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

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*Abstract:**Enterobacter* sp. 3TP2A isolated from petroleum station was found to produce a novel, highly inducible mesophilic intracellular β-galactosidase in the presence of lactose upto 76.5 U/mg.The enzyme was purified to 17.3-fold after gel permeation chromatography with a yield of approximately 11%. The optimium pH and temperature values of purified enzyme were found to be 8.0-9.0 and 35 oC, respectively. The Molecular weight of the enzyme was approx. 60 kDa of a single band by both SDS-PAGE and native-PAGE. The enzyme was inhibited by Zn2+ and EDTA, while Cu2+ had strong inhibitory effect even at low concentrations. Activation by Mg2+ and inhibition by EDTA show that the enzyme is metal-dependent or a metalloenzyme. The enzyme was slightly activated by β-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 mg) and 0.104 mM, respectively. The results indicated that the β-galactosidase *Enterobacter* sp. 3TP2Amay well be a good candidate for use in biotechnology, particularly in the area of environment and health.

*Key words:* β-galactosidase*, Enterobacter*, purification, characterization, inhibition.

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INTRODUCTION

*Enterobacter* is Gram-negative genus, and is facultative an aerobic, rod-shaped, non-spore-forming belongs 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 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 *E. cloacae*, of which genome was completed. 1-5

β-Galactosidase or lactase (EC.3.2.1.23) is well known to hydrolyze the milk lactose into monosaccharides, as well as catalyzing mixture of different galactosides. β-Galactosidase possesses a 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 the species belong to *Clostridia* and *Bacteriodes* in the gut and are now considered as a prebiotic food ingredient.7 The lactose intolerance syndrome is caused by reduction or loss of lactase activity in the intestinal brush border. When lactose is not indigested and when it passes to 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 (like *coliforms*, fecal *coliforms*, and *E. coli* are generally used for nursing the microbiological safety of recreational water and water deliveries). Some methods for detection of coliforms and *E. coli* are depended on enzymatic hydrolysis of fluorogenic or chromogenic substrates for β-galactosidase and β-glucuronidase. More applications of β-galactosidases, such as the preventing lactose from being crystallized in the frozen and condensed milk products, lactose hydrolysis in whey can cause reduction of water whey pollution, and also it will increase the sweetening properties by hydrolysis products, glucose and galactose.12

β-Galactosidase can be obtained from various sources such as plants, animals and microorganisms. But microorganisms, bacteria, yeast and fungi are regarded as an appropriate source for industrial applications. Microorganisms compared to other sources have many advantages such as higher multiplication degree and easy handling in the laboratory. Because β-galactosidase is of commercial importance, a great number of microorganisms have been evaluated as potential sources of this enzyme. To produce -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* HL9615, *Enterobacter agglumerans* B116, *Alicyclobacillus acidocaldarius* subsp. *rittmannii* 17, *Thermotoga maritima* 18, *E. cloacae* B5 19, *Streptococcus mitis*20, *Bifidobacterium longum* subsp. *longum*21, *Bacillus subtilis* 22, *Lactobacillus* sp.23, *Escherichia coli*24, *Bacillus licheniformis* KG925, *Kluyveromyces marxianus* DIV13-247 26, *Anoxybacillus* sp. KP1 27, *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 by using both Bruker Daltonik MALDI Biotyper and 16S rRNA sequence analysis.

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

The phylogenetic identification carried out using the BLASTN[29](http://www.ncbi.nlm.nih.gov/pubmed/9254694?ordinalpos=5&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) program by using the 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 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 oC in sterile Eppendorf tube before use. Cultures were grown in 25 mL Nutrient Broth (NB) in 100 mL flask, incubated at 30 oC for 15 h, in shaker water bath at 120 rpm. The bacterial culture was centrifuged at 10.000 rpm at 4 oC for 10 min. The pellet was re-suspended with 0.1 M Sodium phosphate buffer (Na2HPO4 / NaH2PO4) pH 7.0, after which the cells were sonicated. After centrifugation at 10.000 rpm at 4 oC for 10 min, the supernatant was used as crude enzyme for 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*

25 mL Nutrient Broth, 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 ,72 h). The OD for growth determination was measured at 600 nm. The lactose was sterilized under (UV) for 10 min before added to the medium. At each time intervals, 1.5 mL of culture was taken, centrifuged (10.000 rpm) at 4 oC for 10 min. 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 production of β-galactosidase.

*β-galactosidase Assay*

One mL of reaction solutions were 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 M sodium carbonate (Na2CO3) and then the absorbance was measured at 420 nm. 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 *o-*NPG per mi­n under the assay conditions at 35°C. The concentration of protein was determined by using the method of Lowry. 31

*Purification of β-galactosidase*

β-Galactosidase purification was carried out by using the following methods: The crude extract having β-galactosidase activity was precipitated by using ammonium sulfate up to a final concentration of 70% (w/v) 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 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 dialysed enzyme solution (2.5 mL) was applied to a column (1.5 x 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 were collected for the enzyme activity (A420 nm) and protein content (A280 nm) determination. The fractions containing β-galactosidase activity were pooled, concentrated by ultrafiltration 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 oC. To determine the optimum pH, the β-galactosidase activity was tested within the range of pH from 4.0 to 11.0 at 35 oC. 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 carried out as described above.

*SDS-PAGE and Native PAGE*

The native PAGE was performed under mild denaturing conditions (0.01% sodium dodecyl sulphate (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 for using the methods described by Gul-Guven et al.17. The molecular weight of the enzyme was estimated by SDS-PAGE, carried out according to Laemmli.32 Reference marker including proteins with various subunit molecular weights were pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephospate isomerase (26.6 kDa).

*Thermal and pH Stability*

The purified enzyme was incubated in 1 mL of 0.1 M sodium phosphate buffer pH 8.0 at different times (10, 20, 30, 60, 90,120,150 and 180 min) at 35 oC and 45 oC, 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 on purified β-galactosidase activity were tested, which were P-chloromercuribenzooic acid (0.2, 0.4, 1, 2 mM), Iodoacetamide, Dithiothreitol, β-meraptoethanol and N-ethylmaleimide (1, 2, 4, 8 mM), as well as the effects of metal ions (metal chelator EDTA, CaCl2, CuCl2, ZnCl2, MgCl2) with various concentrations (1, 2, 5, 10, 20 mM). All assays were carried out under standard conditions as described above. The N-ethylmaleimide were dissolved in Ethanol. The pure enzyme in 0.1 M sodium phosphate buffer (pH 8.0) and inhibitors were pre-incubated at 35 ºC for 15 minutes, then added *o*-NPG solution and waited 10 min, stopped with 2 M Na2CO3 after which they were spectrophotometrically measured at 420 nm. The temperature was kept constant during all activity measurements. Activity of samples without addition of inhibitors was taken as 100% activity.

*Enzyme kinetics*

*K*m and *V*max values of the β-galactosidase were determined by changing the o-NPG substrate concentrations, where Michaelis-Menten plot was constructed to calculate the *K*m and *V*max. *o*-NPG substrate concentrations used were as 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4 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 was prepared under sterile condition in 0.1 M sodium phosphate buffer pH 8.0, then 5 U/mL of enzyme was added and incubated at 35 °C. After each time intervals (0, 1, 2, 4, 6, 8 and 10 h), the samples were removed and measured for the amount of glucose production that converted to determine the rate of lactose hydrolysis. GOD-POD (glucose oxidase peroxidase) method was used to periodically monitor 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 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 to be a strain of *E. cloacae* (Supplementary 1). 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 Figure 1b, 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 to as much as 76.5 U/mg after 24h. The time course experiments showed that incubation time of 24 h was most appropriate to determine the bacterial growth and the maximum production time of β-galactosidase (Figure 1a and 1b). 



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

The figure 1 shows that the production and the growth decreases by time which might be due to the depletion of nutrients and lactose available to microorganism or the end products of glucose and galactose may inhibit the enzyme production and activity, as well as due to the β-galactosidase denaturation caused by interaction with other components in the medium. These results were agreement with Ghatak et al. 34, showing that maximum enzyme production was obtained after 24 h in *Enterobacter cloacae* ST SJ 6 strain, as well as in *Lactobacillus* sp. 23. It can be clearly seen that the lactose could induce the β-galactosidase in *Enterobacter* sp. 3TP2A. We have therefore tested various lactose concentrations and showed that the maximum β-galactosidase production was obtained after 24 hours of incubation with 2% lactose concentration at 30 oC (Figure 2). *Escherichia coli* is already known to possess 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, studying the different concentrations of lactose for β-galactosidase production in *E. coli.*



**Figure 2:** Effect of different lactose concentrations on production of β-galactosidase in *Enterobacter* sp.3TP2A

*Purification of β-galactosidase*

Steps of β-galactosidase purification from *Enterobacter* sp. 3TP2A were as follows (Table 1): The crude extract obtained after 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 elutions of the major peaks with sodium phosphate buffer (0.1 M pH 8.0), β-galactosidase was purified by 17.3 fold with a specific activity of 3991 units, while the percentage recovery was 11% (Table 1).

**Table 1:** Steps of β-galactosidase purification from *Enterobacter* 3TP2A.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Purification steps of β-galactosidase** | | | | | |
|  | **Total protein (mg)** | **Total activity (U)** | **Specific activity**  **(U/mg)** | **Purification (fold)** | **Yield (%)** |
| **Crude extract** | 244 | 56306 | 231 | 1 | 100 |
| **Ammonium sulphate 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 some purification studies on *Enterobacter* strains, though with lower yield 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 at about 19 fold from the 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.

*SDS-PAGE and native PAGE*

Analysis and characterization of the purified β-galactosidase from *Enterobacter* sp. 3TP2A were carried out by SDS-PAGE and Native- PAGE. The molecular weight analysis of the β-galactosidase showed a single band of protein, and its molecular mass was found to be approximately 60 kDa (Figure 3a). Native gradient PAGE (Figure 3b) also showed a single enzyme apparent at the same location.



**Figure 3.** SDS-PAGE CBB-staining **(a)** 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), triosephospate isomerase (26.6 kDa) ]; lanes 2, 3 and 4 for CBB-staining are crude extract, ammonium sulphate precipitation/dialysis and sephadex G-75 steps, respectively. **b:** Lanes 2, 3 and 4 for BNG-staining are crude extract, ammonium sulphate precipitation/dialysis and sephadex G-75 steps, respectively.

Natarajan et al. 14 found similar result for the molecular weight of the 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 of 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 β-galactosidase from *Enterobacter cloacae* SJ 6 and determined a single band of 340 kDa, while the molecular weight of the enzyme in *Enterobacter cloacae* B5 was calculated as 442 kDa 19. It is interesting to note that all β-galactosidases studied so far in *Enterobacter* sp. possess much higher molecular weight 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 kD 24, *Bifidobacterium infantis* HL96, which is a close genus to *Enterobacter,* with a expected molecular mass of 113 kDa 15, *Streptococcus mitis* with 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 kDa 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 temperature between 10 to 35oC (reaching optimum at 35 oC) as shown in Supplementary 2a. However, enzyme activity sharply decreased with further increase in temperature up to 60 oC. The results were in agreement with Lu et al. 16 and 19, stating that the optimal temperature for β-galactosidase enzyme from *Enterobacter agglomerans* B1 was 37-40 oC and35 oC for *Enterobacter cloacae* B5, respectively.

As shown in Supplementary 2b, 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 *Enterobacter cloacae* was found as 9.0 carried out by Ghatak et al. 10 and 37. In a study performed by Lu et al. 16, the β-galactosidase enzyme from *Enterobacter agglomerans* B1was highly active in the pH range of 7.5-8.0. Also studied by Lu et al. 19, the β-galactosidase enzyme from *Enterobacter cloacae* B5 was highly active and stable at 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 oC and 45 oC) for different periods of times from 10 min up to 180 min. As shown in Supplementary 3a, it was clear that at temperature 35 oC the enzyme activity was stable under all tested time intervals, whereas at all-time intervals tested the enzyme activity decreased at 45 oC. The β-galactosidase was totally inactivated after 120 min. Saishin et al. 21 studied that the purified β-galactosidase from *Bifidobacterium longum* subsp. JCM 7052 was stable during 5 h incubation at 35 oC, but very instable higher than 40 oC. Similar results were reported by Tryland and Fiksdal 11, where the β-galactosidase of *Klebsiella pneumoniae* subsp. *pneumoniae*, *Yersinia intermedia,* *Rahnella aquatilis* and *Enterobacter cloacae* were not stable at 44.5oC and the activity at this temperature was less than the activity obtained at 35 oC. El-kader et al. 22 found that the partial purified β-galactosidase from *Bacillus subtilis* was stable at 30-35oC, while there was a decrease in the activity of enzyme by increasing temperature up to 60oC. As shown in Supplementary 3b, the enzyme from the strain 3TP2A appears to be 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, Cu2+ and EDTA had an inhibitory effect on theβ-galactosidasepurified from *E. cloacae* (Supplementary 4). EDTA inhibited the enzyme activity (upto 76%) and Cu2+ had strong inhibitory effect on β-galactosidase even at low concentrations (96.9%). However, Mg2+ caused activation of the purified enzyme. Ca2+ did not effect enzyme activity to a great extent, causing deactivation of the enzyme at 20 mM (only 16%), while Zn2+ at 1, 2 and 5 mM inhibited enzyme activity (32, 27, 8%, respectively). Increase in the concentration of Mg2+ causing activation upto 47% and also inhibition by EDTA show that the enzyme is metal-dependent or a metalloenzyme. The deactivation in the presence of EDTA is probably due to causing inavailability of metals as activators or co-factors, as well as protection of sulfhydryl groups at the active site of β-galactosidase. Gul Guven et al. 39 had previously showed that a purified β-galactosidase, belonging to GH42 family, in the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* subsp. *rittmannii* isolated from Antarctica was inhibited by high concentrations of Ca+2 (33% at 10 mM), Zn+2 (86% at 8 mM) and Cu+2 (87% at 4 mM), but slightly activated by Mg2+ (13% at 20 mM).

As shown in Supplementary 5, the enzyme was completely inhibited by N-ethylmaleimide (100%), but not affected by DTT. The enzyme was slightly affected by β-mercaptoethanol, enhancing β-galactosidase activity by 14% at 8mM. The table also shows that the Iodoacetamide had a slight effect on β-galactosidase activity (upto 13%). Inhibition by p-chloromercuribenzoic acid (PCMB) to a great extent upto 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 the Iodoacetamide which is an alkylating reagent through SH group had little effect on the enzyme. Similar results were also reported by Gul Guven et al. 39 on β-galactosidase in *Alicyclobacillus acidocaldarius* subsp. *rittmannii*: reagents containing SH groups such as 2-mercaptoethanol and 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 the dithiotreitol (DTT) did not affect β-galactosidase of *Enterobacter cloacae*, as also demonstrated in our study.

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

*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 (Supplementary 6). The *V*max was found as 0.701 (μmol/ min mg) and *K*m was found as 0.104 mM, indicating a great affinity for its substrate. *V*max and *K*m for β-galactosidase enzyme from *Streptococcus thermophilus* were calculated as 2.8U/ml 3.05 mM, respectively 40. Chakraborti et al. 41 determined *K*mand *V*max values for purified β-galactosidase from *Bacillus* sp. MTCC 3088 for ONPG as 6.34 mM and 9351 IU/ml, 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 *Lactobacillu*s sp. at different concentration of the substrate ONPG, calculating the *V*max as 16x10-3(mol/ min/ mgprotein) and *K*m as 50x10-3 (moles/min).

*Lactose hydrolysis by the purified enzyme*

The experiments of lactose hydrolysis were carried out using lactose under optimized conditions (pH 8.0 and 35oC). The results are presented in Figure 4. It was found that the time for lactose hydrolysis continues 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 on whey and milk. Similarly, Ghatak et al. 10 reported hydrolysis of milk lactose using immobilized β-galactosidase from *Enterobacter cloacae* under optimized conditions (PH 9.0 and 50 oC) and found that about 46.34% lactose in milk was hydrolyzed at 8 h operation using a continuous packed bed reactor system. Moreover, we have previously used a novel yeast strain *Kluyveromyces marxianus* DIV13-247 which also hydrolysed lactose solution in whey and milk to a great extent 26.



**Figure 4.** Lactose hydrolysis using purified β-galactosidase

CONCLUSION

In this study, a mesophilic *Enterobacter* sp. 3TP2Aisolated from 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 was succedded to 17.3 fold after gel permeation chromatography with a yield of approximately 11%. The β-galactosidase from *Enterobacter* sp. 3TP2Amay have an application potential and need a further study for utilizing in biotechnology, to develop commercially usable enzyme, for example immobilized, since immobilization methods for use in continuous bioreactors are less explored. Moreover, β-galactosidase, which has been already used as sensors of pollution (like *coliforms*, fecal *coliforms*) for nursing the microbiological safety of recreational water and water deliveries, may also be utilised for other pathogen species such as those of *Enterobacter* genus throughenzymatic hydrolysis of fluorogenic or chromogenic substrates.

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