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PURIFICATION AND CHARACTERIZATION OF β-GALACTOSIDASE FROM *Enterobacter* sp. 3TP2A

Bestoon AHMED SHAIKHAN

MASTER THESIS

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Enterobacter sp.3TP2A BAKTERİSİNDEN İZOLE EDİLEN β-GALAKTOSİDAZ ENZİMİNİN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

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ABSTRACT

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In this study, a mesophilic *Enterobacter* sp. 3TP2A isolated from petroleum station in Batman in the southeast of Turkey was identified and found to produce a high amount of mesophilic β-galactosidase. The lactose was found to increase the β-galactosidase production to a great extent, meaning that this enzyme is inducible. An intracellular β-galactosidase from *Enterobacter* sp. 3TP2A was purified and characterized. The enzyme was purified by ammonium sulphate precipitation 70%, dialysis, ultrafiltration and finally using sephadex G-75 chromatography method. The enzyme was purified to 17.3-fold after gel permeation chromatography with a yield of approximately 11%. The purified enzyme was found to be stable in pH 8.0 and temperature of 35 ^{o}C. The molecular weight of the purified enzyme was found to be about 60 kDa by both SDS-PAGE and native-PAGE.

The effects of various metal ions at different concentrations of $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, and EDTA (1, 2, 5, 10 and 20 mM) were tested. EDTA and Cu^{2+} had an inhibitory effect on the β-galactosidase purified from *Enterobacter* sp. 3TP2A. EDTA inhibited the enzyme activity (upto 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 effect enzyme activity to a great extent, causing deactivation of the enzyme at 20 mM (only 16%), while Zn^{2+} at 1, 2 and 5 mM inhibited enzyme

activity (32, 27, 8%, respectively). Increase in the concentration of Mg^{2+} causing activation upto 47% and also inhibition by EDTA show that the enzyme is metal-dependent or a metalloenzyme. Also determining the effect of different concentration of inhibitors on purified enzyme; PCMB (0.2, 0.4,1, 2 mM) , Iodo, DTT, β-mer, N-Ethyl, (1, 2, 4, 8 mM). The enzyme was completely inhibited by N-Ethyl (100%), but not affected by DTT. The enzyme was slightly affected by β-mer enhancing β-galactosidase activity at 8mM with 14% . The Iodo had a slight effect on β-galactosidase activity (upto 13%). PCMB inhibited the enzymatic activity to a great extent upto approx. 87%.

The Lineweaver-Burk plot was linear, suggesting a simple Michealis-Menten kinetics. The V_{max} was found as 0.701 (μmol/ min mg) and K_m was found as 0.104 mM. It was also found that the time for lactose hydrolysis continues up to 10 h with the reaction catalyzed by purified β-galactosidase.

The aim of this study was to purify and characterize the mesophilic β-galactosidase from *Enterobacter* sp. 3TP2A and then to test for use in biotechnology such as lactose hydrolysis. The results obtained indicated that this species may well be a good candidate.

Key words: β-galactosidase, *Enterobacter cloacae* 3TP2A, purification, characterization, inhibition and lactose hydrolysis.

ÖZET

Enterobacter sp. 3TP2A BAKTERİSİNDEN İZOLE EDİLEN β-GALAKTOSİDAZ ENZİMİNİN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

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Bu çalışmada, Türkiye'nin Güneydoğu Bölgesi'nde yer alan Batman ilindeki bir akaryakıt istasyonundan izole edilen mezofilik *Enterobacter* sp. 3TP2A bakterisinin yüksek miktarda mezofilik β-galaktosidaz enzimi ürettiği görülmüştür. Ayrıca, laktozun β-galaktosidaz üretimini önemli ölçüde arttırdığı görülmüş olup bu bulgu, bu enzimin endüke edilebilir bir enzim olduğunu göstermektedir. *Enterobacter* sp. 3TP2A bakterisinden izole edilen intraselüler β-galaktosidaz saflaştırılıp karakterize edildi. Saflaştırma işlemi %70 amonyum sülfat çöktürmesi, diyaliz, ultrafiltrasyon ve son olarak da Sephadex G-75 kromatografisi yöntemi kullanılarak gerçekleştirilmiştir. Enzim, jel geçirgenlik kromatografisi kullanılarak yaklaşık %11 verimle 17.3 kat saflaştırıldı. Saflaştırılan enzimin pH 8.0 ve 35 ^{o}C'de stabil olduğu ve moleküler ağırlığının ise SDS-PAGE ve native-PAGE metodlarına göre yaklaşık 60 kDa olduğu tespit edilmiştir.

Çeşitli metal iyonların farklı $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$ ve EDTA (1, 2, 5, 10 ve 20 mM) konsantrasyonlardaki etkileri incelenmiştir. EDTA ve Cu^{2+}'nın *Enterobacter* sp. 3TP2A'dan saflaştırılan β-galaktosidaz üzerinde inhibe edici bir etkiye sahip oldukları belirlenmiştir. EDTA'nın enzim aktivitesini %76'ya kadar inhibe ettiği ve Cu^{2+}'nın β-galaktosidaz üzerindeki inhibe edici etkisinin düşük konsantrasyonlarda bile güçlü olduğu görülmüştür (96.9%). Bununla birlikte, Mg^{2+}'nın saflaştırılan enzimin aktivasyonuna sebep

olduğu görülmüştür. Ca^{2+} ise enzim aktivitesini önemli ölçüde etkilememiş olup 20 mM (yalnızca 16%)'de enzimin deaktivasyonuna sebep olmuştur. Ancak, Zn^{2+} 1, 2 ve 5 mM'de enzimin aktivasyonuna sebep olmuştur (sırasıyla, %32, %27, %8). Mg^{2+} konsantrasyonundaki artışın 47%'ye kadar enzim aktivasyonuna sebep olması ve EDTA tarafından inhibe edilmesi, bu enzimin metal-bağımlı veya metaloenzim olduğunu göstermektedir. Buna ilaveten, farklı konsantrasyonlardaki inhibitörlerin saflaştırılan enzim üzerindeki etkileri şu konsantrasyonlarda incelenmiştir: PCMB (0,2, 0,4, 1, 2 mM) , Iodo, DTT, β-mer, N-etil, (1, 2, 4, 8 mM). Enzim, N-etil tarafından tamamen inhibe edilmesine karşın (%100), DTT tarafından etkilenmemiştir. Enzim, β-galaktosidaz aktivitesini 8 mM'de %14 oranında arttıran β-mer tarafından hafifçe etkilenmiştir. Iodo da β-galaktosidaz aktivitesini hafifçe etkilemiştir (%13'e kadar). PCMB ise enzimatik aktiviteyi %87'lere varan inhibisyonla büyük oranlarda etkilemiştir.

Lineweaver-Burk grafiği lineer olarak belirlenmiş olup bu sonuç basit bir Michealis-Menten kinetiğine işaret etmektedir. V_{max} 0.701 (μmol/ dak mg) ve K_m 0.104 mM olarak tespit edilmiştir. Ayrıca, laktoz hidrolizi süresinin, saflaştırılan β-galaktosidaz tarafından katalize edilen reaksiyondan dolayı, 10 saate kadar çıktığı gözlemlenmiştir.

Bu çalışmanın amacı *Enterobacter* sp. 3TP2A'dan mezofilik β-galaktozidaz'ı saflaştırmak, karakterize etmek ve laktoz hidrolizi gibi biyoteknoloji alanında kullanımını test etmekti. Elde edilen bulgular bu enzimin iyi bir aday olabileceğini göstermiştir.

Anahtar kelimeler: β-galaktosidaz, *Enterobacter cloacae* 3TP2A, saflaştırma, karakterizasyon, inhibisyon ve laktoz hidrolizi

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ABBREVIATION AND SYMBOLS

α	: Alpha
APS	: Amonium per-sulphate
^{o}A	: Angstrom
β	: Beta
β-mer	: Beta-mercaptoethanol
%	: Percent
BNG	: 6-Bromo-2-naphthyle-β-D-galactopyronoside
BSA	: Bovine serum albumin
$CaCl_2$	: Calcium Chloride
CBB	: Coomassie brilliant blue
cm	: Centimeter
$CuCl_2$	: Copper chloride
Da	: Dalton
DTT	: Dithiothreitol
EDTA	: Ethyldiamine tetra aceticacid
FCR	: Folin-ciocalteu reagent
g/L	: Gram/litre
h	: Hour
Iodo	: Iodoacetamide
IPTG	: Isopropyβ-D-1-thiogalactopyronoside
kDa	: Kilodalton
K_m	: Mechaelis constant
L	: Litre
M	: Molarity
mg	: Milligram
$MgCl_2$	: Magniesium chloride
min	: Minute

mL	: Mililitre
µL	: Microlitre
mM	: Milimolar
NaCl	: Sodium chloride
NaoH	: Sodium hydroxide
Na_2CO_3	: Sodium bicarbonate
NB	: Nutrient broth
N-Ethyl	: N-Ethylmaleimide
nm	: Nanometer
^oC	: Degrees centegrade
OD	: Optical density
ONPG	:*o*-Nitrophenol- β-D-galactopyronoside
PABTG	: *p*-aminobenzoic-1-thio-β-D-galactopyranoside
PCMB	: p-Chloromercuribenzoic acid
pH	: Power of hydrogen
rpm	: Rotation per minute
SDS	: Sodium dedocyl sulphate
SDS-PAGE	: Sodium dedocyle sulphatepolyacrilamide gel electrophoresis
TEMED	: Tetramethylethylenediamine
U	: Unit
UV	: Ultraviolet
U/µL	: Unit/microlitre
V	: Volt
V_{max}	: Maximum reaction velosity
w/v	: Weight/volume
$ZnCl_2$	: Zinc chloride

1.INTRODUCTION

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 (Sanders et al. 1997, Ren et al. 2010).

Species of the *Enterobacter* are extensively encountered in nature, but they are also pathogens: *E. cloacae* and *E. hormaechei* are most often secluded from human clinical specimens. Therefore, the most common *Enterobacter* sp. is *E. cloacae* , affecting only nosocomial infections and on the antibiotic resistance features of these microorganisms, there has been a lot of publishing. In spite of the significance of *E. cloacae* as a nosocomial pathogen, the factors and the pathogenic mechanisms which are contributing in such disease associated with the *E. cloacae* complex have not been understood yet; this could be caused by the insufficiency and the dispersion of information obtainable. Its capacity to form biofilms and to secrete different cytotoxins (enterotoxins, pore-forming toxins, hemolysins) is important for its pathogenicity (Mezzatesta et al. 2012, Davin-Regli 2015).

β-galactosidase or lactase (EC.3.2.1.23) hydrolyzes the sugar lactose of milk into monosaccharides, glucose and galactose and also catalyzes mixture of different galactosides. Among enzymes which are of few industrially importance, β-galactosidase discovers wide application in several main areas, pharmaceutical, health, food, technology and environment. Concerning health issues large number of population undergoes from lactose intolerance syndrome all over the world. Primary lactose intolerance has a high degree of race dependence that in the USA exists about 95%-100% of American Indians, 80%- 90% of Blacks, Mediterraneans, Asians and Jews and 50% of individuals of northern and central European origin. The reduction or loss of lactase activity in the intestinal brush border causes Lactase deficiency. When lactose is not indigested and when it passes to the large intestine, it will be converted into acids and carbone dioxide (CO_2) through fermentation by intestinal microflora, so this will cause giddiness, headache and nausea, it could also cause tissue dehydration, poor calcium absorption, generation of hydrogen and carbon dioxide gases, abdominal pain, diarrhea, bloating, flatulence, blanching, and cramps

as well (Kaur et al. 2006, Chen et al. 2009, Amir and Whorwell 2009, Patil et al. 2011, Ghatak et al. 2013, Princely et al. 2013).

More applications of β-galactosidases, such as the preventing lactose from being crystallized in frozen and condensed milk products, whey can cause reduction of water pollution, and also it will increase the sweetening properties of lactose (Soliman 2008).

The lactose hydrolyzing enzyme, β-galactosidase will make the reaction between the disaccharide molecules (lactose) and water easier, after that the oxygen bridge will be cleaved and will result in the production of two simple sugars (glucose and galactose) (Kara 2004) . The extent of these indications is variable and indeed most individuals can endure a moderate amount of lactose in their diet (Lifran et al. 2000).

β-galactosidase can be obtained from various sources such as plants, animals and microorganisms. But microorganisms are regarded as an appropriate source for industrial applications. Among bacteria, yeast and fungi, bacteria are the most suitable because they are generally regarded as safe (GRAS). To produce β-galactosidase, it is important to select a microorganism with great potentiality (Todorova-Balvay et al. 2006, Osiriphum and Jaturapiree 2009, Natarajan et al. 2012).

β-galactosidase is of highly technological importance. Microorganisms have various advantages over other obtainable sources such as easy handling, higher multiplication degree, and high production revenue. Because of commercial interest in β-galactosidase, a huge number of microorganisms have been measured as potential sources of this enzyme (Panesar et al. 2010).

Despite producing many commercial β-galactosidases, mostly from yeast and fungi, the practical application of these enzymes still has various technical problems. For example, the most of the discovered β-galactosidases (optimum temperatures above 30°C) do not act well for hydrolysing lactose at low temperatures of 0-10°C at which milk is usually stored and kept to prevent it from being spoiled (Asraf and Gunasekaran 2010).

On the other hand, a transgalactosylation reaction can also be catalyzed by lactase enzyme, which cause the formation of di, tri, or higher galacto-oligosaccharides (GOS). GOS were found to stimulate the growth and establishment of *Bifidobacteria* in the human intestine and overpower potentially harmful bacteria such as *Clostridia* and *Bacteriodes* species in the gut and are now considered as a prebiotic food ingredient (Hsu et al. 2007).

In addition to the microorganisms used to produce the β-galactosidases, they have various nutritional requirements and thus they produce enzymes rather than β-galactosidases like proteolytic and lipolytic enzymes which can produce inferior organoleptic properties or other quality defects in milk/milk products (El-Kader et al. 2012). In the present study, a strain (3TP2A) of *E. cloacae* identified by Bruker Daltonik MALDI Biotyper, as well as by 16S rRNA gene sequence analysis was used to purify mesophilic β-galactosidase, after which the purified enzyme was characterized and tested for use in lactose hydrolysis.

1.INTRODUCTION

2. PREVIOUS STUDIES

2.1.The genus *Enterobacter*

Enterobacter is gram-negative genus which is a common bacteria, is facultative an aerobic, rod-shaped, non-spore-forming belongs to the family *Enterobacteriaceae*. There are two famous sorts of *Enterobacter*: *Enterobacter cloacae* and *E. aerogenes* which have taken on clinical importance as unprincipled bacteria and have appeared from intensive care patients pathogenic as nosocomial pathogens, particularly on mechanical ventilation (Mezzatesta et al. 2012).

E. cloacae and *E. aerogenes* have already been reported as significant opportunistic and multi-resistant bacterial pathogens for humans throughout the last three decades in hospital zones. The spreading of *Enterobacter* sp. is related to the occurrence of terminated regulatory cascades that powerfully control the membrane permeability confirming the bacterial protecting and the expressing of detoxifying enzymes involved in antibiotic inactivation or degradation. In addition, these bacterial species can acquire many genetic mobile basics that strongly give antibiotic resistance. Furthermore, this specific fitness help them to colonize some environments and hosts quickly and efficiently get used to their metabolism and physiology to exterior conditions and environmental stresses (Davin-Regli 2015).

2.1.1. *Enterobacter cloacae*

E. cloacae was first called as *Bacterium cloacae* and as *Cloaca cloacae* but when the genus was established it was renamed as *E. cloacae*(Table 2.1). *E. cloacae* is ever-present in nature and is known to be causes of many diseases in various plants like onion, ginger, papaya, and macadamia (Gillespie and Hawkey 2006, Nishijima et al. 2007).

The properties of *Enterobacter* are similar to *Klebsiella* and could be recognized by motility and certain biochemical reactions. The motility properties are used to differentiate

motile *E. cloacae* from non-motile *Kleibsella* sp. whereas they could not be distinguished biochemically and morphologically from each other (Muhialdin 2014).

E. cloacae is among the difficult-treated pathogens which are basically resistant to penicillins, cephalosporins (first and second-generation) and amoxicillin/clavulanic acid (AMC) owing to the chromosomal formation of Amp C β-lactamases (cAmpC) (Hilty et al. 2013). *E. cloacae,* as the typical species of *Enterobacter*, is a dominant nosocomial pathogen because of showing antibiotics highly resistance (Ren et al. 2010).

E. cloacae is a universal bacteria that lives in aquatic and terrestrial environments (water, sewage, soil, and food).The species appears as commensal microflora in the intestinal areas of humans and animals. It is also pathogen in plants and insects. This variety of habitats is reflected by the genetic diversity of *E .cloacae*. Recently, (multi locus sequencing typing) MLST and (Pulsed-field Gel Electrophoresis) PFGE epidemiological methods data showed world circulation of several widespread clonal complexes (Mezzatesta et al. 2012, Izdebski et al. 2015).

Table 2.1. Classification of *Enterobacter cloacae* (from Humann et al. 2011).

Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Enterobacteriales*
Family	*Enterobacteriaceae*
Genus	*Enterobacter*
Species	*Enterobacter cloacae*

E. cloacae cause various medical contaminations, intravenous and other hospital equipment's and devices (Dugleux et al. 1991). This bacterium can colonize on operative cleaning solutions and various surgical instruments causing nosocomial outbreaks (Wang et al. 2000). It has been reported for the past decades that the bacteria are considered as nosocomial pathogens in newborn units and several infection outbreaks (Fernandez-Baca et al. 2001, Pestourie et al. 2014).

Nowadays variability among strains is less common and outbreaks resulted from clonal *E. cloacae* hyper-producing Am PC β-lactamase and ESBL(extended spectrum β-lactamase) carrier isolates are defined from neonate specimens, adults urines/feces samples or from environmental samples (Pestourie et al. 2014).

The genetic heterogeneity of the nomenspecies *E. cloacae* is famous. *E. nimipressuralis, E. kobei*, *E. hormaechei*, *E. asburiae*, *E. dissolvens*, *E. cancerogenus* and *E. asburiae* are basically associated with it and are involved in the so-called *E. cloacae* complex (Hoffman and Roggenkamp 2003).

2.2. Industrial enzymes in biotechnology

Enzymes have been industrialized since 1874 for the first time by a chemist Christian Hansen. The chemist employed the first enzyme used for industrial purposes called rennet which was derived from dried claves, stomachs with saline solution (Binod et al. 2013).

The term enzyme which means "in leaven" originated in a Greek word *ευζυμο*, that term was first used by a professor of physiology, Wilhelm Friedrich K¨uhne, at the University of Heidelberg in 1877. Currently it is one of the most widely used molecules after civilization of the ancient human (Gurung et al. 2013).

The sixth edition of the International Union of Biochemistry (I.U.B.) and Molecular Biology was published in 1992, has 3196 different enzymes. The international union of biochemistry introduced the standards for classifying enzymes which recommend that enzyme names originated in both the type of reaction catalyzed and the substrate acted upon. Enzymes could be divided into six parts based on the enzyme commission, oxidoreductase (EC 1), transferase (EC 2), hydrolase (EC 3), lyase (EC 4), isomerase (EC 5) and ligase (EC 6) (Gurung et al. 2013).

Biotechnology is currently using a wide range of enzymes manufactured on a commercial scale utilizing purposely screened microbial. The bio-catalysts of enzymes have an important role in all stages of biochemical reactions and metabolism. The contribution of certain enzymes as organic catalysts is of special interest in different processes on industrial scales. Superior enzymes are such microbial enzymes that are derived from microorganisms and they are employed for application purposes in industrial scales. However, the enzymes have been obtained from microorganisms since the 20th

century, characterization of properties, studies on their isolation, their application in bio-industry and manufacturing on bench-scale to pilot-scale have been developed. Moreover, a number of enzymes originated microbial sources have been employed commercially in various processes. Certain microorganisms such as bacteria, fungi and yeasts have been found in several global studies for the economically viable prepared bio-synthesis of many enzymes contributing in various commercial applications (Nigam 2013).

Enzymes could be used as biocatalysts in conventional catalytic reactions either in immobilized forms or in free and this is based on the specificity of enzyme. Biotechnology has been developed in a way that different enzymes which are being designed or purposely engineered based on the requirements of a procedure. Particular catalytic reactions are performed by several established class of enzymes which have also established their roles in designated bio-processes. Furthermore, several new enzymes have been designed with the involvement of biochemical-reaction engineering and protein-engineering. Certain molecular techniques have recently been engaged to improve and develop the performance and quality of microbial enzymes for their industrial applications more widely (Chirumamilla et al. 2001).

Microbial enzymes play an essential role in the industrial developing bioprocesses. Applications are currently involved in several markets, for instance textiles and detergents, chemical, pharmaceuticals, beverages and food, biofuels, leather, paper and pulp. At present, there is a necessity for improving more versatile enzymes in an attempt to develop more sustainable and industrial economically competitive production. Novel molecular techniques and diversity in microbial, such as genomics and metagenomics, are being exploited to determine new microbial enzymes with catalytic properties which later could be modified/ improved by various approaches based on rational, semi-rational and random directed development. The industrial enzymes are mostly found in recombinant forms synthesized in both bacteria and fungi (Adrio and Demain 2014).

Microorganisms can produce two types of enzymes; the one is retained inside the cell wall called intracellular while the other one is released into the growth medium called

extracellular. Glucose isomerase acts as the intracellular enzyme when it converts glucose into fructose and plays a vital role in the food industry. This type of enzyme needs to be activated by a particular molecule. In contrast, an average signal molecule cannot activate the enzyme since it cannot pass through the cell wall (Prasad 2014).

The extracellular enzymes are mostly used in industry including certain vital enzymes for instance, β-galactosidases, proteases, xylanases, keratinases and amylases.

Amylase*:* this is an essential enzyme due to its specific application in the starch conversion process in the industry (Nigam 2013).

Xylanase*:* Hemicellulose constitutes mainly of agricultural residues and plants along with pectin, lignin and cellulose (Nigam and Pandey 2009).

Keratinases*:* Keratin is considered as fibrous structural and insoluble protein that is a constituent of wool and feathers. The protein could chiefly be found as by-product keratinous wastes which represent the main source of proteins and amino acids that may possible be used as a source of nitrogen for plants or for animal feeds (Gushterova et al. 2005).

Proteases: They are hydrolytic enzymes, belong to the largest group of enzymes and are the most commercially-applicable enzymes, among the enzymes within this group the microbial proteases have been widely studied. Proteases prepared from microbial systems are of three types: acidic, neutral and alkaline. Alkaline proteases are well-organized under alkaline pH conditions and involve a serine residue at their active site (Gupta et al. 2002, Mukherjee et al. 2008, Vijayalakshmi et al. 2011).

2.2.1. The use of β-galactosidases in biotechnology

β-galactosidases is applied in the industrial in two ways; the use of immobilized enzyme bioreactors or utilizing β-galactosidase in a solution called free enzyme method (Gänzle et al 2008). The free enzyme technique is easily applied while reusing soluble enzyme is difficult due to its high costs of enzyme provisions and also high disadvantages (Gosling et al. 2010, Oliveira et al. 2011).

The provided entire cells can be applied as an alternative way to make less the technical and economical efforts relating to the extraction and purification/ isolation of enzyme. The main disadvantage is that lactose permeates through cell membranes very poorly; however chemical agents, such as chemical agents or detergents could be used to improve the microbial cells permeability (Panesar et al. 2006).

The hydrolyzed products such as hydrolysis of lactose derived from whey are sweeter; therefore they could be applied for developing additives for animal and human food. In addition to this, bioethanol could be obtained from the converted monosaccharaides after fermentation by utilizing proper microorganism and gasoline could also be blended with the produced bioethanol following proper purification. Accordingly, the promising process of hydrolyzed lactose has been found in several discoveries for many years for several reasons. However, the less fermentable lactose comparing to other sugars is an obstacle for the utilization of whey as it results in crystalizing at low temperature. The whey lactose could be hydrolyzed to galactose and glucose for improving that issue (Jurado et al. 2002, Matioli et al. 2003, Das et al. 2015).

The hydrolysis of lactose in whey and other dairy products can be accomplished in two approaches; acidic hydrolysis and enzymatic hydrolysis. The enzymatic hydrolysis is more desired than the other way as it allows milder conditions of temperature and pH, without causing any bad flavor, color and odor. Moreover, the acidic method results in protein denaturation finding in lactose solution and produces uninterested by products

(Sener et al. 2006, Demirhan et al. 2010). Table 2.2. Shows the biotechnological applications of β-galactosidase in bacteria.

Table 2.2 Biotechnological applications of β-galactosidases: lactose hydrolysis in milk and cheese whey and synthesis of GOS or other lactose/galactose derivatives by transgalactosylation reactions. (c) Cold-active enzymes; (m) mesophilic; (t) thermophilic enzyme (Oliveira et al. 2012).

β-galactosidases

Hydrolysis by β-galactosidases		Transgalactosylation by β-galactosidases	
Milk	**Acid whey**	**GOS**	**Other lactose or galactose derivatives**
Sweet whey			
Arthrobacter sp(c). *Bacillus staerothermophilus(t)* *Alicyclobacillus acidocaldarius(t).*	*Lactobacillus bulgaricus*	*Lactobacillus planetarium.* *Bifidobacterium infantis.* *Geobacillus staerothermophilus.* *Bacillus staerothermophilus(t)*	**Lactosucrose:** *Bacillus circulans* **Galactosyl trehalose** *Escherichia coli* **Galactosylmannitol, Galactosyle sorbose, Galactosyle salisin:** *Enterobacter cloacae B5* **(**m)

Applications of β-galactosidase are involved in dairy industry for managing the lactose intolerance (Mozumder et al. 2011). Lack of ability to hydrolyze a sugar type,

lactose, found in dairy products called lactose intolerance; this is a worldwide population problem due to the deficiency of β-galactosidase enzyme (Vasiljevic and Jelen 2002).

Mechanism of lactose hydrolysis and absorption and pathogenesis of diarrhea in intolerance is displayed in (Figure 2.1) the chief symptoms of lactose intolerance contain gassiness, swelling, diarrhea and abdominal pain. The undigested lactose passing from the small intestine into the colon will cause these symptoms. In the colon, the fermentation of unabsorbed lactose is caused by the bacteria normally present there will produce short chain fatty acids and gases (carbon dioxide and hydrogen). Flatulence, bloating and swelling pain might be resulted from Gas production. Unabsorbed lactose also has an osmotic effect in the gastrointestinal tract, drawing liquid into the lumen and resulting in diarrhea (de Vresa et al. 2001).

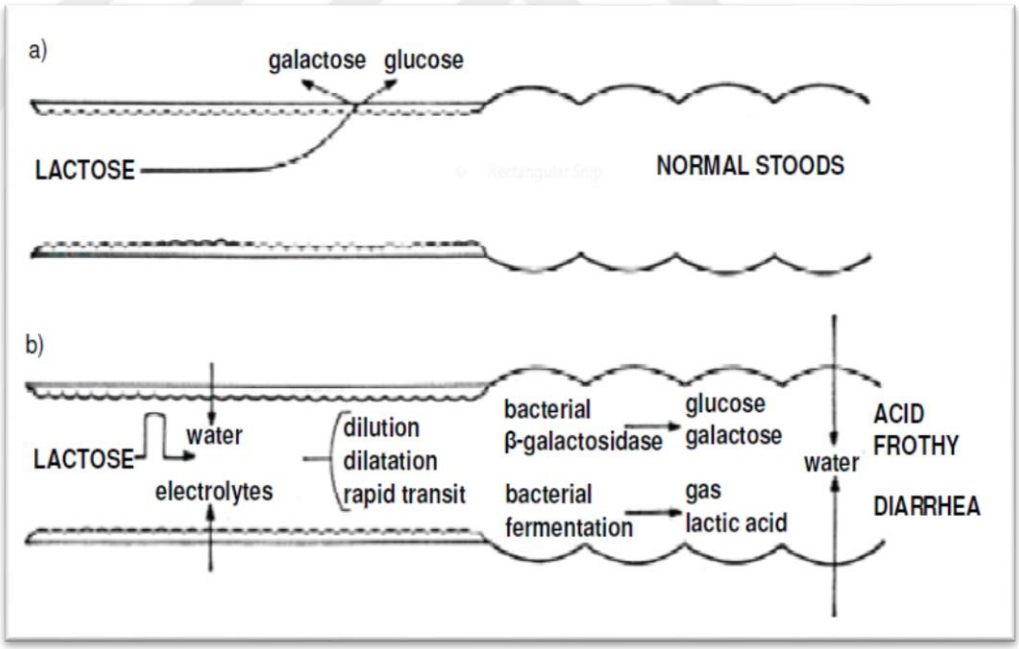


Figure 2.1. a) Mechanism of lactose hydrolysis and absorption.

b) Pathogenesis of diarrhaea in lactose intolerance (Kern and Struthers 1966, Üstok 2007).

2.3. Properties of β-galactosidase

β-galactosidase, an enzyme which has both historical and scientific importance. It is found in many organisms. The β-galactosidase encoded by the lac Z gene of the lac operon of *E. coli* has been very widely studied. It catalyzes β-D-galactopyranoside collapse and galactosyl transfer. Jacob and Monod used it to study introduction of gene expression of the lac operon. They won a Nobel Prize for this work (Huber 2013).

It was investigated that the β-galactosidase is one of the most important enzyme used in food processing, which catalyses the lactose hydrolysis to its component monosaccharides, glucose and galactose (Figure 2.2). It is most frequently identified as lactase. The enzyme has been insulated and cleansed from a wide range of microorganisms but it is most commonly used. β-galactosidases are resulting from yeasts and fungal bases. The optimum pH for lactose hydrolysis is the main variance between yeast and fungal enzyme. The β-galactosidase application for lactose hydrolysis in milk and whey has technological, nutritional and environmental applications to human life (Panesar et al. 2006).

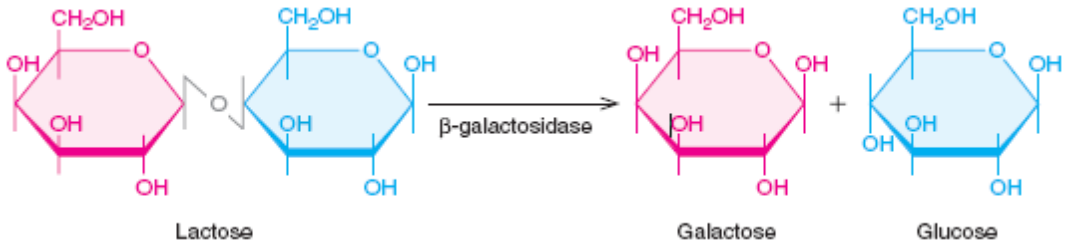
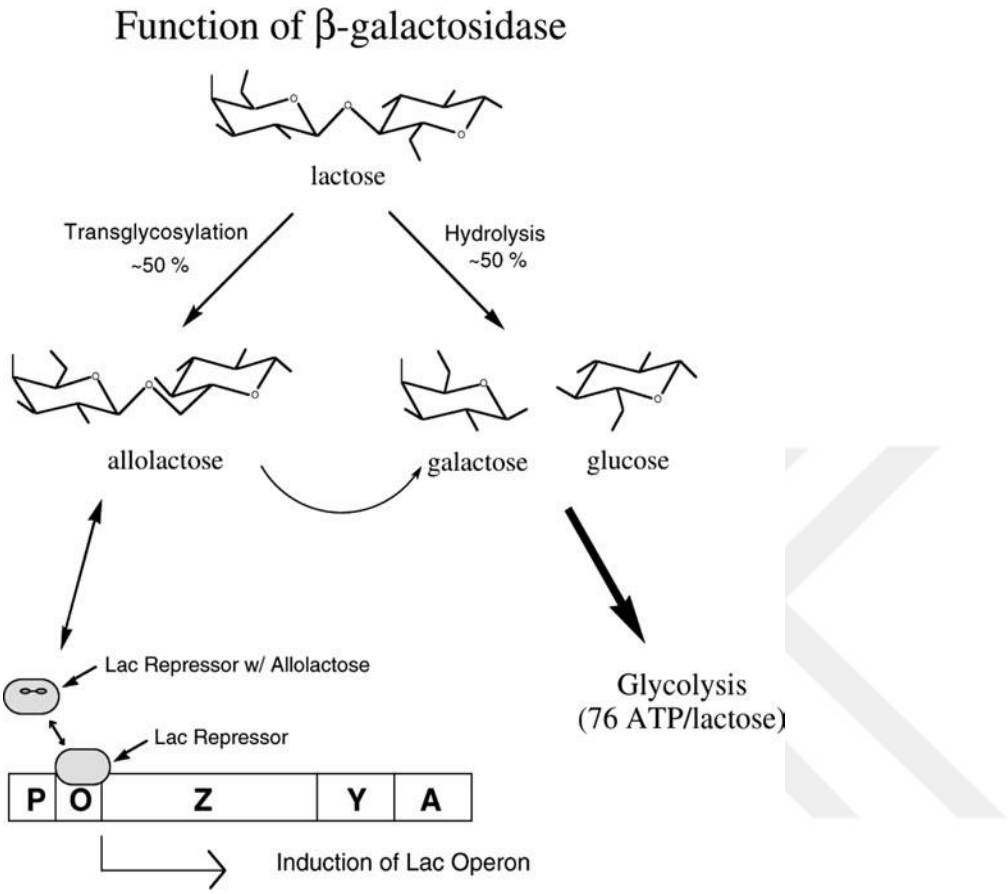


Figure 2.2: β -galactosidase hydrolysis of lactose (Weaver 2004).

β-galactosidase is a tetramer which is composed of four chains of indistinguishable polypeptide and each one formed in 1023 amino acids. The earliest structure of crystal was settled in a crystalized monoclinic formula with four asymmetric tetramers. The structure later will be refined to a single orthorhombic tetramer crystal with 1.7 ^{o}A resolution in the asymmetric unit. The technical superior of last form has been used for following practical

and structural studies. In single monomer, the 1023 amino acids constitute five domains with well-defined structure. The third domain centrally located (residues 334–627) is known as TIM (triose phosphate isomerase) or a8b8 barrel with the active place forming a deep pit at the end of this barrel on C-terminal (Juers et al. 2002).

β-galactosidase has three enzymatic activities (Figure 2.3), (Juers et al. 2012). The first one, it can catalyze the lactose to glucose and galactose, which can then enter glycolysis. Secondly, the enzyme can catalyze the transgalactosylation of lactose to allolactose and the last one, the allolactose can be cleaved to the monosaccharides. It is allolactose which fixes to lac Z repressor and makes the positive feedback loop by which the amount of β-galactosidase is regulated in the cell. In many regards, β-galactosidase is best documented for its reaction with X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside).X-gal is a soluble colorless compound that involves galactose binded to a replaced indole. The specificity of β-galactose is great for the galactose part of its substrates but β-galactose has little specificity for the remainder. Thus, it hydrolyzes X-gal, releasing the substituted indole that unexpectedly dimerizes to give an insoluble, forcefully blue result. On growth medium containing X-gal, colonies of *Escherichia coli* that have an active β-galactosidase become blue because of this reaction.



From Huber et al. (1976).

Figure 2.3: Graphic summarizing the β-galactosidase roles in the cell. The enzyme can hydrolyze lactose to glucose and galactose, it can transgalactosylate to form allolactose, and can hydrolyze allolactose. The presence of lactose can cause the synthesis of allolactose which binds to the lac repressor and reduces its affinity for the lac operon. This allows the synthesis of β-galactosidase, the product of the lac Z gene in turn.

As shown in Table 2.3. There are many sources of suppliers of β-galactosidases from bacteria to fungi as commercial preparations.

Table 2.3:A list of sources and suppliers for commercial β-galactosidase preparations.

Bacterial source	Trade name	Supplier
Bacillus sp.	Novozym 231	Novozymes A/S, Bagsvaerd, Denmark
Escherichia coli	β-galactosidase	Sigma-Aldrich, UK
Yeasts source		
Kluyveromyces lactis	Lactase	SNAM progetti, Italy
	β-galactosidase	Sigma-Aldrich, UK
Saccharomyces fragilis	β-galactosidase	Sigma-Aldrich, UK
Kluyveromyces sp.	Lactase	Enzyme development Corporation, New York USA
Candida pseudotropicalis	Neutral lactase	Pfizer, Milwaukee, USA
Fungi source		
Aspergillus niger	Lactase	Valio Laboratory, Finland
Aspergillus oryzae	Lactase 2214C	Rohm, Darmstadt, Germany
	β- galactosidase	Sigma-Aldrich, UK

From: (Mahoney 1997, Roy and Gupta 2003, Jurado et al. 2004).

2.3.1. Psychrophilic β-galactosidase

Microorganisms of Psychrotrophic and Psychrophilic play a crucial role in our environment as the temperature of our planet is mostly cold (under 5°C). These microorganisms can generally be applied in biotechnological processes under low temperature, however they are considered as the chilled food spoilage. The metabolic processes can be retained at the high rates in the cold condition when the enzymes from psychrophiles show high activity at low temperature. The enzyme activity is usually reflected in low optimum temperature compared with such corresponding enzymes from thermophiles/mesophiles. These enzymes are known as thermolabile because they are inactivated rapidly and show a high specific activity at higher temperatures, in conjunction with their manufacturers they can have an critical character as biocatalysts in food processing and in biotechnology (Karasová et al. 2002).

The isolated enzymes of cold-adapted β-galactosidase from psychrophilic microorganisms are active at the range of temperature between 0 and 30 ^{o}C. These potential enzymes are not only employed in biotechnological purposes but also in the applications of industry. The properties of enzyme structure with high flexibility make the cold-adapted enzymes to be unfolded at low to moderate temperatures, and this can cause the inactivation of enzyme. This enzyme inactivation at moderate temperature .The inactivation of enzymes at moderate temperatures is suitable in experimental laboratories; however it also confines reactions to low temperatures (Fan et al. 2015).

2.3.2. Mesophilic β-galactosidase

It has been reported that the β-galactosidases are derived mainly from mesophilic microorganisms; accordingly, low thermo-stability of these enzymes are the main drawback. Some studies approved that reactions catalyzed at high temperature could have two advantages. The initial reaction rate becomes higher by improving reaction kinetics, and also increased substrate solubility can cause a higher volumetric throughput (Kong et al. 2014).

The enzymes of *E.coli* beside the β-galactosidases as a molecular genetics tool have been deeply studied after discovering the lactose operon. However, the industrial enzyme usage is restricted in the applications of food due to not considering safe, but it is still commercially used for analytical applications. Thus, microorganisms selection based on not harm for human use and also capability for synthesizing high β-galactosidase throughput are essential duty. In this regard, the rich sources of β-galactosidases for the efficient usage in food applications are Lactic acid bacteria including a diverse group of *Lactobacilli*, *Streptococci* and *Lactococci,and Bifidobacterium*, which are called GRAS organisms (Generally Recognized as Safe) (Princely et al. 2013).

Jacob et al. (1961) Investigated that β-galactosidase derived *E. coli* showing a particular place in the practice of molecular biology and the history. It has a key player in the regulation of gene expression in Jacob and Monod's development of the operon model. A distinguishable blue reaction product indicates its presence and that could be a good worker in certain molecular biology procedures such as cloning.

E. coli β-galactosidase is a single tetramer composed of four symmetrical 1023-amino acid chains, each of which contains five domains. The active site located on the third one that comprises an eight-stranded α/β barrel. This site involves elements from other subunits and other domains. The polypeptide chains with the N-terminal region formed from interfaces of the single subunit. These all features together offers a basic structure for the known property of α-complementation. Covalent galactosyl formation makes the progression of catalytic activity when it intermediates with Glu537, and comprises 'deep' and 'shallow' modes of binding substrate (Matthews 2005).

Late lactose-fermenting strains of the following usually prompt lactose-fermenting species and genera , namely, *Escherichia coli ,Citrobacter,cloacae*, and *Klebsiella*, are *o*-NPG positive. Also *o*-NPG positive are certain lactose-variable or non-lactose-fermenting genera and species, namely, *Dispar, Hafnia, Serratia, Vibrio* and *Aeromonas*. Non-lactose fermenting species and genera, *Salmonella, Proteus , Providencia, Alkalescens*

, *Pseudomonas* and *Moraxella* are *o*-NPG Negative. Those species and genera which gave variable results to *o*-NPG tests are set out below Table 2.4. (Lapage and Jayaraman 1964).

Table 2.4: Results of some species and genera with lactose fermentation.

Bacteria	Results
Shigella flexneri	non-lactose fermenting→negative
Shigella sonnei	late lactose fermenting →positive non-lactose fermenting →negative
Shigella dysenteriae 1	non- or late-lactose fermenting→positive
Shigella dysenteriae 2	non-lactose fermenting→negative
Shigella bodyii 9	late lactose fermenting →positive Non-lactose fermenting→negative or weak positive

Modified from Lapage and Jayaraman (1964).

The most isolated bacteria as producer was diagnosed as *Bacillus subtilis.* Furthermore, certain environmental and nutritional conditions for the enzyme production via the selected isolate were studied of changed media comprise as pH of the medium 7.0, yeast extract 10 g/L, lactose 5 g/L and the incubation for 48 hours at the temperature 30 °C was considered as the most appropriate conditions for the β-galactosidase production (El-Kader et al. 2012).

2.3.3. Thermophilic β-galactosidases

A number of isolated β-galactosidases described as thermostable have been formerly achieved from both mesophilic *Archaeabacteria* and *Eubacteria*.They were distinguished and utilized for lactose hydrolysis and in GOS production (Petzelbauer et al 2001, Ansari et al 2012). Though, these thermostable enzymes are mostly produced at very low amount by *archaeabacteria* and thermophilic bacteria; for which reason they could not

be purified easily. Therefore, they could be synthesized on a large scale at industrial scale by employing recombinant techniques in mesophilic hosts (Demirjian et al. 2001, Ansari et al. 2012). The efficacy of recombinant β-galactosidases with thermostable achieved from *Geobacillus stearothermophilus, Sulfolobus solfataricus, Thermotoga maritima, Pyrococcus furiosus* and *Thermus* sp. had already been reported in the production of GOS pursuing at high temperatures. They demonstrated few advantages over innate enzymes comprising large-scale production, developments in their activity and their simplicity of purification (Akiyama et al. 2001, Bruins et al. 2001, Placier et al. 2009).

2.4. Bacterial sources of β-galactosidase

The β-galactosidase enzyme can be synthesized by a large group of bacteria such as *Bacillus stearothermophilus* and *Streptococcus thermophilus*, which are potentially employed as bacterial sources. The enzyme derived from *Escherichia coli* could be used as a model for comprehending how the catalytic mechanism of β-galactosidase acting, whereas the host coliform causes toxicity problems and make it does not suitable in food applications. For this reason, the enzyme from *E. coli* is not normally preferred in the industry of food (Panesar et al 2010). Bacterial sources are desired due to their fermentation easily, high stability and activities of the enzyme. Several bacterial species have been employed by a number of dairy industries for producing β-galactosidase enzyme; these species belonging to genera of *Bifidobacterium* and *Lactobacillus* (Sumathy et al. 2012). A few β-galactosidases properties from bacterial sources are presented in (Table 2.5).

Table 2.5 : Some properties of β-galactosidases from bacterial sources.

Bacteria	Enzyme temp.	Enzyme pH	M.w kDa	Reference
Bacillus sp.	60 °C	8.0	484	Chakraborti et al. 2000
Bifidobacterium infantis HL96	37 °C	-	113	Hung et al. 2001
Enterobacter agglomerans B1	37-40 °C	7.5-8.0	248	Lu et al. 2007
Streptococcus mitis	30-40 °C	6-6.5	268	Campuzano et al. 2009
Bacillus sp. BPTK4	55 °C	7.0	65	Natarajan et al. 2012
Bacillus subtilis	35 °C	8.0	120	El-kader[1] et al. 2012
Bacillus subtilis	30 °C	7.0	-	El-kader[2] et al.. 2012
Lactobacillus sp.	37 °C	7.2	116	Sumathy et al 2012
Enterobacter cloacae	50 °C	8.5-9.5	-	Ghatak et al. 2013
Escherichia coli	35 °C	7.0	-	Khedr et al. 2013
Streptococcus thermophilus A5	40 °C	7.2	-	Princely et al. 2013

Accounting for 0.1–1% of the bacterial species in environment could be cultivated by employing conventional methods. Hence, an auspicious source is considered as the metagenome used for new β-galactosidases discovery with industrially favorable properties (Erich et al. 2015).

The optimized ultrasonication approaches has been used for disrupting the extra cells of *E. coli* for the β-galactosidase release (Panesar et al. 2010). The cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* were treated for the β-galactosidase release using a high-pressure homogenizer, a high-speed bead mill and sonication (Bury et al. 2001). Bacterial sources of β-galactosidases are shown in (Table 2.6).

Table 2.6: Bacterial sources of β-galactosidase.

Bacteria
Alicyclobacillus acidocaldarius subsp. *rittmannii*
Arthrobacter sp.
Bacillus acidocaldarius, B. circulans, B. coagulans, B.subtilis, B. megaterum, B. stearothermophilus
Bacteriodes polypragmatus
Bifidobacterium bifidum, B. infantis
Clostridium acetobutylicum, C. thermosulfurogens
Corynebacterium murisepticum
Enterobacter agglomerans, E. cloaceae
Escherichia coli
Klebsiella pneumoniae
Lactobacillus acidophilus, L. bulgaricus,L. helviticus, L. kefiranofaciens, L. lactis, L. sporogenes, L. themophilus, L.delbrueckii
Leuconostoc citrovorum
Pediococcus acidilacti, P. pento
Propioionibacterium shermanii
Pseudomonas fluorescens
Pseudoalteromonas haloplanktis
Streptococcus cremoris, S. lactis, S. thermophius
Sulfolobus solfatarius
Thermoanaerobacter sp.
Thermus rubus, T. aquaticus
Trichoderma reesei
Vibrio cholera
Xanthomonas campestris

From (Panesar et al. 2006, Gul-Guven et al. 2007, Panesar et al. 2010, Prasad et al. 2014).

2.5. Purification of β-galactosidase

Different techniques are currently applied for the improvement and purification of several bioproducts. The selection of unit operations appropriate for each process stage along with its intrinsic characteristics is required for a purified bioproduct achievement. Various feasible strategies could also be combined with a few techniques. If the unit operations are employed very well, the resulting by product and the development of the process will be impacted. The whole downstream processing costs around two-thirds of entire process costs (Lemes et al. 2014). The production of bioproducts in the industry is not a simple task as it needs a big effort in process improvement. Therefore, the purification strategies are necessary to be investigated because of employing low-cost technologies. The traditional methods are involved in isolating and purifying proteins and include several steps, such as the precipitation of ammonium sulfate, dialysis, ion and affinity chromatography and finally the product concentration (Oliveira et al. 2012). Hence, there is a requirement to examine the optimization and application of alternative methods that could increase the purification process efficiency, and also improve the separation process with a low-cost (Ahmad et al. 2010).

A number of techniques and procedures have been published for the β-galactosidase purification. Though, the evaluation of a single purification sequence is specified in the most existing studies in the literature and it is also mot considered the technique with peculiarities hindering the scale-up method and compromise the efficiency of the process (Lemes et al. 2014).

2.5.1. Sonication

The bacterial cell walls can be disrupted by one of the most widely used techniques called sonication. One of an effective way of cell breakage is the production of sound waves with high frequency for applying to microbial cells. The differences in local transient pressure can break cell walls and the mechanism of micro-cavitation is believed to involve in. The cell breakage efficiency can be influenced by several factors such as, the volume of material processed, the exposure duration and the power output of the instrument. The

treated volume in a given time is not preferred specifically, for instance, using high pressure extrusion. The produced heat is necessary to be avoided by cooling. Ultrasound is referred to frequency of sound waves higher than 15-20 kHz, and this result in cell inactivation, whereas the application of higher acustic power inputs along with the ultrasound can result in microbial cells destruction in suspension (Dinnison 1999, Kara 2004, Prasad 2014).

2.5.2. Ammonium sulfate precipitation

This is a specific step carried out in a more general method termed salting out. The widely used technique is the addition of neutral salts for precipitation of fractionating proteins. The protein is not denatured after the precipitation and also its activity is improved by the pellet re-dissolving. Moreover, the proteins can be stabilized by adding the salts for avoiding bacterial contamination, proteolysis or denaturation. Therefore, the salting out is considered as a critical step carrying out either before or after centrifugation to store an extract overnight (Amersham 2001).

2.5.3. Dialysis

Dialysis can be described as the solutions of different concentrations diffuse through semi-permeable membranes acting as the boundary between them. Certain average pore size can be found in the membrane acting as an inert sieve. The distributed fibers randomly can form the pores synthesizing the semi-permeable dialysis tubing or the dialysis membrane, which is normally produced from cellulose acetate and contains pores ranging from 1 to 20 nm in diameter (Harris 1989).

2.5.4. Ultrafiltration

Ultrafiltration is a technique related to dialysis, and can also be used to desalt protein solutions, effect buffer exchange, or concentrate protein solutions. It is more expensive than dialysis, however, as special equipment and membranes are required. In this technique, pressure is applied to the solution to cause a bulk flow of water and dissolved

low molecular weight solutes, through the membrane, while high molecular weight solutes are retained (Dennison 1999).

2.5.5. Gel permeation chromatography (GPC)

A form of partition chromatography which is used for separating molecules of various sizes called Gel permeation chromatography (GPC). This type of chromatography can be simply termed as gel chromatography or in other forms such as, molecular sieve chromatography, gel exclusion chromatography and gel filtration. This chromatography type basically works on the partitioned molecules among stationary phase of defined porosity and solvent. A porous gel matrix in bead form surrounded by solvent in a packed column is used for the separation process (Swadesh 2000,Amersham 2001).

2.5.6. Affinity chromatography

The above aforementioned chromatographic methods are relied on the protein with the gross physicochemical properties. Though, activity of protein is biologically more subtle and also relies on the very complementary specific and steric relationship between a substrate (or inhibitor) and the active site, or a ligand and a binding site. This biospecific correlation between a protein and a ligand can be exploited by affinity chromatography to choose an interested protein in a single step from a crude mixture (Harris 1989).

The β-galactosidase purification was achieved from gram-negative of *Pseudoalteromonas haloplanktis* TAE 79 strain to homogeneity. The sequence of the NH_2-terminal amino acid and nucleotide of the purified enzyme specify 1,038 amino acids with a calculated *M*r of 118,068 constitute the β-galactosidase enzyme subunit. Furthermore, this enzyme has certain structural properties with β-galactosidase from *Escherichia coli* which includes 51% amino sequence identity, related subunit mass, comparable optimal pH value, protection of amino acid residues comprised in catalysis and needed for divalent metal ions, and it also varies by changing optimum activity towards low temperatures and stability at lower thermal, and by a higher catalytic efficiency on natural and synthetic substrates.

Furthermore, the enzyme is also characterized by activation of particular thermodynamic parameters and by a higher pI (7.8). The β-galactosidase from *P. haloplanktis* was released in *E. coli*, and also properties presented by the recombinant enzyme are similar to those of the wild-type enzyme. Intrinsic fluorescence spectroscopy is employed to monitor unfolding heat-induced and show values of lower melting point for recombinant β-galactosidase associated with the mesophilic enzyme and *P. haloplanktis* wild-type. The assessed lactose hydrolysis in milk reveals β-galactosidase derived *P. haloplanktis* which is capable to outperform the current β-galactosidase employed commercially from *Kluyveromyces marxianus* var. lactis, and it has been suggested to hydrolyze lactose using the cold-adapted β-galactosidase in processing dairy products in refrigerated plants. (Hoyoux et al. 2001).

The purification of *β*-galactosidase has also been accomplished from psychotropic such as *Pseudoalteromonas* sp. and the enzyme isolated form Antarctica. A rapid purification scheme could be used to achieve a high throughput of purification by employing extraction in an aqueous two-phase system ultrafiltration techniques and interaction of hydrophobic chromatography (Fernandes et al. 2002).

The *E. coli* K-12 strain LC110 β-galactosidase has been purified and characterized. Strain LC110 is a Lac^+ revertant of a mutant with a removal of the lac Z β-galactosidase gene. Its new evolved β-galactosidase (ebg) activity was shown to be due to a discrete protein, immunologically unrelated to lac Z β-galactosidase (Arraj and Campbell 1975).

Enterobacter agglomerans B1 was served to obtain a newly purified and a homodimer of 248 kDa transglycosylating β-galactosidase. The optimum temperature and pH for the hydrolysis of *o*-NPGal were 37-40 ^oC and 7.5-8.0, while 114 and 0.06 mM were the K_m values for lactose and *o*-NPGal. The enzyme formed galacto oligosaccharides in around 38% yield at the concentration of lactose 12.5% (w/v). Once *o*-NPGal is used as a donor, glycosyl can be catalyzed by the enzyme and transfer to a series of acceptors, comprising aromatic glycosides, cyclitol, hexahydroxy alcohol, β or α-disaccharides, hexose and pentose. Therefore, this makes the enzyme to contribute as a robust synthetic

tool for the preparation of chemicals containing galactose. Thermal asymmetric interlaced PCR (TAIL-PCR) and degenerate PCR were capable to clone the gene encoding this enzyme. It demonstrated that the expressed 1029 amino-acid protein in *Escherichia coli* was encoded by an open reading frame (ORF) of 3090 nucleotides. The Natural and recombinant enzymes reported the same activities of transferase (Lu et al. 2007).

2.6. Lactose hydrolysis by β-galactosidase

Lactose is a disaccharide sugar composed of galactose and glucose that is originated in milk. Lactose-free milk and dairy products have a significant market, which can be gained by enzymatic hydrolysis using β-galactosidases. The sweetness and solubility of lactose is faint compared to other sugars, including glucose, galactose, fructose and sucrose (Gänzle et al. 2008, Oliveira et al. 2011). That is why, lactose hydrolysis reduces precipitation problems and improves the sweetening control, thus increasing the food applications of lactose solutions, for example sucrose or starch syrups are substituted by lactose solutions in the confectionary and ice-cream industries. (Siso 1996, Oliveira et al. 2011).

Cheese whey and milk are the main sources of lactose, which is a primary by-product of cheese manufacturing. During the production of 1 kg of cheese about 9 L of whey stream are created, amounting to over 160 million ton of whey produced worldwide each year (Guimarães et al. 2010). The capacity of Whey's organic is extraordinary (biochemical oxygen demand of 30–50 g/L and chemical oxygen demand of 60–80 g/L), largely because of the lactose content (Guimarães et al. 2010). In this regard, lactose hydrolysis by β-galactosidases again plays a vital part by extending whey's applications. Particularly, whey fermentations can be prolonged away from somewhat limited capacities of lactose-consuming microorganisms, to the use of lactose-hydrolyzed whey as feedstock for the production of added-value molecules or bulk supplies by lactose-negative microbes. For instance, *Saccharomyces cerevisiae* wild strains (lactose negative) can be used to

produce ethanol from hydrolyzed whey, although catabolite repression-resistant mutants should be applied in order to avoid glucose-galactose diauxy (Oliveira et al. 2011).

The lactose hydrolysis can be accomplished by employing either enzyme or acid hydrolysis. The acid hydrolysis includes lactose heating with acid reagents for instance H_2SO_4, tetraoxosulphate (VI) acid, a cationic exchange resin as acid form called a solid acid or as a free acid form. This is a slight complex process from a mechanistic perspective. This is partly because of further degradation of the monosaccharide products into uninterested chemicals, in which the expected side reactions based on, among additional factors, the composition of lactose such as whey permeate and its source (Cote et al. 2004). The hydrolysis of lactose with free enzyme is not applicable because its high costs and the final products can be contaminated with interference of foreign protein (Olafadehan et al. 2010).

Table 2.7: Shows various β-galactosidase applications (Jurado et al. 2002, Kara 2004).

1. Elimination of lactose intolerance
2. Galacto-oligosaccharides formation during lactose hydrolysis for favor the intestinal bacterial microflora growth.
3. Development in the technological and sensorial characteristics of daily foods
4. Greater biodegradability of whey

2.7. Summary about subject

Paauw et al. (2008) performed the multi locus sequence analysis and comparative genomic hybridization is based on a mixed genome array, which is an influential technique for the purpose of species assignment studying inside the *Enterobacter cloacae* complex. The *E. cloacae* complex is revealed to be evolutionarily shared into two clades that are genetically different from each other. The younger first clade is genetically more identical, contains the *E. hormaechei* species and it is the most frequently cultured *Enterobacter* species in hospitals. The second and older clade is made up of several (sub) species that are genetically more heterogonous. Genetic indicators were recognized that could distinguish between the two clades and cluster.

Humann et al. (2011) claimed that the members of the *E. cloacae* are hard to differentiate between biochemical tests and phylogenetic studies by using multi locus sequence analysis, strains of the same species isolates into numerous clusters. There are only a few perfect *E. cloacae* genome structures and very little knowledge about the mechanism of pathogenesis of *E. cloacae* on plants and humans.

It is also identified that *E. cloacae* is a well-known nosocomial pathogen that causing bacteremia, endocarditis, septic arthritis, and skin/soft tissue infections, and lower respiratory tract-urinary tract and intra-abdominal infections (Fata et al. 1996).

It was also reported by Wang (2015) that acute infection was caused by *E. cloacae* after anterior vertical decompression and fusion. This infection was restricted by quality of an early diagnosis sooner or later and the correspondingly-adopted anti-infection, internal fixation removal, and drainage treatments. It is believed that wound infection is caused by the therapeutic principle for *E. cloacae* after anterior cervical internal fixation is essentially reliable with that for other bacterium-caused wound infections after spinal internal fixation.

It was found that the polysaccharides produced by *E. cloacae* with anti-cancer and apoptosis-inducing properties have appeared as one of the ideal candidates for cervical cancer therapy (Jin et al. 2010). Cao et al. (2010) reported that by inhibition of cancer

growth by polysaccharides produced by *E. cloacae* is mostly through the stimulation of humoral and cell-mediated protection.

It was investigated that the genome of *E. cloacae* ATCC 13047 has virulence properties identified to be important in the onset of infection. There are seven loci encoding proteins for fimbrial biosynthesis and six genes encoding adhesion / invasin like proteins on the chromosome. This strain (ATCC 13047) consists of two loci encoding iron-chelating compounds and three genes encoding hemolysin-like proteins. All genes that are required for the biosynthesis of pseudaminic acid are held by the O antigen gene cluster, which belongs to the family of nonulosonic acid (NulO).The chromosome of *E. cloacae* ATCC 13047 can transfer seven operons involved in toxic heavy-metal resistance (Lwis et al. 2009, Ren et al. 2010).

There has been a study carried out by Hoffmann et al. (2003) that the 16S ribosomal DNA (rDNA) sequences of *E. cloacae* did not form a clear cluster but built an irregular tree, in which the clusters of *E. cloacae* strains interfused with those of *E. aerogenes, Escherichia coli, Citrobacter* species, and *Leclercia* species. This mirrored the genetic heterogeneity of the nomenspecies but did not tolerate its systematic classification.

There have been many studies on characterization and possible use of β-galactosidase in various bacteria species including those belong to *Enterobacter*. A summary of several different bacterial species are given below.

Huber (2013) studied that the *Escherichia coli* β-galactosidase enzyme was comprised of four indistinguishable monomers, each with 1023 amino acids structured into five domains plus an α-peptide. β-galactosidase was active just as a tetramer.

β-galactosidase is encoded by two genes iso enzymes ; β-gal I and β-gal III, from *Bifidobacterium infantis* HL96, which is a close genus to *Enterobacter*, were shown on 3.6 and 2.4kb DNA fragments, correspondingly, by nucleotide sequence examination of the two fragments. β-galI (3,069 bp) encodes a 1,022-amino-acid (aa) polypeptide with a expected molecular mass of 113 kD (Hung et al 1998). *Bifidobacterium* sp. also discovered that most of the strains have more than one β-galactosidase isoenzyme. Earlier studies

displayed the existence of three isoenzymes (β-galI,II, and III) in *B. infantis* HL96, which had high transgalactosylation activity compared with that of 29 selected strains of *Bifidobacteria* (given as unpublished data). To detect an enzyme with forceful transgalactosylation activity and to get a better knowing of the molecular organization of the genes coding for lactose application in a suitable probiotic strain, β-galactosidase genes of HL96 were cloned (Roy et al. 1994, Hung et al. 2001).

Chen et al. (2008) reported about a thermostable β-galactosidase gene bga in *Bacillus stearothermophilus*, which was cloned and expressed in *Bacillus subtilis* WB600. It was studied that the recombinant enzyme was cleaned by a combination of heat treatment, ammonium sulfate fractionation, gel filtration and ion exchange chromatography techniques.

The stability and the activity of the incompletely purification of β-galactosidases from *Thermus* sp strain T2 and *Kluyveromyces fragilis* was formerly studied and compared (Ladero et al. 2002).

It was studied by Campuzano et al. (2009) that a *Streptococcus mitis* genomic DNA fragment carrying the SMT1224 gene encoding a supposed β-galactosidase which was identified, cloned and expressed in *Escherichia coli*. This gene encoded a protein 2,411 amino acids long with an expected molecular mass of 268 kD. This original enzyme signified the first β-galactosidase having a flexible structure with a choline-binding domain, an uncommon property which could also be valuable for some biotechnological applications.

Fukuda et al (1976) purified an intracellular-β-galactosidase to 4400-fold from a culture filtrate of *Escherichia freundii* with 45% improvement. The enzyme preparation was practically excluded from exoglycosidases, sulfatase, and proteases. This enzyme hydrolyzed several keratan sulfates, endoglycosidically releasing oligosaccharides of numerous molecular sizes.

Natarajan et al. (2012) examined β-galactosidase in *Bacillus sp*.BPTK4 separated from the dairy industry sewage in Chennai. Biochemical tests and 16S rRNA sequencing was used for the approval of the strain BPTK4 as *Bacillus subtilis*. The strain BPTK4 was measured for its probiotic nature consuming antibiotic markers. The description of the enzyme and optimization of the making medium were implemented for the maximum activity and production of β-galactosidase.

The inhibition biosynthesis of β-galactosidase and α-glucosidase (EC 3.2.1.20) by phenolic compounds (phenol, 4-chlorophenol, 2-chlorophenol,4-bromophenol and 3,5-dimethylphenol) in *E. coli*, *Bacillus* and *Pseudomonas* species detached from petrol factory wastewater was evaluated by Nweke et al. (2011) . At appropriate concentrations, phenols disallowed the production of β-galactosidase and α-glucosidase.

β-galactosidase was used by Tryland and Fiksdal (1998) 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.

To optimize the process of lactose hydrolysis using immobilized and free β-galactosidase to yield glucose and galactose, some method was exercised by changing three parameters, temperature (15–45 °C), solution pH (5–9) and β-galactosidase enzyme concentration (2– 8 mg/mL) for free mode of analysis , while sodium alginate concentration (2–4%), calcium chloride concentration (3–6%) and enzyme concentration (2–8 mg/mL) for immobilized procedure (Das et al. 2015).

Prieto et al (2014) studied on the use of pre-treated carbon for β-galactosidase adsorption. Adsorption experiments were implemented to study the properties of the pre-treatment of carbon, temperature, initial β-galactosidase and carbon concentrations in the immobilization kinetic and effectiveness. The pre-treatment of carbon improved the required capacity of carbon. The results gained in this study are favorable, because the

carbon is a low cost aid and can be used to immobilize β-galactosidase in an easy and quick means, being probable to get similar proficiency of expensive helps.

A microtiter assay for drug evaluation was improved by Mcfadden et al. (1997) by using a strain of *Toxoplasma gondii* that shows bacterial β-galactosidase. By using chlorophenol red-β-D galactopyranoside (CPRG) as the substrate for β-galactosidase, a colorimetric readout can conclude the efficiency of a drug against the parasite. Drugs recognized to have activity against *T. gondii* (specifically, pyrimethamine, sulfadiazine, atovaquone, and clindamycin) were assessed, and efficiencies were determined by CPRG cleavage. This test deals a high-throughput and nonradioactive alternative for the identification of anti-*T. gondii* compounds.

It was studied by (Li et al. 2009) A β-galactosidase gene (TM−0310) of *Thermotoga maritima* MSB8 was expressed in *Escherichia coli*. Heat treatment and Ni-NTA affinity chromatography can purify the recombinant β-galactosidase (designated BgalB) to homogeneity. BgalB is appropriate with the glycoside hydrolase family 42. Its molecular mass was projected to be 78 kDa and 76 kDa by SDS–PAGE and gel filtration, respectively. The enzyme was optimal at pH 5.5, and it was rather stable over the pH range 5.0–11.4 at 70 ^{o}C. It was optimally active at 80 ^{o}C and was stable up to 75 ^{o}C. Moreover, BgalB showed wide substrate specificity with a favorite for *p*-nitrophenyl-β-galactopyranoside (*p*-NPGal). K_m values of the purified enzyme for *p*-NPGal, *o*-nitrophenyl- β-galactopyranoside (*o*-NPGal) and *p*-NP-β-fucopyranoside were 2.7 mM, 12.5 mM and 1.4 mM, respectively. These properties can make this enzyme an interesting candidate for biotechnological uses. This is the first testimony of the family 42 β-galactosidases from *T.maritima*.

It was studied that by (Kong et al 2014). A fresh β-galactosidase gene (Tnap1577) from the hyperthermophilic bacterium *Thermotoga naphthophila* RUK-10 was cloned and conveyed in *Escherichia coli* BL21 (DE3) cells to create β-galactosidase. The recombinant β-galactosidase was purified in three phases: heat treatment to deactivate *E. coli* proteins,

Ni-NTA affinity chromatography and Q-sepharose chromatography. The optimum temperatures for the hydrolysis of o-nitrophenyl-β-D-galactoside (o-NPG) and lactose with the recombinant β-galactosidase were discovered to be 90 ^{o}C and 70 ^{o}C, respectively. The resembling optimum pH values were 6.8 and 5.8, respectively. The molecular mass of the enzyme was projected to be 70 kDa by SDS-PAGE analysis. Thermostability studies showed that the half-lives of the recombinant enzyme at 75 ^{o}C, 80 ^{o}C, 85 ^{o}C and 90 ^{o}C were 10.5, 4, 1, and 0.3 h, respectively. Kinetic studies on the recombinant β-galactosidase discovered K_m values for the hydrolysis of *o*-NPG and lactose of 1.31 mM and 1.43 mM, respectively. These values are noticeably lower than those stated for other hyperthermophilic β-galactosidases, representing high essential affinity for these substrates. The recombinant β-galactosidase from *Thermotoga naphthophila* RUK-10 also displayed transglycosylation activity in the production of alkyl galactopyranoside. This extra activity proposes the enzyme has possible for wider biotechnological applications after the degradation of lactose.

A psychrophilic gram-negative bacterium, *Rahnella sp*. R3, was employed by Fan et al. (2015) for the first time to isolate a new gene, which serves as a cold-adapted β-galactosidase (R-β-Gal) encoding. *Escherichia coli* BL21 (DE3) expressed recombinant R-β-gal belonging to the glycosyl hydrolase family 42, which was then purified and characterized. The enzyme was appeared to be active at 4 ^{o}C and a homo-trimer in solution. Moreover, metal ions was not necessary to activate the enzyme, in contrast its activity was slightly enhanced by Ca^{2+}, Mn^{2+} and Mg^{2+}, while inactivated by Al^{3+}, Zn^{2+} and Fe^{3+}. The values of K_m for lactose was 2.2 mM and 6.5 mM for *o*-NPG and were both exhibited at 4^{o}C by the purified enzyme.

The lactase activity expressed by *Lactobacillus* bacteria was investigated by Mozumder et al (2012) who isolated it from yogurts obtained in Dhaka city. The cultured strains on selective de man Rogosa and sharp Agar (MRS) agar media were identified as gram positive, fermentative, catalase negative and lactase producer. It has been revealed that enzymes synthesized by lactobacilli could form glucose from substrate lactose in media

with modified lactose by utilizing lactase assay Kit Glu IB and 17.25 mg/mL was the highest concentration of protein recorded in the supernatant of culture media isolated from *L. lactis*. The lactase enzyme was expressed by *L. bulgaricus* strain recorded specific activities (50.04 U/mg) and the highest total activities (850.69 U/L). Ammonium sulphate precipitation was employed to further purify the crude extract exhibited the highest activity subsequently the exchange of anion column chromatography (DEAE cellulose). Fold purification and final specific activity of lactase approached to 1.47 and 62.80 U/mg respectively.

Isolated and purified β-galactosidase was achieved from *Streptococcus thermophilus* A5 by the process called fermentation. Several techniques were employed to accomplish the purified enzyme such as, dialysis, precipitation of ammonium sulphate, gel filtration chromatography by utilizing SDS-PAGE, Sephadex G-100 and certain determined properties of the enzyme for instance, kinetic parameters temperature and pH. The highest productivity of isolated A5 was 7.76 U/mL while its temperature optima and pH were at 40ºC and pH 7.2, respectively. The apparent K_m and V_{max} were found to be 3.05 mM and 2.8 IU/mL, respectively. Specific activity of β-galactosidase recorded 119.38 while its fold purification was fond to be 1.13 (Princely et al. 2013).

El-Kader et al. (2012) isolated over fifty bacterial isolates from various sources such as, agricultural soil, commercial yoghurt and commercial cow milk, and analyzed for their production of β-galactosidase. *Bacillus subtilis* was identified as the most producer bacterial isolate. Furthermore, some of environmental and nutritional conditions were studied for the enzyme synthesis by employing the selected isolate. Certain suitable conditions for the β-galactosidase production in the modified media were required such as yeast extract (10g/L), lactose (5g/L), pH of the medium 7.0 and the temperature of incubation at 30°C for 48 hours.

Khedr et al. (2013) carried out the development of genetic program to improve the production of β-galactosidase enzyme by inducting the mutation of selective strain. UV was employed for the treatment of about 500 isolated mutants for 1, 3, 5 and 7 minutes. β-galactosidase activity differentiates mutants in to either active or inactive. Different concentrations of lactose (5, 10 and 15 g/L) were assessed to determine their effects on the production of beta-galactosidase. The highest production rate of the producer mutants was achieved by using only 10 g/L lactose. Moreover, the production of β-galactosidase by tested mutants reached the highest rate at pH 7 and also at 35°C. It was demonstrated that free glucose had a positive relation with β-galactosidase after hydrolyses of lactose. The mutant M3 was considered as the best β-galactosidase producer as its production was threefold higher than wild types.

The β-galactosidase enzyme was employed for partial purification, further extraction and intracellular β-galactosidase characterization from isolated *Bacillus subtilis* form the soil field of agriculture. The enzyme production and bacterium *Bacillus subtilis* growing in the media containing lactose and yeast extract were accomplished. The precipitation of enzyme was carried out by applying firstly ammonium sulphate following by fractionation and finally implemented Native-PAGE electrophoresis. The molecular weight recorded 120kDa when the optimal activity of the enzyme was generated at pH 8.0 and 35^{o}C (El-Kader et al. 2012).

The β-galactosidase was purified and characterized from bacterium *Bifidobacterium longum* subsp under the study of Saishin et al. (2010). Gum arabic was used to grow the strain *longum* JCM 7052. Polyacrylamide gel electrophoresis was provided to achieve 77 and 110 kDa of the enzyme molecular masses by applying both with and without sodium dodecyl sulfate. The purified β-galactosidase showing by MALDI-TOF-MS was the product of a homologue of the lacA1 gene originated in *B. longum* genome subsp. *longum* DJO10A. 4-nitrophenyl (NP)-β-D-galactopyranoside could be hydrolyzed by this β-galactosidase but neither gum arabic nor lactose. In addition, the 4-NP-β-D-galactopyranoside recorded 0.42 ± 0.015 mM as the value of Km. Galactose and melibiose produced from transglycosylation which was derived from 4-NP-β-D-galactopyranoside

not from lactose. The β-galactosidase maximum activities were accomplished pH 7.0 and 50~55°C and the enzyme also was found to be stable for 5 hours at 35°C while it was steadily inactivated when the temperature was over 40°C. Galactose acted as an inhibitor of active competitive, and K_i was 5.8 ±0.38 mM that refers to constant of its inhibition.

The purified β-galactosidase was accomplished by consecutive column chromatography from *Corynebacterium murisepticum* (inducible by galactose and lactose) on DEAE-cellulose (DE52), DEAE-Sephadex A-50 and sephadex G-200. The molecular mass of the enzyme with homologous dimer subunits was found to be 100,000 daltons. The enzyme with K_m values for the o-nitrophenyl β- D-galactopyranoside (*o*-NPG) and the substrates lactose were 4.4 mM and 16.7 mM, respectively and its low affinity showed for the substrates (Priyolkar et al. 1988).

The production of galacto-oligosaccharide from lactose was catalyzed by an extracellular β-galactosidase which then was harvested from *Bacillus* sp. MTCC 3088 in the late stationary-phase. The purified enzyme was carried out in 36.2-fold by various procedures such as ion exchange, hydrophobic interaction, gel filtration chromatography and $ZnCl_2$ precipitation with total recovery of 12.7%. The estimated molecular mass of the purified enzyme demonstrating by employing gel filtration run on sephadex G- 200 packed column was around 484 kDa while by sodium dodecyl sulfate-polyacrylamide gel electrophoresis which demonstrated the molecular masses of the subunits were found to be 41.2, 45.7, 72.5, 86.5 and 115 kDa. Moreover, polyacrylamide gel electrofocusing determined the isoelectric point of the native enzyme about 6.2. The optimal temperature and pH were 60°C and 8 (Chakraborti et al. 1999).

Recently, a study has been carried out by He et al. (2015) on *Lactobacillus kefiranofaciens* ZW3 achieved from grains of Kefir which is rich in lactose hydrolytic activity. Isolated β-galactosidase gene (*lacLM)* with a heterodimeric Lac LM-type from ZW3 was involved of two overlapping genes *lacM* (960 bp) and *lacL* (1884 bp) encoding small and large subunits valued molecular masses of 35,682 and73,620 Da, respectively. The recombinant proteins such as LacM, LacL and LacLM were purified and characterized

after expressing by *Escherichia coli* BL21 (DE3). The properties of hydrolytic activity and lower thermostability were revealed by the recombinant large subunit after comparing the results with the recombinant holoenzyme. Furthermore, the pH and optimal temperature of the large subunit showed 8.0 and 50^{o}C while holoenzyme displayed 7.0 and 60^{o}C, conversely activity was not exhibited by the recombinant small subunit. Therefore, the great improvement in the thermostability and activity of the large subunit was concluded when the recombinant small subunit was mixed it with.

Gram-negative anaerobe, *Thermoanaerobacter* as an extremely thermophilic was used to isolate β-galactosidase after purifying it through DEAE cellulose by using chromatography (Panesar 2010).

An intracellular β-galactosidase was under study of Gul-Guven et al. (2007) from *Alicyclobacillus acidocaldarius,* acts as thermoacidophilic, subsp. *rittmannii* purified by employing different methods such as, ion-exchange, gel permeation, ammonium sulphate used for precipitation, and preparative electrophoresis and affinity chromatography.

The β-galactosidase with transglycosylation activity produced by an isolated strain of *E. cloacae* B5 from the soil was studied by Lu et al. (2008). Galacto-oligosaccharides were synthesized by its freeze-thawed cells when this saccharide produced a high throughput of 55% from 275 g/L lactose for 12 hours at the temperature 50^{o}C. A new purified β-galactosidase with ability to transfer glycosyl was achieved from the strain. The molecular mass of the homotetramer enzyme was found to be about 442 kDa. The hydrolysis activity of *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPGal) showed at the temperature 35 ^{o}C and optimal pH between 6.5 and 10.5. A wide range of acceptor specificity was demonstrated by the enzyme for catalyzed glycosyl and transglycosylation from *o*-NPGal to different chemicals for instance, mannitol, inositol, melibiose, trehalose, sucrose, xylose, cellobiose, rhamnose, sorbose, arabinose, mannose, fructose, glucose, galactose and salicin, leading to yields new saccharide ranging from 0.8% to 23.5%. A

cloned gene was used to encode the enzyme and also the recombinant enzyme, which released from *Escherichia coli*, showed the same activity of transglycosylation as the natural enzyme.

The enzymatic methods were also studied by a researcher Lu et al. (2010) and his coworkers for synthesizing new galactosyl derivatives and the purified β-galactosidase as a robust biocatalyst from *E. cloacae* B5 was proved by the same researcher for galactosyl transfer using an appropriate donor onto various substrates. A number of novel galactose containing chemicals (GCCs) was expected to be prepared by the same methods and therefore, more libraries of diverse galactosylated compounds are likely to be developed in which whether the compounds could show their inherent values or act as intermediates as valuable products for further modification.

Ghatak et al. (2010) isolated and identified a bacterial strain called *Enterobacter cloacae* from soil, and this bacterium showed its ability to produce both extracellular and intracellular β-galactosidase. The thermostablility of the intracellular enzyme was approved and also its optimum pH and temperature for the reaction of enzyme with substrate were recorded at 9.0, 50 °C, respectively for 5 minutes by utilizing a substrate termed *o*-NPG. The production of the maximum β-galactosidase using 50 mL medium in a shake flask was found at pH 7.0, 30 °C and for 72 hours incubation in 250 mL erlenmeyer flask. The enzyme activity was only stimulated by Mg^{2+}. The enzyme catalytic activity was stimulated by cetyl trimethyl ammonium bromide while inhibited by EDTA. Moreover, the activity of enzyme was able to retain until temperature 55 °C and incubate for one hour. The crude intracellular enzyme showed its maximum activity 14.35 IU/mg of protein. The β-galactosidase with the values of K_m and V_{max} were found to be 2.805 mM and 37.45×10^{-3}mM/min/mg by employing a substrate, *o*-NPG, at 50°C.

The thermoacidophilic bacterium isolated from Antarctica *Alicyclobacillus acidocaldarius* subsp. *rittmannii* which is a family member of the GH42 was used to isolate the purified β-galactosidase. Different concentrations of the enzyme reaction product

glucose did not exhibit the effect on the enzyme, while the other reaction product galactose extremely inhibited it after utilizing substrates such as lactose and *o*-NPG. The analyses of Linewever-Burk was plotted from both lactose and *o*-NPG hydrolysis results demonstrated acted as an inhibitor in a mixed-type of the purified β-galactosidase. Mg^{2+} (13% at 20 mM) slightly activated the enzyme, but higher concentrations of Cu^{+2} (87% at 4 mM), Zn^{+2} (86% at 8 mM) and Ca^{+2} (33% at 10 mM) were capable of inhibiting it. The metal ion chelators EDTA and 1,10- phenanthroline equal to 20 mM could not considerably alter the enzyme activity, specifying that the enzyme is not characterized with metalloenzyme. The β-galactosidase activity was found to be enhanced by DTT and 2-Mercaptoethanol, whereas its activity (97% at 1 mM; 99.7% at 2 mM) was fully inhibited by *p*-chloromercuribenzoic acid (PCMB), and this indicates that the reagents were capable of modifying at least one residue of the vital cystine in the active site of β-galactosidase. Moreover, the β-galactosidase was slightly suffered from the effects of N-Ethylmaleimide and Iodoacetamide, while it was strongly inhibited (19.8% at 1 mM; 71.9% at 10 mM) by phenylmethylsulfonyl fluoride (PMSF) with serine participation in the activity of the enzyme (Gul Guven et al. 2011).

Activity of lactase was studied in the existence mono and divalent metal ions, and also its cofactors and several reducing agents were under the same study. K^{+} and Mn^{2+} showed higher activation effects than the rest of cations, and Dithiotrethiol significantly exhibited as inhibitor. The inhibition type was not considered as non-competitive because of the inhibition constant, 12.15 mM. The determination of hydrolysis reaction of the kinetic Lactase model was achieved. The inhibition effect of galactose exhibited more comparing to the products of glucose and the inhibition type was identified as non-competitive. The inhibition constant of galactose in lactose substrate was found to be 78.7mM (Madani et al. 1999).

The investigation of the β-galactosidase interaction with copper oxidenanoparticles (CuO NPs) is considered as the primary objective of this study. The β-galactosidase conformational changes involved by CuO NPs binding has been revealed using circular

dichroism (CD) spectroscopic, fluorescence and Steady-state absorption techniques. In the present case, the results reduced by temperature dependent fluorescence specify a static quenching mechanism. Van der Waals forces and the principal role of H-bonding between CuO NPs and β-galactosidase binding process are delineated by the binding thermodynamic parameters. Isothermal titration calorimetry (ITC) was used to study the binding and the result discovered that the complexation is enthalpy driven, the hydrogen bond formation between CuO NPs and β-galactosidase is indicated by the $\Delta H^{\circ} < 0$, $\Delta S^{\circ} < 0$. The native disrupted conformation of the protein on binding with CuO NPs is revealed by a reduced functionality protein CuO NPs conjugate system in comparison to CuO NPs and the native protein displayed a competitive inhibition mode. It is generally believed that the native structure modification is less associated with the formation of H-bond occurs with NPs. Investigation of size distributions and morphological features were achieved by using dynamic light scattering (DLS) and transmission electron microscopy (TEM). Furthermore, the addition of CuO NPs accounts for the b-galactosidase unfolding results in the significant increase in the Rh. A small perturbation in the structure of protein caused thermal and chemical b-galactosidase unfolding in the presence of CuO NPs. The critical consequences of this modification in nanoparticle functional activity associated with β-galactosidase should be taken into consideration because of employing these nanoparticles for the purpose of therapeutic and diagnostic (Rabbani et al. 2014).

The encoded β-galactosidase by two overlapping genes, *lacL* and *lacM,* is considered as a heterodimeric that purified from *Lactobacillus reuteri* L103. Polypeptides are encoded by *lacM* (960 bp) and *lacL* (1887 bp) genes and their molecular masses of 35,682 and 73,620, respectively. The sequences of *lacM* and *lacL* with deduced amino acid represent significant identity with the β-galactosidases sequences from *Escherichia coli* and other lactobacilli. The lacLM genes with the coding region were successfully cloned and overexpressed *E. coli* by employing the T7 RNA polymerase promoter acting as an expression system. The co-expression of *lacM* and *lacL,* and *lacL* expression alone along with activity staining of both recombinant and native β-galactosidases recommended a translational coupling between *lacM* and *lacL,* demonstrating that the necessary of both

genes for the production of a functional β-galactosidase. The apparent homogeneity of recombinant β-galactosidase was accomplished after purifying, characterizing as well as comparing to with the native β-galactosidase from *L. reuteri* L103 (Nguyen et al. 2007).

Wheatley et al. (2015) reveled that a specific monovalent cation (M^+) , which is either K^+ or Na^+, is a requirement of many enzymes for the highest activity. However, the study of transport proteins with high selectivity M^+ sites has been carried out. Moreover, lower selectivity and less characterize are generally monitored in enzyme M^+ binding sites. At this point, the study of model enzyme *E. coli* β-galactosidase with the M^+ binding site is determined, in which it shows 10 fold selective for Na^+ higher in compare with K^+. Obtaining data from computational models and X-ray crystallography, the predomination of electrostatic environment in describing the Na^+ selectivity is discovered. This lower selectivity site become significant on the electrostatic environment rather subtle influences, involving the M^+ effects of the induced polarization on the coordinating ligands and the consequence of second coordination shell residues on the primary charged distribution ligands. This effort can expand the understanding of ion selectivity in proteins to indicate new mechanisms essential for the M^+ selectivity sites in enzymes.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

List of chemicals which were used during experiments are given in appendix A.

3.1.2 Equipments

List of Equipments which were used during experiments are given in appendix B.

3.1.3 Bacterial strain

The bacterial strain used throughout this study was *Enterobacter* sp. 3TP2A which was isolated and identified by Dr. Ömer ACER from petroleum station in Batman in the southeast of Turkey.

3.1.4 Media

All the required culture media, their composition were given in Appedix C.

3.1.5 Buffers and reagents

List of the buffers and reagents, which were used during experiments, their composition and preparation are given in Appendix D.

3.2 Methods

3.2.1 Classification of 3TP2A strain as *Enterobacter cloacae* by MALDI

The strain 3TP2A was classified by 16S rRNA sequence analysis (isolated and identified by Dr. Ömer ACER and Prof. Dr. Kemal GÜVEN previously) also identified by using Bruker Daltonik MALDI Biotyper in Microbiology Department of Medical School in Dicle University in the present study.

3.2.2. Cultivation of strain and preparation of crude enzyme

Stock cultures of this strain were stored at -20 ^{o}C in sterile Eppendorf tube before use (0.5 mL of activated culture plus 0.5 mL of Glycerol) . Culture activation was done by transferring 1 mL stock culture into 25 mL sterile Nutrient Broth (NB) in 100 mL flask, followed by incubation at 30 ^{o}C for 15 h, in shaker water bath at 120 rpm (overnight incubation) and then 1 mL of pre-culture was transferred into 25 mL sterile NB for subculture incubation at 30 ^{o}C for 15 h, in shaker water bath at 120 rpm (overnight incubation).

The culture was centrifuged at 10.000 rpm at 4 ^{o}C for 10 min and the supernatant was separated from pellet. The pellet was re-suspended with 0.1 M Sodium phosphate buffer (Na_2HPO_4 / NaH_2PO_4) pH 7.0 and the cells were sonicated 10 times (1min each time and kept on ice for 2 min). The lysates were centrifuged at 10.000 rpm at 4 ^{o}C for 10 min. The supernatant was used as crude enzyme for detection of β-galactosidase activity. These crude extracts were used for purification steps.

3.2.3 β-galactosidase assay

One mL of reaction solutions were prepared by adding 100 µL of crude enzyme and 50 µL of 60 mM *o*-nitrophenyl-*β*-D-galactopyranoside (*o*-NPG, Sigma, dissolved in phosphate buffer) into 850 µL of 0.1 M sodium phosphate buffer (pH 9.0). The samples

were then incubated for 10 min at 30 °C. The enzyme reaction was stopped by the addition of 500 µL of 2 M sodium carbonate (Na_2CO_3) and 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 30°C.

3.2.4 Protein assay (Lowry method)

The concentration of protein was determined by using the method of Lowry (Lowry et al. 1951) also by using Bovine Serum Albumin (BSA) as the standard and optical density of the reaction mixture was measured at 660 nm by the spectrophotometer. The soluble protein amount was calculated from the standard curve as mg of protein per mL of test samples. The order of experimental procedures is as follows:

500 µL distilled water for blank.

50 µL enzyme solution +450 µL distilled water

↓ transfer 5 mL alkaline solution (Appendix D 2.3)

Incubate at 40 ^{o}C for 15 min.

↓

500 µL FCR (1:1 dilution)

↓

Incubate at room temperature in dark place for 30 min.

↓

Measure at 660 nm.

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

25 mL Nutrient broth was used in 150 mL flask in the absence and presence of 1% lactose throughout the time course experiments.

The strain in stock was firstly cultivated at 30 ^{o}C in shaker water bath at 120 rpm for 15 h (overnight incubation). Then, the inoculations (5 mL) from pre-culture were performed in NB and NB containing 1% lactose for 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, 15 mL of culture were taken, centrifuged (10.000 rpm) at 4 ^{o}C for 10 min. Pellets were collected and 5 mL of sodium phosphate buffer was added, vortexed and then sonicated 10 times (1 min sonication, 2 min kept on ice to avoid heating). The samples were centrifuged and the supernatant was used as crude enzyme.

3.2.6 Effect of temperature on β-galactosidase activity

Crude β-galactosidase of *Enterobacter* sp. 3TP2A was used in order to determine the optimum temperature. 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 (10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 ^{o}C) for 10 min, and then the reaction was stopped with 2 M (Na_2CO_3) followed by reading absorbance at 420 nm.

3.2.7 Effect of pH on β-galactosidase activity

Crude β-galactosidase of *Enterobacter* sp. 3TP2A was used in order to determine the optimum pH . To determine the optimum pH, the β-galactosidase activity was tested within the range of pH from 4.0 to 11.0 in the presence of *o*-NPG (60 mM). 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. Composition and preparation of buffers were defined in Appendix D 1. The reaction solution was incubated for 10 min, and then stopped with 2 M Na_2CO_3 followed by reading absorbance at 420 nm.

3.2.8 Effect of lactose concentration on production of β-galactosidase

25 mL Nutrient Broth cultures inoculated with 1 mL pre-culture of *Enterobacter* sp. 3TP2A strain was used in 150 mL flask containing various concentrations of lactose (0.5, 1, 1.5, 2, 2.5, 3, 3.5 %), incubated for 24 h at 30 ^{o}C in water bath shaker (120 rpm). After centrifuging of the samples (10.000 rpm) at 4 ^{o}C for 10 min. the supernatants were removed and 5 mL of Na_2HPO_4 / NaH_2PO_4 buffer (pH 9.0) was added to each sample, mixed by a vortex and centrifuged. Described in section 3.2.2 .

3.2.9 Purification of β-galactosidase

Purification of β-Galactosidase enzyme was carried out by using the following methods

3.2.9.1 Production of crude extract

350 mL of Medium with 2% lactose was centrifuged for 10 min at 10.000 rpm at 4 ^{o}C and added 12 mL of distilled water, sonicated 10 times (1 min sonication 2 min wait on ice) and centrifuged for 10 min at 10.000 rpm at 4 ^{o}C. As a result, 1 mL out of 14 mL crude extract was saved in freezer.

3.2.9.2 Ammonium sulphate precipitation:

The crude extract with β-galactosidase activity was precipitated by using ammonium sulfate by adding over period of time slowly on ice with a constant stirring up to a final concentration of 70% (w/v). The centrifuged precipitate (10.000 rpm, 10 min, 4°C) was re-dissolved in a small volume of 0.1 M phosphate buffer pH 9.0 and the precipitate was dialyzed against 0.1 M phosphate buffer (pH 9.0) overnight in fridge. Finally, the dialyzed samples were concentrated under nitrogen flow using an ultrafiltration system. They were then used for further purification.

3.2.9.3 Gel permeation chromatography

Sephadex G-75 (Sigma) was used for gel permeation chromatography. To prepare the column; 5 g of sephadex G-75 resins into 150 mL of 0.1 M Tris-acetate (pH 7.0). It was dissolved and allowed to swell for at least 3 hours at 20 °C or 1 hour at 90 °C. Firstly, the column was washed with ultrapure water, then with purified water. Then it was washed with sodium phosphate buffer (pH 9.0) and carefully filled. Once separation of the sample is complete, the gel should be washed with 2 column volumes of 0.2 M NaOH or a solution of non-ionic detergent, rinsed with water, and re-equilibrated with 2-3 column volumes of buffer.

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 buffer. The samples were eluted with the same buffer at a flow rate of 3 mL/ min fractions were collected for determination of enzyme activity (A420 nm) and protein content (A280 nm). The fractions containing β-galactosidase activity were pooled, concentrated by ultrafiltration used for further studies.

Figure3.1: Gel permeation chromatography

3.2.9.4 Affinity chromatography:

The active pool from gel permeation chromatography (2 mL) was applied onto *p*-Aminobenzyl-1-thio-β-D-galactopyranoside (PABTG-agarose) equilibrated with 0.1 M sodium phosphate buffer (pH 9.0). The column was washed with the same buffer until the flow-through was complete. A linear gradient of 0.1–1.0 M NaCl in sodium phosphate buffer was applied at a flow rate of 0.2 mL/min (1 mL fractions). The column was washed with 10 mM and 100 mM sodium borate buffer (pH 9.0). The fractions were measured at 280 nm for determination of amount of protein and enzyme assays were measured at 420 nm. The active pools, which did not bind to the ligand on the affinity column, were dialyzed in sodium phosphate buffer and concentrated by ultrafiltration. The enzyme did not bind to the affinity adsorbent on the column as the enzyme was eluted from the column at very low NaCl concentrations.

3.2.10 Enzyme characterization

3.2.10.1 SDS-PAGE and native PAGE

The PAGE was performed under mild denaturing conditions (0.01% sodium dodecyl sulphate (SDS) using two parallel continuous 7% gels. After electrophoresis, the protein bands were detected either by staining with Coomassie Brilliant Blue (CBB) R-250 or by 6-bromo-2-naphthyl-galactopyranoside (BNG) staining for β-galactosidase activity using the methods described by Gul-Guven et al. (2007). The molecular weight of the subunits was estimated by SDS-PAGE using a vertical gel electrophoresis system. SDS-PAGE was done according to Laemmli (1970). Purified enzyme extracts obtained from each step was mixed with sample buffer [10 mL glycerol, 5 mL 2-mercaptoethanol, 30 mL 10% SDS, 12.5 mL 0.5 M Tris buffer (pH 6.8) and 100 mg bromophenol blue in a total volume of 100 mL]. Reference marker including proteins with various subunit molecular weights [Sigma SDS7B2: α2-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)]. All samples and marker were boiled for

3–5 min and applied to the 7% resolving gel with 5% stacking gel. After running the gel, the proteins were stained by Coomassie Brilliant Blue for 6–8 h.

The native PAGE was incubated in 0.025% 6-bromo-2-naphtyl-β-D-galactopyranoside (BNG) in 0.1M sodium phosphate pH 8.0 containing 10% methanol (v/v). The incubation was allowed to proceed for 30 min at 35 ^{o}C, followed by 2–5 min in a solution 0.125% of Diazo-blue in distilled water. A purple band on gel indicated the presence of enzyme. The reaction was terminated by rinsing and fixing the gel in 7.5% acetic acid, after which the gels were dried.

3.2.10.2 Thermal stability

The purified enzyme (10 µL) was incubated in 1 mL of 0.1 M sodium phosphate buffer pH 9.0 at different times (10 , 20, 30, 60, 90,120,150 and 180 min) at 35 ^{o}C and 45 ^{o}C, 60 mM *o*-NPG was added to each sample. After 10 min, the enzyme activity was stopped by 2 M Na_2CO_3 .

3.2.10.3 pH stability

The pH stability of purified β-galactosidase was investigated in the pH range of 4.0-11.0 using 0.1 M of the following buffer systems: sodium citrate (4.0, 5.0), sodium phosphate (pH 6.0, 7.0, 8.0, 9.0) and NaOH /Glycine (pH 10.0, 11.0) buffer systems. The100 µL buffer solutions mentioned above incubated at 35 ºC for 15 min, then purified enzyme (10 µL) was mixed with different buffer solutions. After 30 min incubation, 900 µL phosphate buffer and 50 µL *o*-NPG were added respectively and incubated at 35 ºC for 10 min, after that the reaction was stopped by 2 M Na_2CO_3 and the β-galactosidase activity was read at 420 nm, under standard assay conditions.

3.2.10.4 The effects of inhibitors

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). 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 Na_2CO_3 after which they were spectrophotometrically measured at 420 nm. The temperature was kept constant during all activity measurements.

3.2.10.5 Effect of metals on β-galactosidase activity

The effects of metal ions (metal chelator EDTA, $CaCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$) on purified β -galactosidase activity were tested. The enzyme was pre-incubated for 15 min at 35 °C with various metal concentrations (1, 2 , 5, 10, 20 mM), and then adding the substrate *o*-NPG, incubated 10 min under standard conditions. Activity of samples without addition of metal ions was taken as 100% activity.

3.2.10.6 Effect of substrate concentration (*o*-NPG) on β-galactosidase activity

Purified β–galactosidase of *E. cloacae* strain was purified as described in section 3.2.9. The effect of *o*-NPG concentrations on β-galactosidase activity was determined. Kinetic constants, K_m and V_{max}, of the β-galactosidase were determined by changing the o-NPG substrate concentrations. *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. Michaelis-Menten plot and Eadie-Hofstee plot were constructed to calculate the K_m and V_{max}.

3.2.11 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.

4. RESEARCH FINDINGS

4.1. Classification of 3TP2A strain as *Enterobacter cloacae* by MALDI

The strain was checked for its purity. For this purpose we decided to re-identify the bacteria which was isolated from petroleum station in Batman in the southeast of Turkey, also used throughout this study. It was found to produce β-galactosidase. The classification results are shown in Table 4.1.

Table 4.1: Classification results of the strain identified by Bruker Daltonik MALDI Biotyper.

Analyte name	Analyte ID	Organism (Best match)	Score value	Organism (Second best match)	Score value
A 1 (+++) (A)	**KH 1**	*Enterobacter cloacae*	2.423	*Enterobacter asburiae*	1.991
A 2 (++) (B)	**KH 2**	*Enterobacter cloacae*	2.257	*Enterobacter ludwigii*	2.039

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

The phylogenetic identification neighbors was originally implemented by the BLASTN (Altschul et al. 1997) program against the database having type strains with genuinely published prokaryotic names and representatives of uncultured phylotypes (Kim et al. 2012). The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm (Myer and Miller 1988), which was applied atthe EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012). Table 4.2 shows 16 S rRNA gene sequencing.

Table 4.2: 16 S rRNA gene sequencing (611 bp) for 3TP2A strain.

TTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGG
TAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGA
AACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATT
AGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGA
TGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCC
TTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCA
ATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGT
CAAGTCGGATGTGAAATCCCCGGGCTCAACC

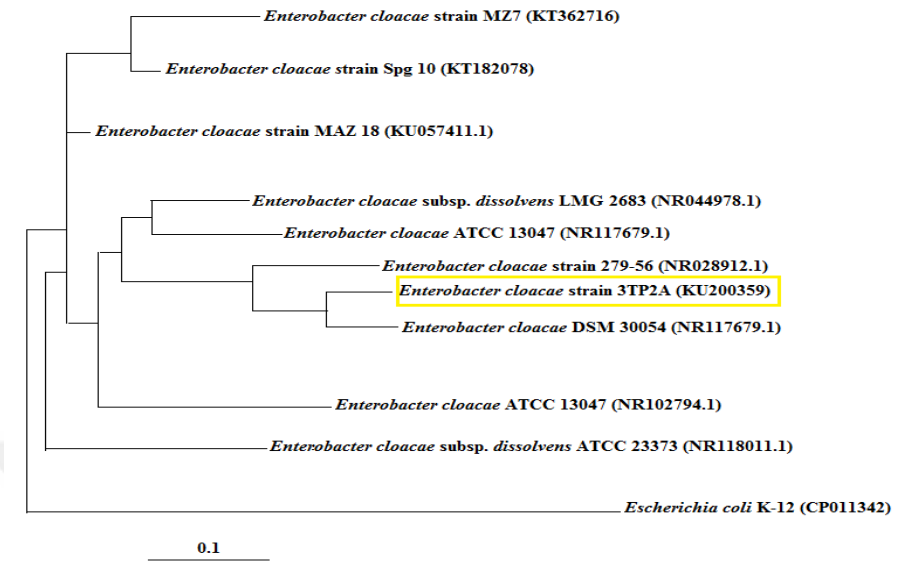


Figure 4.1. Phylogenetic analysis of 16S rRNA gene sequence similarities of *Enterobacter* sp. 3TP2A based on the BLAST result using the neighbor-joining method. Scale bar represents 0.1 substitutions per nucleotide position. The organisms and GeneBank accession numbers of analyzed sequences are given in parenthesis.

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

As β-galactosidase from *Enterobacter* sp. 3TP2A is an intracellular enzyme, it requires cell disruption for its release. However, it is very closely related to the physiological state of the cells and growth conditions applied. For example, cells harvested during logarithmic phase of growth are more easily disrupted than those harvested during stationary phase.

4.3.1. Growth of *Enterobacter* sp. 3TP2A

Using a wide variety of the incubation time starting from 3 h to 72 h as shown in the Figure 4.2.a, the optimum time for growth of *Enterobacter* sp. 3TP2A was found to be 24 h in the medium with 1% lactose concentration. The red line showed optical density of

bacterial growth in Nutrient Broth (NB) with 1% lactose concentration, whereas the blue line showed optical density of bacterial growth in NB without lactose.

a)

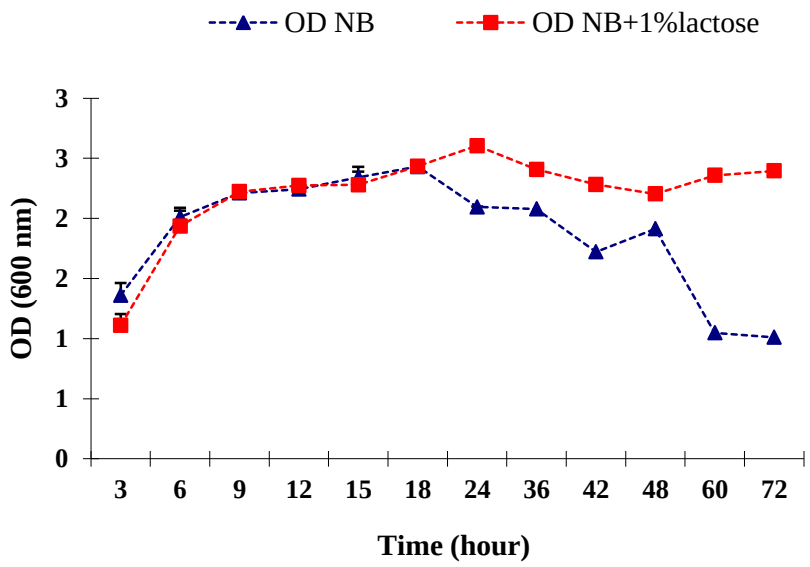
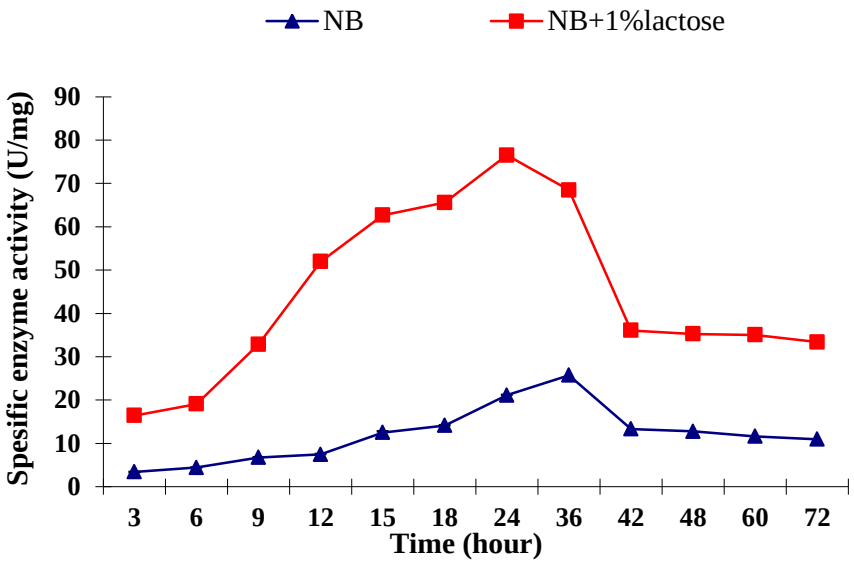


Figure 4.2 Effect of incubation time on growth (a) and the production of β-galactosidase (b) in *Enterobacter* sp. 3TP2A.

b)



4.3.2. Enzyme activity

As shown in Figure 4.2.b, it was found that the optimum incubation time for maximum production of β-galactosidase (intracellular β-galactosidase) by *Enterobacter* sp. 3TP2A was 24 h. A prolonged incubation time beyond this period did not increase the enzyme yield. The red line showed β-galactosidase production at 1% lactose concentration, whereas the blue line showed enzyme production in NB without lactose. It can be clearly seen that the lactose could induce the β-galactosidase in *Enterobacter* sp. 3TP2A.

4.4. Effect of different lactose concentrations on production of β-galactosidase

The effect of different concentrations of lactose on β-galactosidase production was studied. The results showed its maximum β-galactosidase production after 24 hours of incubation at 2% lactose concentration as shown in Figure 4.3.

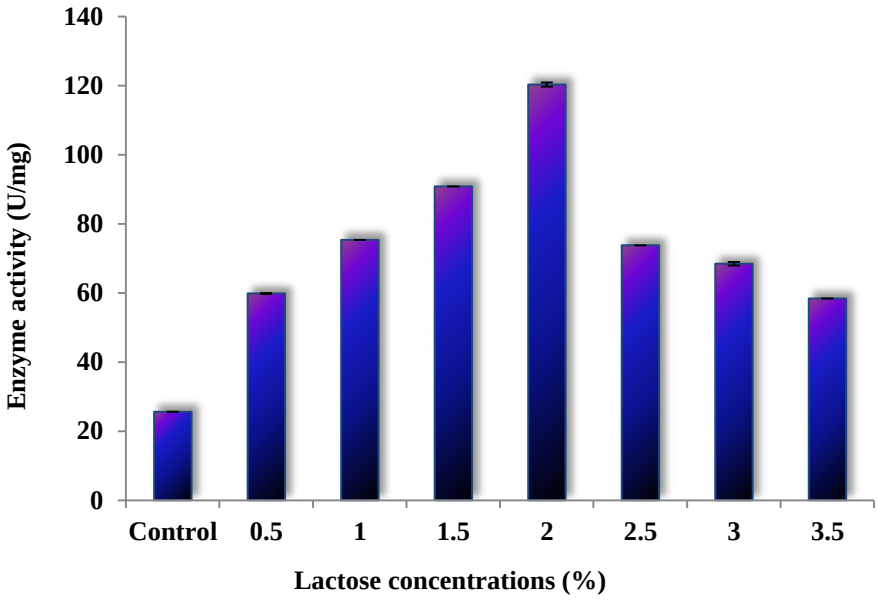


Figure 4.3: Effect of different lactose concentrations on production of β-galactosidase in *Enterobacter* sp.3TP2A

4.5. Effect of temperature on crude β-galactosidase activity

It was observed that the activity of crude β-galactosidase increases at temperature between 10 to 35^{o}C (reaching optimum at 35 ^{o}C) as shown in Figure 4.4. However, enzyme activity sharply decreased with further increase in temperature up to 60 ^{o}C.

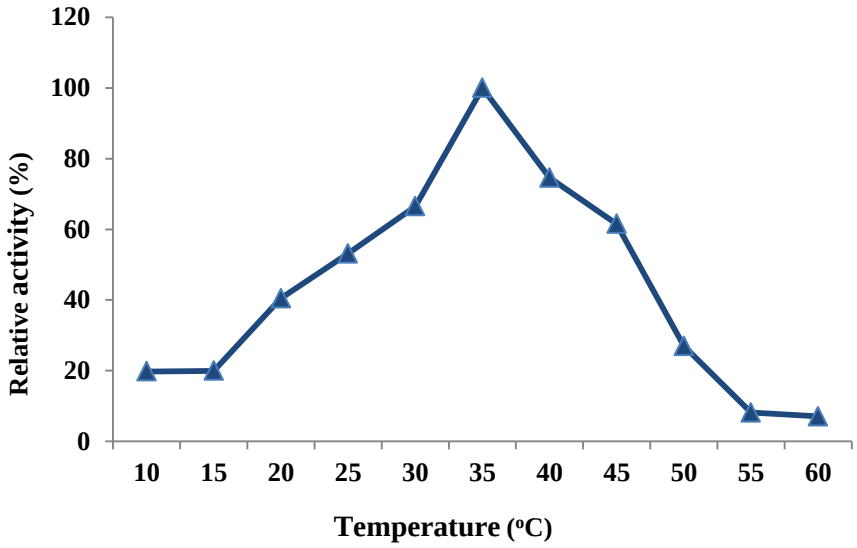


Figure 4.4: Effect of temperature on β-galactosidase activity in *Enterobacter* sp. 3TP2A.

4.6. Effect of pH on crude β-galactosidase

As known, pH influences the velocity of an enzyme-catalyzed reaction. Therefore, it is important to know the effect of pH on activity of an enzyme. The effect of pH on β-galactosidase activity is shown in Figure 4.5, for crude enzyme extracted from *E. cloacae* 3TP2A. As it is seen, the optimum pH for the maximum activity of crude enzyme was found to be pH 8.0-9.0.

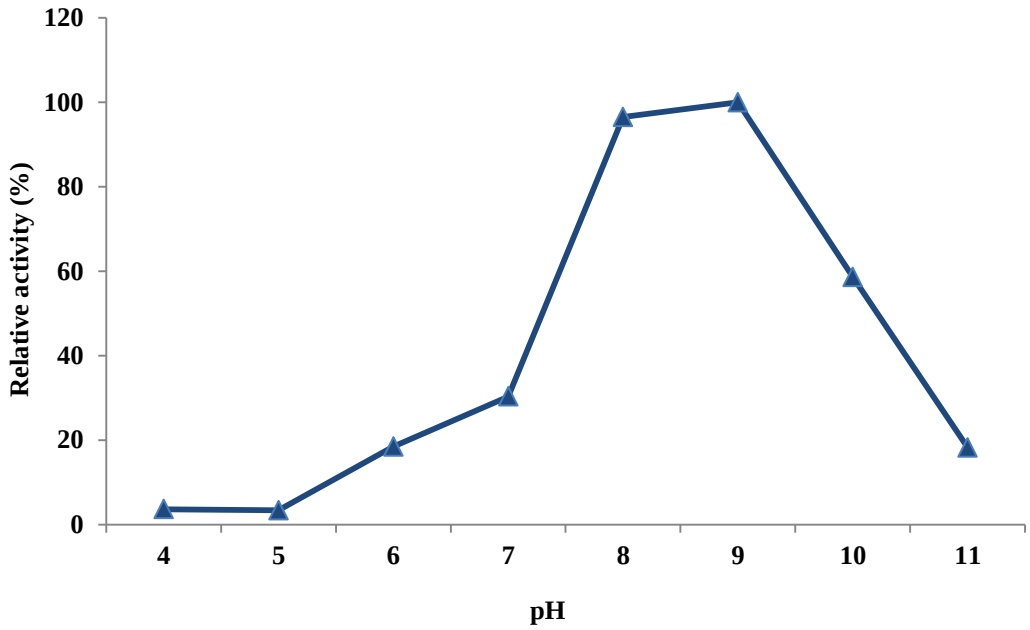


Figure 4.5: Effect of pH on crude β-galactosidase from *Enterobacter* sp. 3TP2A.

4.7. Purification of β-galactosidase

Steps of β-galactosidase purification from *Enterobacter* sp. 3TP2A were as follows (Table 4.3): The crude extract obtained after centrifugation step is regarded as 1 fold purification with a specific activity of 231 units per mg protein. Then, it was precipitated as the clear supernatant by salting out method using ammonium sulfate saturation with 70% concentration followed by dialysis. This step yielded a 3.2 fold purification with a specific activity of 739.1 units per mg protein and the percentage recovery was 42.2 for β-galactosidase. It was then subjected to a purification step using a sephadex G-75 column. The major peaks were eluted with sodium phosphate buffer (0.1 M pH 9.0). By this procedure, β-galactosidase was purified by 17.3 fold with a specific activity of 3991 units per mg protein, while the percentage recovery was 11% (Table 4.3).

Table 4.3: Steps of β-galactosidase purification from *Enterobacter cloacae* 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

4.8. Enzyme characterization

4.8.1. SDS-PAGE and native PAGE

Analysis and characterization of the purified β-galactosidase from *Enterobacter* sp. 3TP2A were carried out by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and native- PAGE. The molecular mass analysis of the β-galactosidase showed a single band of protein, and its molecular mass was found to be approximately 60 kDa (Figure 4.6a). Native gradient PAGE (Figure 4.6b) also showed a single enzyme apparent at the same location.

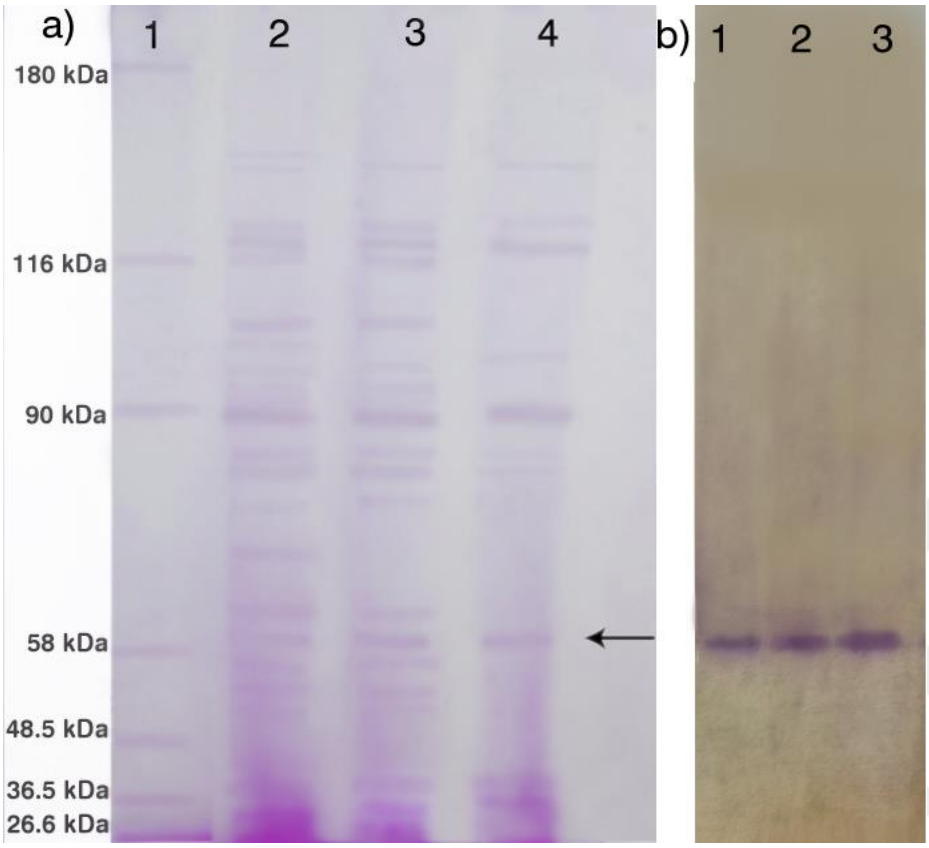


Figure 4.6. SDS-PAGE CBB-staining **(a)** BNG-staining **(b)** analysis of β-galactosidase from *Enterobacter* sp. 3TP2A. **a:** Lane 1, molecular mass markers [Sigma SDS7B2: α2-macroglobulin (180 kDa), β-galactosidase (116 kDa), lactoferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephospate isomerase (26.6 kDa)]; lanes 2, 3 and 4 for CBB-staining are crude extract, ammonium sulphate precipitation/dialysis and sephadex G-75 steps, respectively. **b:** Lanes 1, 2 and 3 for BNG-staining are crude extract, ammonium sulphate precipitation/dialysis and sephadex G-75 steps, respectively.

4.8.2.Thermal 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 ^{o}C and 45 ^{o}C) for different periods of times from 10 min up to 180 min. As shown in Figure 4.7, it was clear

that at temperature 35 °C the enzyme activity was stable under all tested time intervals, whereas at all-time intervals tested the enzyme activity decreased at 45 °C. The β-galactosidase was totally inactivated after 120 min.

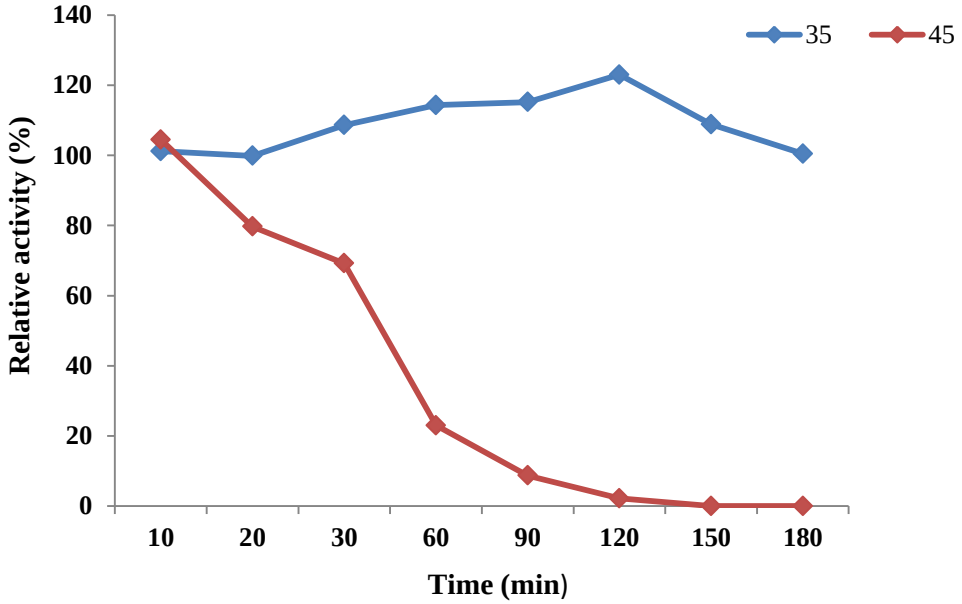


Figure 4.7: Effect of thermal stability on purified β-galactosidase activity from *Enterobacter* sp. 3TP2A.

4.8.3. pH stability

The pH stability profile at 35 °C is shown in Figure 4.8. The enzyme from *E. cloacae* appears to be most stable at pH 8.0 and the stability decreases sharply below and above pH 8.0.

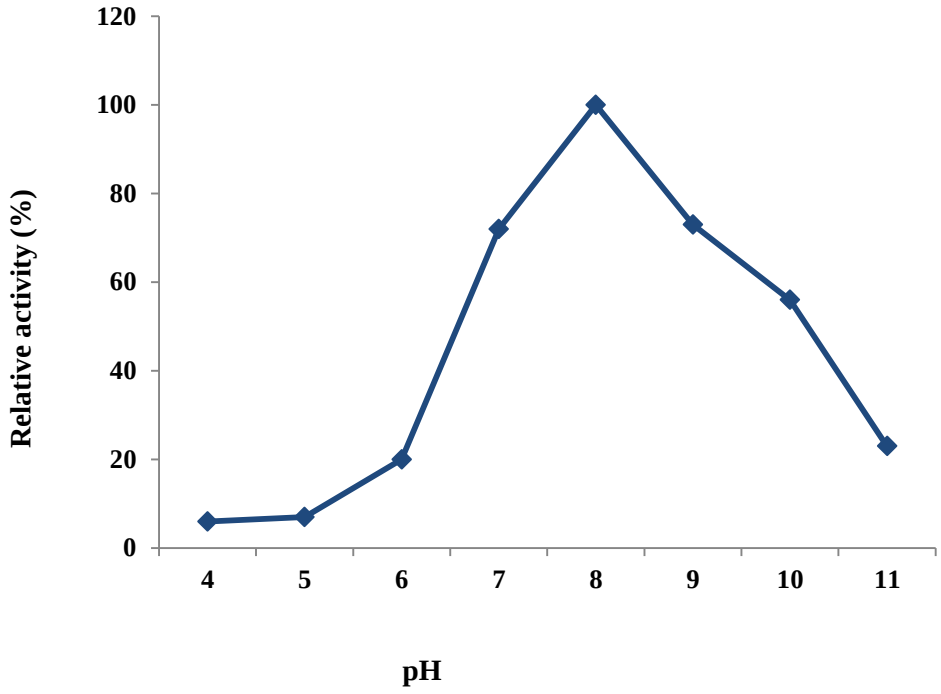


Figure 4.8: Effect of pH on stability of purified β-galactosidase from *Enterobacter* sp. 3TP2A.

4.8.4 Effects of metal ions on purified β-galactosidase activity

Different concentrations of various metals were tested which may have effect on purified β-galactosidase either an activator or inhibitor during the hydrolysis process. The effects of metal ions were examined by adding the chlorides of Ca^{2+} , Mg^{2+}, Zn^{2+} and Cu^{2+} and EDTA at different concentrations to the buffer solution. As can be observed from Table 4.4, EDTA and Cu^{2+} had an inhibitory effect on the β-galactosidase purified from *E. cloacae*. EDTA inhibited the enzyme activity (upto 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 effect enzyme activity to a great extent, causing deactivation of the enzyme at 20 mM (only 16%), while Zn^{2+} at 1, 2 and 5 mM inhibited enzyme activity (32, 27, 8%, respectively). Increase in the concentration of Mg^{2+} causing activation upto 47% and also inhibition by EDTA show that the enzyme is metal-dependent or a metalloenzyme.

Table 4.4: Effect of metal ions on the activity of purified β-galactosidase from *E. cloacae*

Chemicals	Percent activity retained (%)				
	1 mM	**2mM**	**5 mM**	**10 mM**	**20 mM**
Ca^{2+}	94±2.3	95±1.5	100±1.5	105±1.0	84±2.1
Cu^{2+}	4.1±0.1	0	0	0	0
Mg^{2+}	117±1.5	125±2.3	120±0.3	120±1.5	147±2.3
Zn^{2+}	68±1.8	73±0.1	92±2.9	103±2.7	ND
EDTA	32±0.9	29±0.4	27±2.4	25±0.3	24±0.8

ND: Not determined

4.8.5.The effects of some inhibitors on purified β-galactosidase activity

We have also studied the effects of various inhibitors on purified β-galactosidase from *E. cloacae* 3TP2A. 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 at 8 mM with 14% as shown in Table 4.5. The table also shows that the Iodoacetamide had a slight effect on β-galactosidase activity (upto 13%). p-Chloromercuribenzoic acid (PCMB) inhibited the enzymatic activity to a great extent upto approx. 87%.

Table 4.5: Effect of inhibitors on the activity of purified β-galactosidase

Chemicals	Percent activity retained (%)			
	1mM	**2mM**	**4mM**	**8mM**
EtM	0	0	0	0
DTT	100±1.5	102±2.1	97±0.3	108±1.9
β-Mer	102±1.2	99±1.4	ND	114±1.5
Iod	99±3.02	87±4.3	94±1.4	93±1.7
	0.2 mM	**0.4 mM**	**1 mM**	**2 mM**
PCMB	13.7±0.4	13.9±0.5	13.08±0.8	13.3±0.2

ND: Not determined

4.8.6. Effect of substrate concentration (*o*-NPG) on β-galactosidase activity

The purified activity of β-galactosidase was measured at different concentrations of the substrate *o*-NPG. The amount of the substrate varied between (0.05- 4.0 mM) that used in the reaction medium within the assay buffer. A parabolic Michaelis-Menten kinetics is followed for β-galactosidase. The reaction rate increased as increasing substrate (*o*-NPG) concentration. K_m and V_{max} values of β-galactosidase were calculated from the reciprocal plots of substrate. Concentration versus reaction velocity is shown in Figure 4.9. The Lineweaver-Burk plot was linear, suggesting a simple Michealis-Menten kinetics. The K_m was found as 0.104 mM and V_{max} was found as 0.701 (μmol/ min mg).

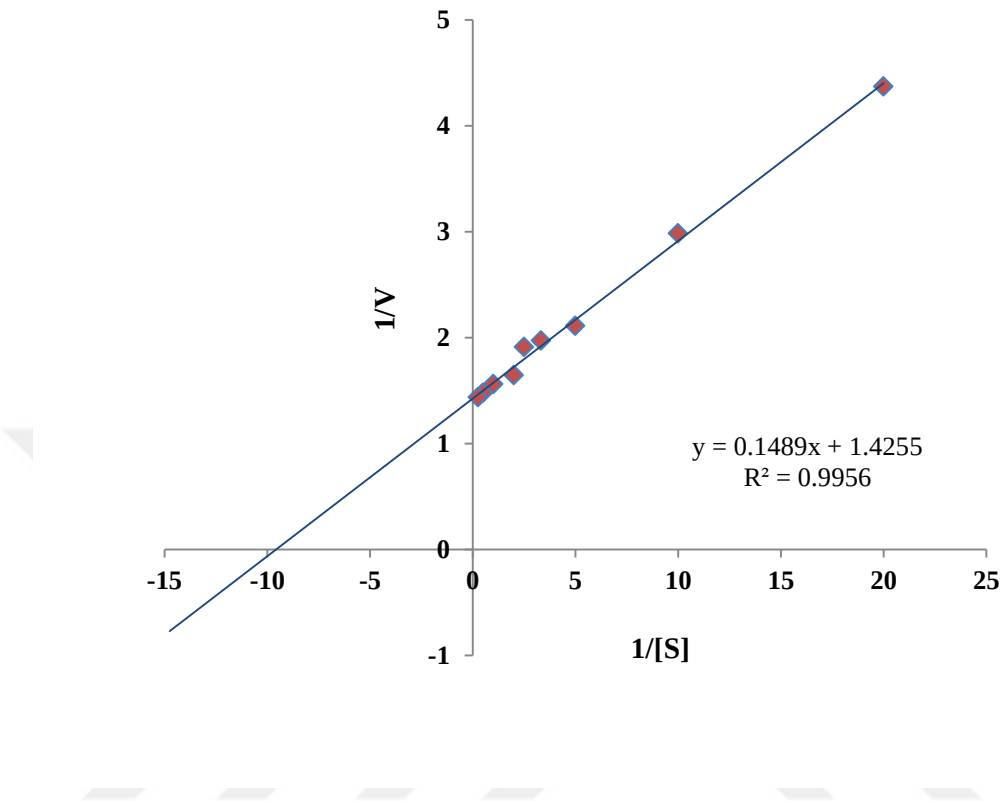


Figure 4.9: Lineweaver-Burk plot of the purified β-galactosidase

4.9. Lactose hydrolysis by the purified enzyme

The experiments of lactose hydrolysis were carried out using lactose under optimized conditions (pH 8.0 and 35 ^{o}C). The results are presented in Figure 4.10. It was found that the time for lactose hydrolysis continues up to 10 h with the reaction catalyzed by purified β-galactosidase.

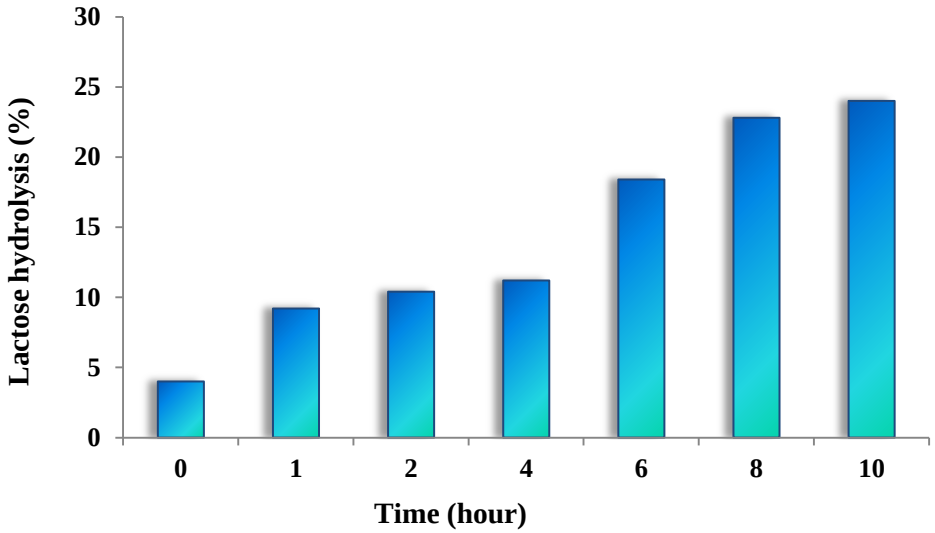


Figure 4.10: Lactose hydrolysis using purified β-galactosidase.

5. DISCUSSION AND CONCLUSION

The aim of this study was to isolate and identify a bacterium that produce β-galactosidase at a large level, as well as purification and characterization of this enzyme. We therefore used a bacterial strain isolated from petroleum contaminated site in Batman, identified both by Bruker Daltonik MALDI Biotyper and by using 16S rRNA sequence analysis (Table 4.1 and Table 4.2). Classification results by both methods showed that the strain was a member of the genus *Enterobacter*, and most likely to be a strain of *E. cloacae*. There was also a study carried out by Sogawa et al. (2011), displaying clinical segregates of the gram-negative rods like *Escherichia coli* and *Pseudomonas aeruginosa* used for evaluating colony to-colony differences of the quick microorganism identification method acting the mixing of MALDI–TOF MS and MALDI BioTyper. Four different colonies grown on the same blood agar plate were designated and were subjected to the identification process arbitrarily. The MALDI–TOF MS mass spectrum patterns of the four colonies were alike in both clinical isolates. The identification index of the MALDI BioTyper software score values were 2.314 to 2.422 (with a mean of 2.371±0.044) for *Escherichia coli* and 2.342 to 2.423 (2.377±0.039) for *Pseudomonas aeruginosa*. On another study (Khalifa et al. 2016) *Enterobacter cloacae* MSR1 exhibited 16S rRNA gene sequences of 99 % homology with *Enterobacter cloacae* subsp. *cloacae* strain DSM 30054T (accession number HE978272), *E. cloacae* strain 5621A (accession number JN644526), *Enterobacter cloacae* strain AB6 (accession number JQ640581) and *E. cloacae* strain E717 (accession number EF059865). As expected there was a substantially low homology (81%) with *Bacillus firmus* strain UST981101-006 (accession number FJ188300), after comparative sequence analysis using BLAST tools in NCBI. The 16S rDNA sequence-based phylogenetic tree analysis revealed that *Enterobacter cloacae* MSR1 belonged to the family *Enterobacteriaceae* and grouped with *Enterobacter cloacae* clade. on the other hand reported by (Toczyłowska-Mamińska et al. 2015). A pure colony of *E. cloacae* was isolated from the gut of termite *Coptotermes curvignathus* and the identity of the strain was confirmed by the PCR and BLAST analysis. The BLAST analysis of the

amplified 16S rDNA (592 bp) gene fragment showed maximum similarity with *E. cloacae* strain DSM 30054 (99%) belonging to genus *Enterobacterioaceae* .

The time course experiments to determine the bacterial growth and the maximum production time of β-galactosidase (intracellular β-galactosidase) by *Enterobacter* sp. 3TP2A in the presence and absence of lactose showed that the incubation time of 24 h was most appropriate both for the growth and for enzyme production, after which the production and the growth decreases by time (see Figure 4.2). The reason for this 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. It might also be due to the denaturation of the β-galactosidase caused by interaction with other components in the medium. These results were agreement with Ghatak et al. (2010) , showing that maximum enzyme production was obtained after 24 h in *Enterobacter cloacae* ST SJ 6 strain. Moreover, Natarajan et al. (2012) claimed that the incubation time for cultivation of *Bacillus* sp. depicts the characteristics of the culture and was also based on the growth rate and enzyme production. There was a profound influence on the activity of enzyme at 48 h. On the other hand, Sumathy et al. (2012) studied β-galactosidase production in *Lactobacillus* sp. isolated from (de Man, Rogosa and Sharpe) MRS broth containing cheese and milk by spread plate method and colonies were observed successfully after 24 hr of incubation at 37 ^{o}C. The result of the present study is in accordance with the study of Priyolkar et al. (1989). They found that the maximum production of β-galactosidase in *Corynebacterium murisepticum* was obtained in the presence of lactose at 30 ^{o}C. Lactose could induce this enzyme while IPTG was ineffective as a gratuinous inducer below 10^{-1} M. Moreover, El-kader et al. (2012) determined that the optimum incubation time for maximum production of β-galactosidase by *Bacillus subtilis* was at 48h. As described by Lu et al (2009), bacterial strains that could synthesize galactooligoSaccharide (GOS) from lactose were isolated from the soil. (Hung et al. (2001). The time course of GaOS production was also monitored during lactose hydrolysis by β-gal I and β-gal III from *Bifidobacterium infantis* HL96.

β-galactosidase of the studied bacterial strain 3TP2A was found to be inducible by lactose. 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 ^{o}C (Figure 4.3). The results were in agreement similar with the results obtained by Khedr et al. (2013), studying the different concentrations of lactose for β-galactosidase production. Nine selected mutants of *Escherichia coli* were grown in Luria Broth (L.B) medium supplemented with 5 g lactose. The results showed that only one mutant had maximum activity after 24 hours of incubation. 5 mutants of *E.coli* reached their maximum production after 27 h.

E.coli is already known to possess lac-operon system where the β-galactosidase gene is regulated and inducible by lactose. Lactose is split into galactose and glucose by the enzyme called β-galactosidase; and the lac Z gene encodes the enzyme in the lac operon of E. coli. Several proteins are synthesized from a single translated message by an operon representing a set of linked genes which are transcribed together. Prokaryotes are allowed by the "polycistronic" message and the operon structure to regulate a common function shared by the genes. The lac operon, in E. coli, is responsible for producing three genes encoding for the involved enzymes in transacetylase (lac A), permease (lac Y), β-galactosidase (lac Z) and lactose metabolism. These enzymes can normally be found in cells at very low concentrations, while they are induced when the sole carbon source is lactose. The low level of RNA transcript and protein decrease when lactose is metabolized. The repressor protein, lactose, controls the lac operon, and this protein is encoded by a regulated "cis-acting" gene called lac I. The lac promoter is retained in the inactivate form by the negative regulation of the lac repressor in the lack of an inducer (Khedr et al. 2013).

The optimum temperature for β-galactosidase enzyme activity was found as 35 ^{o}C (Figure 4.4). The results were in agreement with Lu et al. (2008) . Stated that the optimal temperature for β-galactosidase enzyme from *Enterobacter cloacae* B5 was 35 ^{o}C. The results were similar to those obtained by Lu et al. (2007), who indicated that the optimal

enzyme activity for β-galactosidase from *Enterobacter cloacae* B1 was 37-40 ^{o}C. El-kader et al. (2012) reported that the maximum activity of partially purified β-galactosidase in *Bacillus subtilis* was at 35 ^{o}C. Tryland and Fiksdal (1997) claimed the optimal β-galactosidase activity of *Escherichia coli* to be 44.5 ^{o}C. Princely et al (2013) found the optimum temperature for partial purified intracellular β-galactosidase from *Streptococcus thermophiles* as 40 ^{o}C. However, Ghatak et al. (2013) reported that optimum temperature for the immobilized β-galactosidase from *Enterobacter cloacae* was 50 ^{o}C.

As can be seen in Figure 4.5, the optimum pH for crude β-galactosidase enzyme from *Enterobacter cloacae* was pH 8.0-9.0. Same findings were observed in another study performed by Ghatak et al. (2013) also performed a study on immobilized β-galactosidase from *Enterobacter cloacae,* and found the optimum pH as 9.0. The earlier findings by Ghatak et al. (2010) on β-galactosidase of *Enterobacter cloacae* was also noted as pH 9.0, studied over a pH range of 6.5–10.0. In a study performed by Lu et al. (2007), 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. (2008), the β-galactosidase enzyme from *Enterobacter cloacae* was highly active and stable at the pH range of 6.5-10.5. El-kader et al (2012) showed that the maximal activity of β-galactosidase of *Bacillus subtilis* at pH 7.5-8.5 (optimum at pH 8.0). Natarajan et al. (2012) reported the maximum production of β-galactosidase enzyme from *Bacillus* sp. at pH 7.0. Princely et al. (2013) claimed that the intracellular β-galactosidase from *Streptococcus thermophiles* exhibited maximum activity at pH 7.2.

Steps of β-galactosidase purification from *Enterobacter* sp. 3TP2A were as follows (Table 4.3): The crude extract obtained after centrifugation step is regarded as 1 fold purification with a specific activity of 231 units per mg protein. Then, it was precipitated as the clear supernatant by salting out method using ammonium sulfate saturation with 70% concentration followed by dialysis. This step yielded a 3.2 fold purification with a specific activity of 739.1 units per mg protein and the percentage recovery was 42.2 for β-galactosidase. It was then subjected to a purification step using a sephadex G-75 column. The major peaks were eluted with sodium phosphate buffer (0.1 M pH 9.0). By this

procedure, β-galactosidase was purified by 17.3 fold with a specific activity of 3991 units per mg protein, while the percentage recovery was 11% (Table 4.3). β-galactosidase was also purified from *Enterobacter cloacae* B5 with an overall 26 fold purification and 3.8% yield (Lu et al. 2009). Furthermore, Lu et al. (2007) 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. Natarajan et al. (2012) reported the purification of β-galactosidase from *Bacillus* sp. following acetone precipitation (3.3 fold), sephadex G-75 (9.5 fold and the percentage recovery of 10.14). On the other hand, Bekler et al. (2015) purified the recombinant β-galactosidase enzyme of *Bacillus licheniformis* strain KG9 in *E.coli* following ammonium sulphate precipitation and dialysis (3.1 fold and 14.7 specific activity U/ml/mg protein with the percentage recovery 54.1). Moreover, Chakraborti et al. (2000) purified β-galactosidase from *Bacillus* sp. MTCC 3088, following precipitation by (1.2 fold and 1441 specific activity U/mg protein) and sephadex G-200 column (purified 36.2 fold and 44399 specific activity U/mg protein with the percentage recovery 12.7). El-kader et al. (2012) studied β-galactosidase produced by *Bacillus subtilis* and reported the purification fold of 0.612, specific activity 0.822 U/mg protein and the percentage recovery as 23.02 with ammonium sulphate precipitation 60-90% . Princely et al (2013) purified β-galactosidase from *Streptococcus thermophiles* using ammonium sulphate (1.13 fold and 119.38 specific activity U/mg protein). Moreover, Mozumder et al. (2012) purified the β-galactosidase from *Lactobacillus lactis,* observing 1.3 fold and specificactivity 55.62 U/ml/mg protein with the yield of 16.29 in the step of 80% ammonium sulphate precipitation and dialysis. Moreover Gul-Guven et al. (2007) purified a β-galactosidase from *Alicyclobacillus acidocaldarius* subsp. *rittmannii* following ammonium sulphate precipitation and dialysis (1.90 fold and 1.31 specific activity U/ml/mg protein with a yield 54.9) and gel permeation chromatography (2.53 fold and 1.75 specific activity U/ml/mg protein with the percentage recovery of 36.9).

Figure 4.6 displays results of electrophoretic analysis of β-galactosidase by SDS-PAGE and native PAGE. The examination of the electrophoretic mobility of β-galactosidase shows a single band and its molecular mass to be approximately 60 kDa. The native PAGE also showed a single protein band at the same location. Natarajan et al (2012) found similar result for the molecular weight of the purified β-galactosidase from *Bacillus* sp. using SDS-PAGE analysis determined as 65 kDa. Chakraborti et al. (2000) showed that the relative molecular weight of purified β-galactosidase from *Bacillus* sp. MTCC 3088 was estimated by gel filtration chromatography on sephadex G-200 column to be 484 kDa. Moreover, Saishin et al. (2010) 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. Sumathy et al. (2012) reported a β-galactosidase with a molecular weight of 116 kDa in *Lactobacillus* sp. Lu et al. (2009) found the molecular masses of the β-galactosidase from *Enterobacter cloacae* B5 determined by SDS-PAGE and native gradient PAGE as 119 and 442 kDa, respectively. Hung et al. (2001) studied gene expression in *Escherichia coli* induced by IPTG and analyzed by both SDS-PAGE and non-denaturing PAGE, showing a protein with an apparent molecular mass of approximately 115 kDa. Lu et al (2007) reported the molecular mass of β-galactosidase from *Enterobacter agglumerans* B1 as 120 and 248 kDa determined by SDS-PAGE and native-gradient PAGE, respectively. This indicated that the enzyme was a dimer with two identical subunits. El-kader et al. (2012) determined the molecular mass of partial purified β-galactosidase from *Bacillus subtilis* as 27.3 kDa analyzed by native PAGE.

The thermal stability of the purified β-galactosidase was determined by exposing the enzyme in the absence of substrates to two different temperatures (35 ^{o}C and 45 ^{o}C) for different periods of times from 10 min up to 180 min. As shown in Figure 4.7, it was clear that at temperature 35 ^{o}C the enzyme activity was stable under all tested time intervals, whereas at all-time intervals tested the enzyme activity decreased at 45 ^{o}C. The β-galactosidase was totally inactivated after 120 min. This result was in agreement with Lu et al. (2007) where the β-galactosidase from *E. cloacae* B1 was stable below 37 ^{o}C. Shaishin et al. (2012) studied that the purified β-galactosidase from *Bifidobacterium longum subsp*.

JCM 7052 was stable during 5 h incubation at 35 ^{o}C, but very instable higher than 40 ^{o}C . Similar results were reported by Tryland and Fiksdal (1998) , where the β-galactosidase of *Enterobacter cloacae, Klebsiella pneumoniae* subsp. *pneumoniae, Yersinia intermedia* and *Rahnella aquatilis* were not stable at 44.5^{o}C and the activity at this temperature was less than the activity obtained at 35 ^{o}C. El-kader et al. (2012) found that the partial purified β-galactosidase from *Bacillus subtilis* was stable at 30-35^{o}C, while there was a decrease in the activity of enzyme by increasing temperature up to 60^{o}C. Natarajan et al. (2012) studied on the temperature stability of purified β-galactosidase from *Bacillus* sp. BPTK4 between the temperature ranges of 35-45 ^{o}C, where it retained 100% of its activity at the temperature 45 ^{o}C. Lu et al. (2009) reported the β-galactosidase from *E. cloacae* B5 was stable below 30 ^{o}C. Ghatak et al. (2013) was found immobilized β-galactosidase from *E. cloacae* to be stable at 20 ^{o}C.

The pH stability profile at 35 ^{o}C is shown in Figure 4.8. The purified β-galactosidase from *E. cloacae* 3TP2A in the present study appears to be most stable at pH 8.0 and the stability decreases sharply below and above pH 8.0.The results were in agreement with Ghatak et al. (2013), reporting that the immobilized β-galactosidase from *E. cloacae* remained stable within the pH range of 8.5-9.5. El-kader et al. (2012), stated that the β-galactosidase was stable at pH 8.0-8.5 from *Bacillus subtilis.* Moreover, Lu et al. (2007) found that the β-galactosidase from *E. cloacae* B1 was stable at pH 7.5 and 10.0. Natarajan et al. (2012) determined the pH stability of purified β-galactosidase from *Bacillus* sp. as 7.0.

Different concentrations of various metals were tested which may have effect on purified β-galactosidase either as an activator or inhibitor during the hydrolysis process. The effects of metal ions were examined by adding the chlorides of Ca^{2+} , Mg^{2+}, Zn^{2+} and Cu^{2+} and EDTA at different concentrations to the buffer solution. As can be observed from Table 4.4, EDTA and Cu^{2+} had an inhibitory effect on the β-galactosidase purified from *E. cloacae*. EDTA inhibited the enzyme activity (upto 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 enzyme activity to a great extent,

causing deactivation of the enzyme at 20 mM (only 16%), while Zn^{2+} at 1, 2 and 5 mM inhibited enzyme activity (32, 27, 8%, respectively). Increase in the concentration of Mg^{2+} 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. The results of the present study is in accordance with the study of Lu et al. (2007), showing the effect of some metal ions on β-galactosidase from *E. cloacae* B1. In their study, Cu^{+} completely inhibited the enzyme activity, while Zn^{+} and EDTA exhibited partial inhibition, Ca^{+} and Mg^{+} increased the activity by 21% and 96%, respectively. On the other hand, Lu et al. (2009) reported that Cu^{+} completely inhibited the β-galactosidase activity in *E. cloacae*, while Mg^{+} and EDTA did not affect enzyme activity. Zn^{+} also exhibited inhibition of enzyme activity by 82%. Ghatak et al. (2013) studied the effect of various metal ions on immobilized β-galactosidase from *E. cloacae* shown in Table 5.1.

Table 5.1 Effect of various metals on immobilized β-galactosidase activity in *E.cloacae*.

Metal ions	**Relative activity (%)**			
	0.5 mM	**1 mM**	**5 mM**	**10 mM**
Ca^{2+}	117.62	87.23	50.99	ND
Cu^{2+}	42.86	40.02	37.11	ND
Mg^{2+}	79.08	87.80	60.71	ND
Zn^{2+}	74.34	68.13	54.23	ND
EDTA	105.12	92.03	87.00	47.01

ND: Not determined

From Ghatak et al. (2013)

In addition, Shaishin et al. (2010) tested the metal chlorides on purified β-galactosidase from *Bifidobacterium longum* subsp. JCM, showing that 1 mM Cu^{2+} inhibited the β-galactosidase activity by 50%, while Ca^{2+}, Mg^{2+} and Zn^{2+} did not cause any effects on the enzyme activity. A metal chelator EDTA also did not affect the purified β-galactosidase activity. Turkiewicz et al. (2003) studied the effect some metal ions on β-galactosidase activity from *Pseudoalteromonas* sp. 22b and reported that 5 mM Mg^{+} ion caused only 15% rise in activity, Ca^{+} have no impact on enzyme activity, Zn^{+} ion reduced significantly between 17%-34% of an initial activity, and Cu^{+} even at 1mM completely inactivate the β-galactosidase in 1min. El-kader et al. (2012) tested the effects of various cations on the activity of β-galactosidase from *Bacillus subtilis* : the highest relative enzymatic activities of *Bacillus subtilis* β-galactosidase were observed in the presence of 0.1mM and 1.0 mM of Mg^{2+} (106% and103%, respectively) and 1.0 mM Ca^{2+} decreased the enzyme activity. The activity of β-galactosidase was completely inhibited by the addition of 1.0 and 10.0 mM Cu^{2+} and EDTA, respectively.

Chakraborti et al. (2000) stated that Ca^{2+} had no inhibition effect even at higher concentration (1-10 mM) on β-galactosidase activity, while Mg^{2+} at higher concentration increased the enzyme activity by 41-42%. It has been also observed that Cu^{2+} has a strong inhibitory effect on β-galactosidase even at a lower concentration. Zn^{2+} at 20 mM showed 66 % inhibition in enzyme activity. EDTA up to 25 mM concentration did not inhibit enzyme activity.

We have also studied the effects of various inhibitors on purified β-galactosidase from *E. cloacae* 3TP2A. 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 at 8mM with 14 % as shown in Table 4.5. The table also shows that the Iodoacetamide had a slight effect on β-galactosidase activity (upto 13%). p-Chloromercuribenzoic acid (PCMB) inhibited the enzymatic activity to a great extent upto approx. 87%. The enzyme inhibited by N-Ethylmaleimide shows that at least one essential cystine residue is modified by the reagent. However, it is interesting to note that Iodoacetamide (the reagent alkylating SH group) had little effect on β-galactosidase.

Similar results were also reported by Gul Guven et al. (2011): reagents containing SH groups such as 2-mercaptoethanol and DTT at 8 mM were found to enhance β-galactosidase activity by 22 and 25%, respectively, indicating the presence of a sulfhydryl group in the active site of β-galactosidase. p-Chloromercuribenzoic acid (PCMB) completely inhibited the enzymatic activity. The enzyme was also slightly affected by N-Ethylmaleimide. In another study, β-galactosidase from *Pseudoalteromonas* sp. 22b was strongly inhibited by 4-chloromercuribenzoic acid (PCMB), and slightly activated by 2-mercaptoethanol, also activated by DTT (Turkiewicz et al. 2003). Lu et al. (2009) showed that the Dithiotreitol (DTT) did not affect β-galactosidase of *Enterobacter cloacae*, as also demonstrated in our study. In another study , β-galactosidase from *Cryptococcus laurentii* OKN-4 not effect by p-Chloromercuribenzoic acid (PCMB) (Outsuka et al. 1990).

The purified activity of β-galactosidase was measured at different concentrations of the substrate *o*-NPG. The amount of the substrate varied between 0.05- 4.0 mM that used in the reaction medium within the assay buffer. A parabolic Michaelis-Menten kinetics is followed for β-galactosidase. The reaction rate increased as increasing substrate (*o*-NPG) concentration. K_m and V_{max} values of β-galactosidase were calculated from the reciprocal plots of substrate. Concentration versus reaction velocity is shown in Figure 4.9. The Lineweaver-Burk plot was linear, suggesting a simple Michealis-Menten kinetics. The V_{max} was found as 0.701 (μmol/ min mg) and K_m was found as 0.104 mM. Lu et al. (2007) found relatively similar results for *o*-NPG hydrolyzed by β-galactosidase enzyme from *Enterobacter cloacae* B1: the K_m and V_{max} values were calculated as 0.06 mM and 0.43 mM/min, respectively. Chakraborti et al. (2000) determined kinetic parameters like V_{max} and K_m for purified β-galactosidase from *Bacillus* sp. MTCC 3088 for *o*-NPG by utilizing Lineweaver-Bruk plots. The K_m and V_{max} values measured for *o*-NPG were 6.34 mM and 9351 IU/ml, respectively. The optimum substrate concentration was found to be 24 mM hydrolyzed by β-galactosidase enzyme from *Streptococcus thermophilus*. V_{max} and K_m were calculated from the lineweaver-Burk reciprocal double plot as 2.8U/ml 3.05 mM, respectively (Pricely et al. 2013).

Lu et al. (2009) also determined the K_m and V_{max} values for *o*-NPG hydrolyzed by β-galactosidase from *Enterobacter cloacae* B5, calculated as 0.01 and 2 mM/min, respectively. The results of the present study were in accordance with the study of Sumathy et al. (2012), 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/ mg protein) and K_m as $50x10^{-3}$ (moles/min).

The experiments of lactose hydrolysis were performed using lactose under optimized conditions (pH 8.0 and 35^{o}C). The results presented in Figure 4.10 showed that the time for lactose hydrolysis continued up to 10 h with the reaction catalyzed by purified β-galactosidase from *Enterobacter cloacae* 3TP2A. Similarly, Ghatak et al. (2013) reported hydrolysis of milk lactose using immobilized β-galactosidase from *Enterobacter cloacae* under optimized conditions (pH 9.0, 50 ^{o}C and dilution rate 4.30 h^{-1}) and found that about 46.34% lactose in milk was hydrolyzed at 8 h operation using a continuous packed bed reactor system. In another study Panesar et al. (2007). Reported that 82.2% hydrolysis of milk lactose at 30 ^{o}C, 7 mL h^{-1} with immobilized yeast cells. On another study (Erich et al. 2015) the initial lactose concentration of the milk was 46±0.5 g L^{-1}. The β-galactosidase *Escherichia coli* BL21 exhibited a better performance in lactose hydrolysis from the beginning. After 60 min, 75% of the milk lactose had already been hydrolyzed with *Escherichia coli* BL21 compared to 67% with *Kluyveromyces lactis* (GODO-YNL2). However, the total conversion of lactose after 25 h was most important . In comparison to M1 leaving lactose amounts well below 50mg L^{-1}, GODO-YNL2 still left about 180 mg lactose L^{-1}. Thus, the recommended level for "lactose-free" labelling (below 100mg L^{-1}) was reached with the novel β-galactosidase *Escherichia coli* BL21.

Conclusion

In this study, a mesophilic *Enterobacter* sp. 3TP2A isolated from petroleum station in Batman in the southeast of Turkey was identified and found to produce a high amount of mesophilic β-galactosidase. The lactose was found to increase the β-galactosidase production to a great extent, meaning that this enzyme is inducible. The β-galactosidase enzyme was then purified and characterized. The crude β-galactosidase displayed optimal activity at pH 9.0 and 35 °C and the purified β-galactosidase was more stable at pH 8.0. Following several steps of purification, the enzyme was purified to 17.3 fold after gel permeation chromatography with a yield of approximately 11%. Analysis of substrate specificity with *o*-NPG and lactose showed that the enzyme has a great affinity for its substrate. Thus, the β-galactosidase from *Enterobacter* sp. 3TP2A may have an application potential within dairy processes and need a further study for utilizing in biotechnology:

1-Determination of the most suitable conditions for the mass production of β-galactosidase in *E. cloacae*.

2-To develop commercially usable enzyme, for example immobilized, for the hydrolysis of lactose in food and pharmaceutical industries.

3-To determine whether β-galactosidase produced in the intestinal inhabitant *E. cloacae* may be used for the development of lactose intolerance and/or in dairy industry such as use in whey industry to produce glucose syrup.

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APPENDIX A.

List of chemicals

No	Chemical	Supplier
1	Nutrient broth agar	Merck
2	Nutrient agar	Fluka
3	β-lactose	Sigma
4	*o*-Nitrophenyl-β-D-galacto pyranoside (*o*-NPG)	Sigma
5	Potassium sodium tartrate (Na-K tartrate)	Merck
6	Copper sulphate penta hydrate ($CuSO_4$. $5H_2O$)	Merck
7	Amonium sulphate ($(NH_4)_2SO_4$	Merck
8	Sephadex G-75	Sigma
9	Sodium chloride (NaCl)	Sigma
10	Methanol	Merck
11	Tris-HCl	Sigma
12	Bromophenol blue	Merck
13	Glycerol	
14	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	Merck
15	SDS protein standards	Sigma SDS7B2
16	Acrylamide	Sigma
17	Ammonium per-sulphate	Sigma
18	Tetramethyleethylenediamine(TEMED)	Sigma
19	Comassie brilliant blue	Sigma
20	Glacial acetic acid	Sigma
21	Acetic acid	Sigma
22	Diazo blue	Sigma
23	Sodium carbonate (Na_2 CO_3)	Merck
24	Sodium hydrogen phosphate Na_2HPO_4	Merck
25	Sodium dihydrogen phosphate NaH_2PO_4	Merck
26	p-chloromercuribenzooic acid (PCMB)	Sigma
27	β-mercapto ethanol	Merck
28	Iodoacetamide	Sigma
29	N-Ethylmaleimide	Sigma
30	Dithiothreitol	Sigma
31	Ethylene diamine tetra acetic acid (EDTA)	Sigma
32	Calcium chloride $CaCl_2$	Merck
33	Magnesium chloride $MgCl_2$	Sigma
34	Zinc chloride $ZnCl_2$	Sigma
35	Copper chloride $CuCl_2$	Merck
36	BNG(6-Bromo-2-naphthyl-β-D-galactopyranoside)	Sigma
37	Folin cioaltue reagent (FCR)	Merck

APPENDIX B.

List of equipments

Name of equipment	Model
1.Julabo	SW 22 shaker
2.Grant	Water bath Ltd 6G
3.Sigma	Centrifuge 2K15
4.Microcentrifuge	Micro
5.Autovortex	Mixer SA2
6.Heidolph Instrument	Mixer
7.Magnatic Stirrer Hot Plate	STUART
8.TotalSTAR (Hood)	AV-100
9.pH meter	Metler-Toledo GmbH
10.Soniprep 150	Ultrasonic Disintefrator
11.Autoclave	HMC HIRAYAMA
12. Spectrophotometer	VARIAN UV-visible 50 tables
13.Balance	GEC AVERY
14.Pippet	Eppendorf
15.Refrigrator	Arçelik
16.Biorad	Universal Hood
17.Cobas	C 111

APPENDIX C.

Media

a) Nutrient agar

Bacto beef extract.....3g

Bacto peptone.....5g

Bacto agar.....15g

Suspend 23 grams in 1 liter distilled or deionized water and mix to dissolve.

Completely sterilize at 121-124 ^{o}C for 15 minutes

pH 7.0 +0.2 at 25^{o}C

b) Nutrient broth

Peptone from meat..... 5.0 g

Meat extract..... 3g

Suspend 8 g in 1 litre of demineralized water; if required dispense into smaller containers; autoclave (15 min at 121^{o}C) pH 7.0 +0.2 at 25^{o}C

APPENDIX D.

Buffers and reagents

1. Buffers:

0.1 M buffers were prepared by titration of 0.1 M conjugate base with its 2.0 M respective acid until desired pH value. The types of buffers used in different pH ranges are:

Sodium phosphate buffer (0.1 M): pH 6.0, pH 7.0, pH 8.0, pH 9.0

Citrate buffer (0.1 M): pH 4.0, pH 5.0, pH 6.0

Glycine / NaOH buffer (0.1 M): pH9.0, pH 10.0, pH 11.0

Sodium borate buffer $Na_2B_4O_7 \cdot 10H_2O$:

10 mM and 100 mM dissolved in distilled water.

2. Chemicals:

2.1 *o*-nitrophenol-β-D-galactopyranoside (*o*-NPG) solution:

(60 mM) *o*-NPG solutions were prepared by addition of solid ONPG in appropriate buffer solution (0.1 M) under the assay conditions. ONPG has low solubility therefore it should be well mixed. This solution should be prepared freshly.

Mw ONPG=301.26 g/L

2.2 Sodium carbonate

2 M sodium carbonate is prepared by dissolving 15.9 g in 50 mL distilled water.

M.W Na_2CO_3 =106 g/L

2.3 Alkaline solution

4% Na-K tartrate 4 g dissolved in 100 mL of distillate water.

2% $CuSO_4.5H_2O$ 2 g dissolved in 100 mL of distillate water.

4% Na_2CO_3 4 g dissolved in 100 mL of distillate water.

2.4 NaCl (0.1 M):

0.5844 g NaCl was dissolved in 10 mL distillate water.

2.5 NaOH (0.1 M):

0.4 g NaOH was dissolved in 100 mL distillate water.

3. Stock solution for inhibitors:

β-Mercaptoethanol

0.2 M β-mercaptoethanol dissolved it in1mL sodium phosphate buffer pH 8.0.

Iodoacetamide

0.2 M Iodoacetamide 0.037 g dissolved it in 1mL sodium phosphate buffer pH 8.0.

Dithiothreitol

0.2 M Dithiotrietol 0.0308 g dissolved it in 1mL sodium phosphate buffer pH 8.0.

P-Chloromercuribenzooic acid

50 mM P-chloromercuribenzooic acid 0.018g dissolved it in1mL sodium phosphate buffer pH 8.0.

N-Ethylmaleimide

0.2 M Ethylmaleimide 0.025 g dissolved it in 1mL Ethanol.

4. Stock solution for metal ions:

EDTA

0.4 M EDTA 0.148 g dissolved it in 1mL sodium phosphate buffer pH 8.0 .

$CaCl_2$

0.4 M $CaCl_2$ 0.044 g dissolved it in1mL sodium phosphate buffer pH 8.0.

$MgCl_2$

0.4 M $MgCl_2$ 0.0813 g dissolved it in1mL sodium phosphate buffer pH 8.0.

$ZnCl_2$

0.4 M $ZnCl_2$ 0.054 g dissolved it in 1mL sodium phosphate buffer pH 8.0.

$CuCl_2$

0.4 M $CuCl_2$ 0.068 g dissolved it in1mL sodium phosphate buffer pH 8.0.

5. Electrophoresis solutions:

30 % Acrylamide / 0.8 % Bis-Acrylamide

30 g Acrylamide and 0.8 g Bis-acrylamide were weighed and completed with distillate water 100 mL, stored in a brown flask at 4^oC.

1.5 M Tris-HCl (pH 8.8)

54.45 g Tris was weighed and completed with distillate water 150 mL, it was arranged to pH 8.8 with 1 N HCl after that final volume was completed with distillate water upto 300 mL stored at 4^oC.

0.5 M Tris-HCl (pH 6.8)

6 g Tris base was dissolved in 60 mL distillate water and was arranged to pH 6.8 with 1 N HCl. Final volume was completed with distillate water up to 100 mL, stored at 4^oC.

10 % APS (Amonium per-sulphate) :

0.1 g APS was dissolved in 1 mL distillate water (it should be fresh).

Running buffer

3 g Tris, 14.4 g Glycine, 0.1 SDS were weighed and completed with distillate water upto 1000 mL and stored at 4^oC.

Loading buffer

7 mL 0.1 M Tris-HCl (pH 6.8), 3.6 mL Glycerol, 1.2 mg Bromophenol Blue were dissolved and then added 10 mL distilled water, it was placed in 1 mL Eppendorf tubes and stored at -70 ^{o}C.

10 % SDS

0.1 g SDS was dissolved in 1mL distilled water.

6-Bromo-2-naphthyl-β-D-galactopyranoside (BNG)

0.025 % 6-Bromo-2-naphthyl-β-D-galactopyranoside (BNG) was dissolved in 0.1 M sodium phosphate buffer containing 10% methanol at pH 8.0.

Diazo-blue B:

0.125 % diazo-blue B was prepared in distillate water.

Coomassie brilliant blue dye (1 Liter) :

2 g Coomassie Brillant Blue (0.2 % w/v)

500 mL Methanol (50 % v/v)

100 mL Glasiyal acetic acid (10 % v/v)

400 mL distillate water.

The chemicals that given above were homogenized in a flask with a magnatic stirrer.

Destain solution (Washing solution) (1 Litre)

50 mL Methanol (5 % v/v)

70 mL Glacial acetic acid (7 % v/v)

880 mL distillate water.

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Education

Country	University	College	Dept.	Program	Graduation year
Turkey	Dicle Üniversitesi	Science	Biology	M.sc	2014-2016
Iraq	SALAHADDIN University	Science	Biology	B.sc	2007-2008

Employment History:

Nanakaly Hospital, Ministry of Health in Kurdistan Region Government start from November 2010 to present time.

Rzgary Hospital Erbil city start of the work on 15.09.2009 until 01.10.2010

Employee of PEPSI COLLA world during one year on 2008-2009 (AL-Hayat company for soft drink-mineral water company)

Languages:

1.Kurdish : Mother tongue.

2.English : Good.

3.Arabic : Good