 0.701 μmol min^{-1} and 0.104 mM, respectively. The results indicated that the *β*-galactosidase *Enterobacter* sp. 3TP2A might well be a good candidate for use in biotechnology, particularly in the area of environment and health.

Keywords: *β*-galactosidase; *Enterobacter*; purification; characterization; inhibition.

INTRODUCTION

Enterobacter is a Gram-negative genus, and is facultative an aerobic, rod-shaped, non-spore-forming belonging to the family Enterobacteriaceae. *Enterobacter* species are universal bacteria that live in aquatic and terrestrial environments (water, sewage, soil and food). *Enterobacter* species are important human opportunistic pathogens, which are in charge of nosocomial infections such as

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urinary tract infections (UTI), neonatal meningitis, cholecystitis and osteomyelitis. The species *E. cloacae* appears as commensal microflora in the intestinal areas of humans and animals. This variety of habitats is reflected by the genetic diversity of *Enterobacter cloacae*, of which the genome was completed.[1–5]

β-Galactosidase or lactase (EC.3.2.1.23) is well known to hydrolyze milk lactose into monosaccharides, as well as catalyzing a mixture of different galactosides. *β*-Galactosidase possesses wide application in several main industrial areas, pharmaceutical, health, food technology and environment. *β*-galactosidase can catalyze two different reactions in organisms, namely hydrolytic and transgalactosylation reactions. It can hydrolyze lactose, mainly being used in food technology for lactose hydrolysis in milk and milk by-products.[6] It can also go through a process called transgalactosylation, taking part in the production of a variety of trans-galactosylation products or prebiotic galacto-oligosaccharides (GOS). It was recently found that these prebiotic products stimulate the growth and establishment of *Bifidobacteria* in the intestine of human and overcome potentially harmful bacteria, such as species belonging to Clostridia and Bacteriodes in the gut and are now considered as prebiotic. Lactose intolerance syndrome is caused by a reduction or loss of lactase activity in the intestinal brush border. When lactose is not indigested and when it passes to the intestine, it causes many symptoms, including giddiness, headache and nausea, abdominal pain, diarrhea, bloating, flatulence, blanching, and cramps.[8–10] Moreover, *β*-galactosidase was used by Tryland and Fiksdal[11] as pointers of pollution (such as coliforms, fecal coliforms, and *Escherichia coli* that are generally used for nursing the microbiological safety of recreational water and water deliveries). Some methods for detection of coliforms and *E. coli* are dependent on the enzymatic hydrolysis of fluorogenic or chromogenic substrates for *β*-galactosidase and *β*-glucuronidase. More applications of *β*-galactosidases, such as the prevention of lactose crystallization in frozen and condensed milk products, lactose hydrolysis of whey can reduce whey pollution of water, and also increase its sweetening properties through its hydrolysis products, glucose and galactose.[12]

β-Galactosidase can be obtained from various sources, such as plants, animals and microorganisms. However, microorganisms, bacteria, yeast and fungi are regarded as appropriate sources for industrial applications. Microorganisms compared to other sources have many advantages, such as higher multiplication degree and easy handling in the laboratory. As *β*-galactosidase is of commercial importance, a great number of microorganisms have been evaluated as potential sources of this enzyme. To produce *β*-galactosidase, it is important to select a microorganism with great potentiality.[12–14]

There have been many studies on characterization of *β*-galactosidase in various bacteria species, including, *Bifidobacterium infantis* HL96,[15] *Enterobacter agglumerans* B1,[16] *Alicyclobacillus acidocaldarius* subsp. *rittmannii*,[17] *Thermo-*

toga maritima,[18] *E. cloacae* B5,[19] *Streptococcus mitis*,[20] *Bifidobacterium longum* subsp. *longum*,[21] *Bacillus subtilis*,[22] *Lactobacillus* sp.,[23] *E. coli*,[24] *Bacillus licheniformis* KG9,[25] *Kluyveromyces marxianus* DIV13-247,[26] *Anoxybacillus* sp. KP1,[27] and *Anoxybacillus ayderensis*.[28]

In the present study, a strain of *Enterobacter* species (3TP2A), identified by Bruker Daltonik MALDI Biotyper, as well as by 16S rRNA gene sequence analysis, was used to purify its mesophilic *β*-galactosidase, after which the purified enzyme was characterized and tested for use in biotechnology.

EXPERIMENTAL

The strain 3TP2A isolated from a petroleum station in Batman in the southeast of Turkey was classified using both a Bruker Daltonik MALDI Biotyper and 16S rRNA sequence analysis.

Identification of Enterobacter sp. 3TP2A by 16S rRNA gene sequence analysis

The phylogenetic identification was carried out by the BLAST[29] program using a database having type strains with genuinely published prokaryotic names and representatives of uncultured phylotypes. The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using a global alignment algorithm, which was applied at the EzTaxon server[30] (http://www.ezbiocloud.net/eztaxon).

Cultivation of strain and preparation of crude enzyme

Stock cultures of this strain were stored at –20 °C in sterile Eppendorf tubes before use. Cultures were grown in 25 mL nutrient broth (NB) in 100 mL flask, incubated at 30 °C for 15 h in shaker water bath at 120 rpm. The bacterial culture was centrifuged at 10.000 rpm at 4 °C for 10 min. The pellet was re-suspended in 0.1 M sodium phosphate buffer (Na_2HPO_4/ /NaH_2PO_4) pH 7.0, after which the cells were sonicated. After centrifugation at 10.000 rpm at 4 °C for 10 min, the supernatant was used as crude enzyme for the detection of *β*-galactosidase activity. These crude extracts were used for both time course experiments and purification steps.

Time course of bacterial growth and production of β*-galactosidase in the presence and absence of lactose*

Nutrient broth (25 mL) inoculated with the bacterial strain 3TP2A was used in 150 mL flask in the absence and presence of 1 % lactose throughout the time course experiments (3, 6, 9, 12, 15, 18, 24, 36, 42, 48 ,60 and 72 h). The *OD* for growth determination was measured at 600 nm. The lactose was sterilized under (UV) for 10 min before adding to the medium. At each time intervals, 1.5 mL of culture was taken and centrifuged (10.000 rpm) at 4 °C for 10 min. The pellets were treated as described above. The same procedures were applied in order to test the effect of various concentrations of lactose (0.5–3.5 %) on the production of *β*-galactosidase.

β-*Galactosidase assay*

One mL of reaction solutions was prepared using 0.1 M sodium phosphate buffer (pH 7.0 or 8.0). The samples were then incubated for 10 min at 35 °C. The enzyme reaction was stopped by adding 500 μL of 2.0 M sodium carbonate (Na_2CO_3) and then the absorbance was measured at 420 nm. A blank containing 1.0 mL of buffer plus substrate was used to correct the thermal hydrolysis of *o*-NPG. The enzyme activity was expressed as *o*-nitrophenol (*o*-NP)

units liberated, where one unit (U) is defined as the amount of enzyme that released 1 μmol of *o*-NP from ortho-nitrophenyl-*β*-D-galactopyranoside (*o*-NPG) per min under the assay conditions at 35 °C. The absorbencies at 420 nm were converted to *o*-nitrophenol concentration using a millimolar extinction coefficient (ε_{mM420}) of 4.376 mM^{-1} cm^{-1} for *o*-NPG. The concentration of protein was determined using the method of Lowry.[31]

Purification of β-*galactosidase*

β-Galactosidase purification was realized by the following methods. The crude extract having *β*-galactosidase activity was precipitated using ammonium sulfate up to a final concentration of 70 % and the centrifuged precipitate (10.000 rpm, 10 min, 4 °C) was re-dissolved in a small volume of 0.1 M phosphate buffer pH 7.0 and the precipitate was dialyzed against 0.1 M phosphate buffer (pH 7.0) overnight in a fridge. Finally, the dialyzed samples were concentrated under nitrogen flow using an ultrafiltration system. Sephadex G-75 (Sigma) was used for gel permeation chromatography. The dialyzed enzyme solution (2.5 mL) was applied to a column (1.5 cm×30 cm) of Sephadex G-75 previously equilibrated with the phosphate buffer. The enzyme fractions eluted with the same buffer at a flow rate of 3 mL min^{-1} were collected for the enzyme activity (A_{420}) and protein content (A_{280}) determination. The fractions containing *β*-galactosidase activity were pooled, concentrated by ultrafiltration and used for further studies.

Effect of temperature and pH on β-*galactosidase activity*

The influence of different temperatures on *β*-galactosidase activity was tested at pH 7.0 using 60 mM substrate (*o*-NPG) concentration. For this, the activity of enzyme was tested at different temperatures between 10–60 °C. To determine the optimum pH, the *β*-galactosidase activity was tested within the range of pH from 4.0 to 11.0 at 35 °C. Three different buffers were used; 0.1 M citrate buffer between pH 4.0 and 6.0, 0.1 M sodium phosphate buffer between 7.0 and 9.0 and 0.1 M NaOH/glycine buffer at pH 10.0–11.0. The assay was performed as described above.

Molecular weight estimation by electrophoresis and gel filtration chromatography

The native PAGE was performed under mild denaturing conditions (0.01 % sodium dodecyl sulfate (SDS) using two parallel continuous 7 % gels. Following electrophoresis, the protein band of β-galactosidase was detected either by staining with Coomassie Brilliant Blue (CBB) R-250 or by 6-bromo-2-naphthyl-galactopyranoside (BNG) staining using the methods described by Gul-Guven *et al.*[17] The molecular weight of the enzyme was estimated by SDS--PAGE, performed according to Laemmli.[32] Reference markers including proteins with various subunit molecular weights were pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triosephospate isomerase (26.6 kDa).

The gel filtration technique was applied to estimate the MW of the enzyme using standard proteins with known MWs: carbonic anhydrase (MW: 29 kDa, Sigma C5024), *α*-amylase (MW: 55 kDa, Sigma A6380) and *β*-galactosidase (MW: 116 kDa, Sigma G-6008). The enzyme purification method was performed exactly the same as above using a Sephadex G-75 column (1.5 cm×30 cm). The dialyzed enzyme solution (1.5 mL) was applied to the column and the enzyme fractions were eluted with the same buffer at a flow rate of 3 mL min^{-1} collected for enzyme activity (A_{420}) and protein content (A_{280}) determination. The K_{av} values for proteins of known molecular weights were calculated using the formula $K_{av} = (V_e–V_0)/(V_T–V_0)$, where V_e is the elution volume of the sample, V_T and V_0 are the total liquid volume and the void volume of the column, respectively. K_{av} values were plotted against the logarithmic values of the

corresponding molecular weights to obtain a calibration curve to estimate the molecular mass of the studied *β*-galactosidase.

Thermal and pH stability

The purified enzyme was incubated in 1.0 mL of 0.1 M sodium phosphate buffer pH 8.0 for different times (10, 20, 30, 60, 90,120,150 and 180 min) at 35 and 45 °C, while the pH stability was investigated in the pH range of 4.0–11.0 using 0.1 M of the buffer systems and by the β-galactosidase activity assay described above.

The effects of inhibitors on β*-galactosidase activity*

The effect of various inhibitors at different concentrations, *i.e.*, *p*-chloromercuribenzoic acid (PCMB, 0.2, 0.4, 1.0 and 2.0 mM), iodoacetamide, dithiothreitol, 2-mercaptoethanol and *N*-ethylmaleimide (1.0, 2.0, 4.0 and 8.0 mM) as well as the effects of metal ions (metal chelator EDTA, $CaCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$) at various concentrations (1.0, 2.0, 5.0, 10 and 20 mM) on the activity of purified *β*-galactosidase were tested. All assays were performed under standard conditions as described above. *N*-Ethylmaleimide was dissolved in ethanol. The pure enzyme in 0.1 M sodium phosphate buffer (pH 8.0) and inhibitors were pre-incubated at 35 °C for 15 min, and then *o*-NPG solution was added. After 10 min, the reaction was stopped with 2.0 M Na_2CO_3 and the solutions were measured spectrophotometrically at 420 nm. The temperature was kept constant during all activity measurements. The activity of samples without the addition of inhibitors was taken as 100 % activity.

Enzyme kinetics

The K_m and V_{max} values of the *β*-galactosidase were determined by changing the o-NPG substrate concentrations, and a Michaelis–Menten plot was constructed to calculate the K_m and V_{max}. The used concentrations of the *o*-NPG substrate were 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 4.0 mM in 0.1 M phosphate buffer at pH 8.0 at 35 °C for 10 min.

Lactose hydrolysis

A solution of lactose 50 g L^{-1} was prepared under sterile condition in 0.1 M sodium phosphate buffer pH 8.0, then 5.0 U mL^{-1} of enzyme were added and incubated at 35 °C. After each time intervals (0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 h), samples were removed and measured for the amount of produced glucose, which was used to determine the rate of lactose hydrolysis. The GOD–POD (glucose oxidase peroxidase) method was used to monitor periodically the glucose released during the reaction.[33]

RESULTS AND DISCUSSION

The aim of this study was to isolate and identify a bacterium isolated from a petroleum station, which produces *β*-galactosidase at a large level, as well as the purification and characterization of this enzyme. Classification results by 16S rDNA sequence-based phylogenetic tree analysis showed that the strain was a member of the genus *Enterobacter*, and most likely was a strain of *E. cloacae* (Fig. S-1 of the Supplementary material to this paper). The identification index of the Bruker Daltonik MALDI Biotyper software score value was 2.423 and also showed high resemblance to *E. cloacae*.

Time course of bacterial growth and production of β*-galactosidase in the presence and absence of lactose*

As can be seen in Fig. 1b, the *β*-galactosidase of the studied bacterial strain 3TP2A was found to be inducible by lactose to a large extent. The lactose was found to increase the intracellular *β*-galactosidase production from 25.7 U mg^{-1} to as much as 76.5 U mg^{-1} after 24 h. The time course experiments showed that an incubation time of 24 h was most appropriate to determine the bacterial growth and the maximum production time of *β*-galactosidase (Fig. 1a and b).

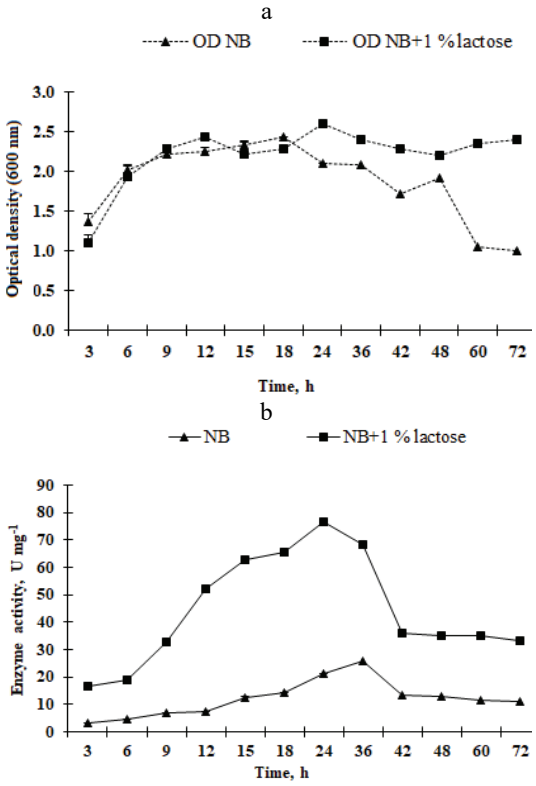

Fig. 1. Effect of incubation time on the growth (a) and the production of *β*-galactosidase (b) in *Enterobacter* sp. 3TP2A.

Fig. 1 shows that the production and growth decrease with time, which might be due to depletion of nutrients and lactose available to the microorganism or the end products of glucose and galactose may inhibit enzyme production and activity, or due to *β*-galactosidase denaturation caused by interaction with other components in the medium.

These results are in agreement with those of Ghatak *et al.*,[34] showing that the maximum enzyme production was obtained after 24 h in *Enterobacter cloacae* ST SJ 6 strain, as well as in *Lactobacillus* sp.[23]

It can clearly be seen that the lactose could induce the β-galactosidase in *Enterobacter* sp. 3TP2A. Therefore, various lactose concentrations were tested and it was shown that the maximum β-galactosidase production was obtained after 24 h incubation with a lactose concentration of 2 % at 30 °C (Fig. S-2 of the Supplementary material). *E. coli* is already known to possess a lac-operon system where the β-galactosidase gene is regulated and inducible by lactose. The results were in agreement with the results obtained by Khedr *et al*.[35] who studied different concentrations of lactose for β-galactosidase production in *E. coli.*

Purification of β-*galactosidase*

The steps of β-galactosidase purification from *Enterobacter* sp. 3TP2A were as follows (Table I): The crude extract obtained after the centrifugation step is regarded as 1-fold purification with a specific activity of 231 units per mg protein. Then, ammonium sulfate precipitation step with 70 % concentration followed by dialysis yielded a 3.2-fold purification with a specific activity of 739.1 units. The percentage recovery of β-galactosidase for this step was 42.2. Sephadex G-75 was used for the next purification step. After elution of the major peaks with sodium phosphate buffer (0.1 M, pH 8.0), the β-galactosidase had been purified by 17.3-fold with a specific activity of 3991 units, while the percentage recovery was 11 % (Table I).

TABLE I. Steps of β-galactosidase purification from *Enterobacter* 3TP2A

Step	Total protein content, mg	Total activity, U	Specific activity, U mg^{-1}	Purification, fold	Yield, %
Crude extract	244	56306	231	1	100
Ammonium sulfate precipitation and dialysis	32.2	23768.4	739.1	3.2	42.2
Sephadex-G75	1.5	6141.1	3991	17.3	11

There have been other purification studies on *Enterobacter* strains, although with lower yields in comparison to the present study. An overall 26-fold purification and 3.8 % yield was previously obtained for β-galactosidase from *Enterobacter cloacae* B5.[19] Furthermore, Lu *et al*.[16] purified the β-galactosidase by about 19-fold from a cell extract with a 1.6 % yield from *Enterobacter agglumerans* B1. There have been many studies on the purification of β-galactosidase in other bacteria, including *Bacillus licheniformis* KG9,[25] *Bacillus subtilis*,[22] *Lactobacillus lactis*[36] and *Alicyclobacillus acidocaldarius*.[17]

Molecular weight estimation by the electrophoresis and gel filtration techniques

Analysis and characterization of the purified β-galactosidase from *Enterobacter* sp. 3TP2A were realized by SDS-PAGE and Native-PAGE, as well as estimated by gel filtration chromatography using a Sephadex G-75 column (Fig. S-3 of the Supplementary material). The molecular weight analysis of the β-gal-

actosidase showed a single protein band, and its molecular mass was estimated as approximately 60 kDa (Fig. 2a). Native gradient PAGE (Fig. 2b) and estimation by gel filtration chromatography also showed an approximate molecular weight of 60 kDa (Fig. S-3 of the Supplementary material).

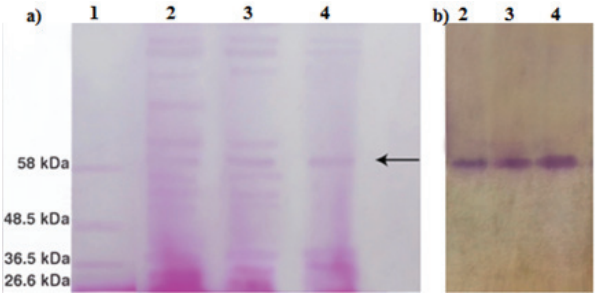

Fig. 2. SDS-PAGE CBB-staining (a) **and** BNG-staining (b) analysis of *β*-galactosidase from *Enterobacter* sp. 3TP2A. a) Lane 1, molecular mass markers: pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triosephospate isomerase (26.6 kDa). Lanes 2–4 with CBB-staining are crude extract, ammonium sulfate precipitation/dialysis and Sephadex G-75 steps, respectively. b) Lanes 2–4 with BNG-staining are crude extract, ammonium sulfate precipitation/dialysis and Sephadex G-75 steps, respectively.

Natarajan *et al.*[14] obtained similar result for the molecular weight of purified *β*-galactosidase from *Bacillus* sp. using SDS-PAGE analysis determined as 65 kDa. The molecular weight of the enzyme was also determined as 68 kDa from *Anoxybacillus* sp. KP1[27] and 90 kDa from *Anoxybacillus ayderensis*[28] by SDS--PAGE. Moreover, Saishin *et al.*[21] determined the molecular mass of the enzyme from *Bifidobacterium longum* subsp. *longum* by SDS-PAGE and Native PAGE, as a single band at 77 kDa. El-kader *et al.*[22] determined the molecular weight of purified *β*-galactosidase from *Bacillus subtilis* as 27.3 kDa analyzed by native PAGE. Sumathy *et al.*[23] reported a *β*-galactosidase with a molecular weight of 116 kDa in *Lactobacillus* sp. Lu *et al.*[16] reported the molecular mass of *β*-galactosidase from *Enterobacter agglumerans* B1 as 120 kDa determined by SDS--PAGE. Ghatak[37] studied the *β*-galactosidase from *Enterobacter cloacae* SJ 6 and determined a single band at 340 kDa, while the molecular weight of the enzyme in *Enterobacter cloacae* B5 was calculated as 442 kDa.[19] Interestingly all hitherto studied *β*-galactosidases in *Enterobacter* sp. possessed much higher molecular weights compared to that of the strain 3TP2A in the present study. There have been many studies on β-galactosidase characterization in various bacteria: *Escherichia coli β*-galactosidase, a monomer with 116 kDa,[24] *Bifidobacterium infantis* HL96, which is a similar genus to *Enterobacter*, with a expected molecular mass of 113 kDa[15] and *Streptococcus mitis* with a molecular mass of 268 kDa.[20] A recombinant *β*-galactosidase gene (TM-0310) from *Thermotoga*

maritima MSB8 expressed in *E. coli* was found to have molecular weights of 78 and 76 kDa by SDS–PAGE and gel filtration, respectively.[18]

Effect of temperature and pH on β-*galactosidase activity*

It was observed that the activity of purified *β*-galactosidase increases at temperatures between 10 to 35 °C (reaching the optimum at 35 °C), as shown in Fig. S-4a of the Supplementary material. However, enzyme activity sharply decreased with further increase in temperature up to 60 °C. The results are in agreement with those of Lu *et al.*,[16,19] stating that the optimal temperature for *β*-galactosidase enzyme from *E. agglomerans* B1 was 37–40 °C and 35 °C for *E. cloacae* B5.

As shown in Fig. S-4b, the optimum pH for the maximum activity of crude enzyme was found to be pH 8.0–9.0. In other studies, the optimum pH for *β*-galactosidase from *E. cloacae* was found as 9.0, as determined by Ghatak *et al.*[10,37] In a study performed by Lu *et al.*,[16] the *β*-galactosidase enzyme from *Enterobacter agglomerans* B1 was highly active in the pH range of 7.5–8.0. According to Lu *et al.*,[19] the *β*-galactosidase enzyme from *E. cloacae* B5 was highly active and stable in the pH range of 6.5–10.5.

Thermal and pH stability

The thermal stability of the purified *β*-galactosidase was determined by exposing the enzyme in the absence of substrates to two different temperatures (35 and 45 °C) for different periods of times from 10 min up to 180 min. As shown in Fig. S-5a of the Supplementary material, it is clear that at a temperature of 35 °C, the enzyme activity was stable under all tested time intervals, whereas the enzyme activity decreased at 45 °C at all-time intervals tested. The *β*-galactosidase was totally inactivated after 120 min. Saishin *et al*.[21] found that the purified *β*-galactosidase from *Bifidobacterium longum* subsp. JCM 7052 was stable during 5 h incubation at 35 °C, but very unstable at temperatures higher than 40 °C. Similar results were reported by Tryland and Fiksdal,[11] where the *β*-galactosidase of *Klebsiella pneumoniae* subsp. *pneumoniae*, *Yersinia intermedia*, *Rahnella aquatilis* and *E. cloacae* were not stable at 44.5 °C and the activity at this temperature was less than the activity obtained at 35 °C. El-Kader *et al*.[22] found that partially purified *β*-galactosidase from *Bacillus subtilis* was stable at 30–35 °C, while the activity decreased on increasing the temperature up to 60 °C. As shown in Fig. S-5b, the enzyme from the strain 3TP2A appears to be the most stable at pH 8.0 and the stability decreases sharply below and above pH 8.0.

Effects of metal ions and some other inhibitors on purified β-*galactosidase activity*

The effects of metal ions as either activators or inhibitors during the hydrolysis process were examined. Among these, Cu^{2+} and EDTA had an inhibitory effect on the *β*-galactosidase purified from *E. cloacae* (Table S-I of the Supple-

mentary material). EDTA inhibited the enzyme activity (up to 76 %) and Cu^{2+} had strong inhibitory effect on *β*-galactosidase even at low concentrations (96.9 %). However, Mg^{2+} caused activation of the purified enzyme. Ca^{2+} did not affect the enzyme activity to a great extent, causing deactivation of the enzyme at 20 mM (only 16 %), while Zn^{2+} at 1.0, 2.0 and 5.0 mM inhibited the enzyme activity (32, 27 and 8 %, respectively). Activation up to 47 % with increasing concentration of Mg^{2+} and also inhibition by EDTA showed that the enzyme is metal-dependent or a metalloenzyme. The deactivation in the presence of EDTA is probably due to it making metals unavailable as activators or co-factors, as well as protecting the sulfhydryl groups at the active site of *β*-galactosidase. Gul Guven *et al.*[39] previously showed that a purified *β*-galactosidase, belonging to the GH42 family, in the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* subsp. *rcittmannii* isolated from the Antarctica was inhibited by high concentrations of Ca^{2+} (33 % at 10 mM), Zn^{2+} (86 % at 8.0 mM) and Cu^{2+} (87 % at 4.0 mM), but was slightly activated by Mg^{2+} (13 % at 20 mM).

As shown in Table S-II of the Supplementary material, the enzyme was completely inhibited by *N*-ethylmaleimide (100 %), but not affected by DTT. The enzyme was slightly affected by 2-mercaptoethanol, enhancing *β*-galactosidase activity by 14 % at 8.0 mM. The Table also shows that iodoacetamide had a slight effect on *β*-galactosidase activity (up to 13 %). Inhibition by *p*-chloromercuribenzoic acid (PCMB) to a great extent up to approx. 87 % and also by *N*--ethylmaleimide shows that at least one essential cystine residue is modified by the reagents. On the other hand, it is interesting to note that iodoacetamide, which is an alkylating reagent through the SH group, had little effect on the enzyme. Similar results were also reported by Gul Guven[39] on *β*-galactosidase in *Alicyclobacillus acidocaldarius* subsp. *rittmannii*: reagents containing SH groups, such as 2-mercaptoethanol and dithiotreitol (DTT) at some concentrations were found to enhance *β*-galactosidase activity, also indicating the presence of a sulfhydryl group in the active site of *β*-galactosidase. As expected, *p*-chloromercuribenzoic acid (PCMB) also completely inhibited the enzymatic activity, while the enzyme was slightly affected by *N*-ethylmaleimide. Lu *et al.*[19] showed that DTT did not affect *β*-galactosidase of *E. cloacae*, as also demonstrated in the present study.

Effect of substrate concentration (ONPG) on β*-galactosidase activity*

The K_m and V_{max} values of the enzyme were calculated from the reciprocal plots of substrate. A simple Michealis–Menten kinetics was observed since the Lineweaver–Burk plot was linear (Fig. S-6 of the Supplementary material). The V_{max} was found to be 0.701 μmol min^{-1} and K_m to be 0.104 mM, indicating a great affinity for its substrate. The V_{max} and K_m for the *β*-galactosidase enzyme from *Streptococcus thermophilus* were calculated as 2.8 U ml^{-1} 3.05 mM, respectively.[40] Chakraborti *et al.*[41] determined K_m and V_{max} values for purified *β*-

-galactosidase from *Bacillus* sp. MTCC 3088 for ONPG as 6.34 mM and 9351 IU ml^{-1}, respectively. Moreover, the results of the present study were in accordance with the study of Sumathy *et al.*,[23] who measured the *β*-galactosidase activity from *Lactobacillus* sp. at different concentration of the substrate ONPG, calculating the V_{max} to be 16×10^{-3} mol min^{-1} and K_m to be 50×10^{-3} mol min^{-1}.

Lactose hydrolysis by the purified enzyme

The experiments of lactose hydrolysis were performed using lactose under optimized conditions (pH 8.0 and 35 °C). The results are presented in Fig. S-7 of the Supplementary material. It was found that the time for lactose hydrolysis continued up to 10 h with the reaction catalyzed by purified *β*-galactosidase, indicating that there is a potential application of the enzyme to be used for lactose hydrolysis of whey and milk. Similarly, Ghatak *et al.*[10] reported hydrolysis of milk lactose using immobilized *β*-galactosidase from *E. cloacae* under optimized conditions (pH 9.0 and 50 °C) and found that about 46.34 % of the lactose in milk was hydrolyzed at 8 h operation using a continuous packed bed reactor system. Moreover, a novel yeast strain *Kluyveromyces marxianus* DIV13-247, which also hydrolyzed the lactose solution in whey and milk to a great extent, was previously used.[26]

CONCLUSIONS

In this study, a mesophilic *Enterobacter* sp. 3TP2A isolated from a petroleum station in Batman in the southeast of Turkey was identified and found to produce a high amount of mesophilic *β*-galactosidase inducible by lactose. The purified and characterized *β*-galactosidase succeeded to 17.3-fold after gel permeation chromatography with a yield of approximately 11 %. The *β*-galactosidase from *Enterobacter* sp. 3TP2A may have application potential and needed further study for utilization in biotechnology to develop a commercially usable enzyme, for example immobilized, since immobilization methods for use in continuous bioreactors are less explored. Moreover, *β*-galactosidase, which has already been used as sensors of pollution (like *coliforms*, fecal *coliforms*) for nursing the microbiological safety of recreational water and water deliveries, may also be utilized for other pathogen species such as those of *Enterobacter* genus through enzymatic hydrolysis of fluorogenic or chromogenic substrates.

SUPPLEMENTARY MATERIAL

Additional data are available electronically from the journal web site: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

ЕФИКАСНА ПРОИЗВОДЊА β-ГАЛАКТОЗИДАЗЕ ИЗ *Enterobacter* sp.

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Enterobacter sp. 3TP2A изолован из нафтне станице ефикасно производи нову мезофилну унутарћелијску β-галактозидазу у присуству лактозе (до 76,5 U mg^{-1}). Ензим је пречишћен 17,3 пута гел хроматографијом, а принос је био око 11 %. Оптимална активност пречишћеног ензима се достиже на pH 8,0–9,0 и на температури од 35 °C. Молекулска маса ензима је око 60 kDa, што је процењено на основу SDS-PAGE, нативне електрофорезе (једна трака) и гел хроматографијом. Ензим инхибирају Zn^{2+} и EDTA, а Cu^{2+} испољава јак инхибиторни ефекат и при ниским концентрацијама. Активација у присуству Mg^{2+} и инхибиција са EDTA доказују да је ензимска активност зависна од метала, односно да је ово металоензим. Ензим у малој мери активира β-меркаптоетанол и благо инхибира јодацетамид. PCMB значајно инхибира ензимску активност, а *N*-етилмалеимид потпуно. Израчунате V_{max} и K_m вредности су 0,701 μmol min^{-1} и 0,104 mM. Резултати су показали да би се β-галактозидаза из *Enterobacter* sp. 3TP2A могла користити у биотехнологији, посебно у области заштите животне средине и здравља.

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