CHARACTERIZATION, CLONING, EXPRESSION AND APPLICATION OF BACTERIAL BETA-GALACTOSIDASE

Onladda Juajun

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

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นางอรลัดา เจือจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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จากการคัดเลือกแบคทีเรีย 8 สายพันธุ์ อาทิเช่น *Pediococcus* spp. และ *Lactobacillus* spp. พบว่า *Bacillus licheniformis* DSM 13 เป็นสายพันธุ์แบคทีเรียที่สามารถสร้างเอนไซม์เบตา-กาแลคโตซิเดสได้สูงสุด โดยเอนไซม์นี้ มีลักษณะเป็นโมเลกุลคู่ สร้างจาก ยีน *lacA* (2055 bp) ซึ่งเมื่อถูกแปลงรหัสเป็นโปรตีน จะมีค่าน้ำหนักโมเลกุลโดยการคำนวณทางทฤษฎีเท่ากับ 78.85 กิโลดาลตัน ยีน *lacA* นี้ถูกโคลนเข้าในพลาสมิด p10HisFLAG ทำให้ได้พลาสมิด pOJBlilacA2 ซึ่งสามารถนำไปแสดงออกให้ได้จำนวนสูงในแบคทีเรีย *Escherichia coli* TOP10 จากนั้นเอนไซม์เบตา-กาแลคโตซิเดสที่ถูกสร้างขึ้นจากวิธีทางพันธุวิศวกรรมนี้ ได้ถูกนำไปทำให้บริสุทธิ์โดยวิธี affinity chromatography ด้วยคอลัมน์ Ni Sepharose 6 fast flow จนบริสุทธิ์ แล้วจึงนำไปวิเคราะห์คุณสมบัติด้านต่างๆ ซึ่งพบว่าเอนไซม์บริสุทธิ์ที่ได้มีค่า specific activity เท่ากับ 271.3 ยูนิตต่อมิลลิกรัมโปรตีน ค่าความเป็นกรดด่างและอุณหภูมิที่เหมาะสมของเอนไซม์เบตา -กาแลคโตซิเดส คือที่ pH 6.5 และ 50 องศาเซลเซียส สำหรับการไฮโดรไลซิสทั้ง *o*-nitrophenyl β-D-galacto-pyranoside (*o*NPG) และ แลคโตส ส่วนค่า K_m ของแลคโตส และ *o*NPG มีค่าเท่ากับ 169.4 และ 13.7 mM ตามลำดับ นอกจาก นี้ยังพบว่า เอนไซม์นี้ถูกยับยั้งอย่างแรงด้วยผลิตภัณฑ์ จากการไฮโดรไลซิส คือ กลูโคสและกาแลคโตส ส่วน monovalent ion (Na^+ และ K^+) ความเข้มข้น 1-100 mM และ divalent cation (Mg^{2+}, Mn^{2+} และ Ca^{2+}) ความเข้มข้น 1 mM สามารถกระตุ้นกิจกรรมเอนไซม์ได้เล็กน้อย ผลการศึกษาพบว่าเอนไซม์นี้มีประสิทธิภาพในการย่อยแลคโตสได้ดี อย่างไรก็ตาม ศักยภาพของปฏิกิริยา transgalactosylation ของเอนไซม์นี้ในการผลิตกาแลคโต-โอลิโกแซคคาไรด์ (GOS) ไม่สูงนัก คือ พบว่ามีการผลิตกาแลคโต-โอลิโกแซคคาไรด์น้อยกว่า 10% (w/w) ของปริมาณน้ำตาลทั้งหมด เมื่อใช้แลคโตสที่ความเข้มข้นเริ่มต้น 200 กรัมต่อลิตร

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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ONLADDA JUAJUN : CHARACTERIZATION, CLONING, EXPRESSION AND APPLICATION OF BACTERIAL BETA-GALACTOSIDASE.
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β-GALACTOSIDASE/ *Bacillus licheniformis* DSM 13/
TRANSGALACTOSYLATION / GALACTO-OLIGOSACCHARIDE

The screening of eight strains of bacteria, such as *Pediococcus* spp. and *Lactobacillus* spp. revealed that *Bacillus licheniformis* DSM 13 could produce the highest amount of β-galactosidase enzyme for lactose hydrolysis. The homodimeric β-galactosidase of *B. licheniformis* DSM 13 was encoded by *lacA* gene (2055 bp), with a translated protein sequence of a theoretical molecular mass of 78.85 kDa. When the *lacA* gene was cloned into p10HisFLAG vector, the recombinant plasmid (pOJBlilacA2) was gained and could be over-expressed in *Escherichia coli* TOP10. The recombinant β-galactosidase could be purified by affinity chromatography using Ni Sepharose 6 fast flow column to become apparent homogeneity. The purified enzyme had a specific activity of 271.3 U/mg proteins. The optimal pH and temperature of β-galactosidase for both *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) and lactose hydrolysis were 6.5 and 50^{o}C, respectively. The K_m values for lactose and *o*NPG were 169.4 and 13.7 mM, respectively; it is strongly inhibited by the hydrolysis products, i.e. glucose and galactose. Monovalent ions（Na^+ and K^+）in the concentration range of 1-100 mM as well as di-valent metal cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}) at the concentration of 1 mM slightly activated the enzyme. This enzyme could be used efficiently for lactose hydrolysis; however, the transgalactosylation potential of this

enzyme for the production of galacto-oligosaccharides (GOS) from lactose was found to be low, with less than 10% (w/w) of total sugars obtained, when the initial lactose concentration was 200 g/L.

School of Biotechnology

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LIST OF ABBREVIATIONS

A	=	Absorbance
μA	=	Microampare
Amp	=	Ampicilin
APS	=	Ammonium persulphate
bp	=	Base pairs
BSA	=	Bovine serum albumin
CE	=	Capillary electrophoresis
CV	=	Column volume
DNA	=	Deoxyribonucleic acid
dNTPs	=	dATP, dCTP, dGTP, dTTP
DTT	=	1,4 Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
GH	=	Glycosyl hydrolase
GOD	=	Glucose oxidase
GOS	=	Galacto-oligasaccharide
g/(L-h)	=	Gram per liter per hour
h	=	Hour
HPAEC-PAD	=	High-performance anion exchange chromatography with pulsed amperometric detection
HPTLC	=	High-performance thin layer chromatography
IEF	=	Isoelectric focusing

LIST OF ABBREVIATIONS (Continued)

IPTG	=	Isopropyl β-D-thiogalactopyranoside
kDa	=	Kilo dalton
(m, μ)L	=	(Milli, Micro) liter
lac	=	Lactose
min	=	Minute
mM	=	Milli molar
Mr	=	Molecular weight
MRS	=	Man rogosa sharp
(c,n) m	=	(Centi, Nano) meter
4MUGal	=	4-Methylumbellriferyl β-D-galactopyranoside
NaOAc	=	Sodium acetic acid
NaOH	=	Sodium hydroxide
^{o}C	=	Degree celcius
OD	=	Optical density
*o*NP	=	*o*-Nitrophenol
*o*NPG	=	*o*-Nitrophenyl *β*-D-galactopyranoside
PCR	=	Polymerase chain reaction
pI	=	Isoelectric point
PMSF	=	Phenylmethylsulphonylfluoride
POD	=	Horseradish peroxidase
rpm	=	Round per minute
s	=	Second

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	=	Super optimal broth with catabolite repression
TAE	=	Tris-acetate-EDTA
TEMED	=	Tetramethylethylenediamine
TLC	=	Thin layer chromatography
U	=	Unit
UV	=	Ultraviolet
kV	=	Kilovolt
v/v	=	Volume by volume
w/v	=	Weight by volume
w/w	=	Weight by weight
X-gal	=	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

1.1 Rational and background

Lactose intolerance or lactose malabsorption is common in the majority adult world's populations because of the decline of the intestinal β-galactosidase (β-gal or commonly known as lactase) activity of the maturing intestine (Turnbull, 2000; Vasiljevic and Shah, 2008) and is often associated with bowel symptoms (Castiglione *et al.*, 2008). The prevalence of lactose intolerance symptoms will be influenced by the population being seen, as adult Asians and Africans have a 90% prevalence (Turnbull, 2000), adults of northern and central Europe and Caucasians in North America and Australia have 5 to 15% of lactose intolerance as well as more over 70% of adults worldwide are lactose malabsorber (de Vrese *et al.*, 2001). These people tend to eliminate milk and dairy products from their diet, and consequently their calcium intake may be compromised (Sanders *et al.*, 2007). Lactase activity is high at birth, decreases in childhood and adolescence, and remains low in adulthood (de Vrese *et al.*, 2001). However, the activity of intestinal lactase in lactose intolerant individuals is usually less than 10% of childhood levels (Buller and Grand, 1990). Most humans, however, quit producing this enzyme in childhood. If these people consume dairy products with lactose, they can develop gastrointestinal symptoms such as abdominal bloating, pain, flatulence, and diarrhea (Sanders *et al.*, 2007). Lactose, sugar in milk, upon ingestion is hydrolyzed by lactase in the brush border membrane of the mucosa of the small intestine into constitutive

monosaccharides (glucose and galactose) which are readily absorbed in the blood system (Vasiljevic and Shah, 2008). Besides lactose maldigestion, crystallization of lactose is a problem in many dairy products such as ice-cream and sweetened condensed milk. Furthermore, disposal of large qualities of lactose-containing by-products from cheese manufactory, whey and whey permeates causes serious environmental problems when high quantities of whey are discharged. Therefore, lactose hydrolyses catalyzed by β-galactosidases is of great importance for the milk and dairy industries.

The enzyme β-galactosidase has been used to hydrolyze lactose in milk to produce lactose free milk products and has become of interest for the production of galacto-oligosaccharides (GOSs) from lactose by transglycosylation reaction (Prenosil *et al.*, 1987). GOS are non-digestible carbohydrates formed by galactose monomers and a unit of terminal glucose and resembling oligosaccharides occurring naturally in human milk can be produced from lactose (Niittynen *et al.*, 2007; Hernandez *et al.*, 2009). The lactose content of bovine milk ranges between 4.4% and 5.2% averaging at 4.8% anhydrous lactose where as 7% of lactose presents in human milk (Gänzle *et al.*, 2008). GOSs have attracted increasing attention because of their presence with different complex structures in human breast milk; therefore, the use of GOS in infant milk formulas is nowadays of great interest (Crittenden and Playne, 1996; Gopal *et al.*, 2001). Qiang et al. (2009) reported that the functional oligosaccharides improve the absorption of water and electrolytes in the small intestine and as a result leads to a reduction in the incidence of diarrhea and its duration. Moreover, they found that fructo- and galacto-oligodaccharide are known for their ability to stimulate the growth of *Bifidobacteria* and *Lactobacilli* and to

inhibit that of potentially pathogenic bacteria, *Enterobacteria*, *Clostridium* and *Salmonella* (Qiang *et al.*, 2009). Among the oligosaccharides, GOSs are one of the top prebiotics produced commercially (Crittenden and Playne, 1996; Sako *et al.*, 1999). GOSs have been considered as prebiotics as they are "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Gibson *et al.*, 2004). Prebiotics have been defined as non-digestible food ingredients that benefit the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that gave the potential to improve host health (Salminen *et al.*, 1998), in addition, they approach to increase the number of beneficial bacteria such as bifidobacteria and lactobacillus in the intestine (Mattila-Sandholm *et al.*, 2002). GOSs are now widely used as low calorie sweeteners, food ingredients, pharmaceuticals and other biologically active compounds and can be synthesized by chemical synthesis, but their preferred mode of synthesis on a preparative scale is by enzymatic catalysis from lactose using an appropriate β-galactosidases (Nakkharat and Haltrich, 2006).

Although the yeast *Kluyveromyces lactis* is still the major commercial source of β-galactosidase because of its dairy environmental habit and outstanding lactose hydrolysis activity, β-galactosidase from *K. lactis* has a major drawback in terms of thermostability (Ganeva *et al.*, 2001), nevertheless β-galactosidase still has been extensively studied in a number of bacteria so far, in addition, *Bacillus* species is one of the most used in this area. *B. licheniformis* is a Gram-positive endospore-forming organism that can be isolated from soils and plant material all over the world (Sneath *et al.*, 1986) and has a long history in the production of proteases, amylases and

lipopeptide surfactants, which are used in detergent production, liquefaction of starch, and oil recovery, respectively (Clerck and Vos, 2004) as well as still has a GRAS (generally regarded as safe) status (Salkinoja-Salonen *et al.*, 1999). *B. licheniformis* strains are listed in the third edition of Food Chemicals Codex (1981) as sources of carbohydrase and protease enzyme preparations used in food processing (de Boer *et al.*, 1994) and never been reported to be pathogenic for either animals or plants and is used extensively for large-scale industrial production of exoenzymes as it can secrete large quantities of proteins of up to 20-25 g/L (Schallmey *et al.*, 2004).

Although, it has been reported about β-galactosidase gene in many published data on different *Bacillus* species, this enzyme is still continuely studying to develop the characteristic for many purposes. Also in this thesis, we described the cloning of β-galactosidase gene from *B. licheniformis* DSM 13 and its expression in *Escherichia coli*. The properties of the recombinant enzyme were also studied. Furthermore, the enzyme hydrolysis and the GOS production were verified.

1.2 The objectives and scope of the research

The aim of this research was to develop enzyme β-galactosidase for the production of a prebiotic, galacto-oligosaccharide (GOS). This could be achieved through the following objectives:

1. To screen for suitable bacteria producing β-galactosidase.

2. To select the most appropriate strains for further study by partial characterization of the crude enzyme.

3. To purify and characterize the properties of the recombinant enzyme (such as optimal pH and temperature, pH and temperature stability, and kinetics).

4. To clone β-galactosidase genes and express in *Escherichia coli*.

5. To utilize the recombinant enzyme to synthesize GOS from lactose.

6. To analyze the properties of GOS such as size and amount.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of β-galactosidase

The enzyme β-D-galactoside-galactohydrolase (EC 3.2.1.23) commonly known as β-galactosidase or lactase. It is involved in lactose utilization by hydrolyzing lactose into glucose and galactose (Figure 2.1) in many species. Hydrolysis of lactose can be carried out by heating at low pH (acid hydrolysis) or by enzymic catalysis with the enzyme β-galactosidase either free in solution or immobilized by one of the several enzyme immobilization methods which are abundant in the literature (Gekas and Lopez-Leiva, 1985).

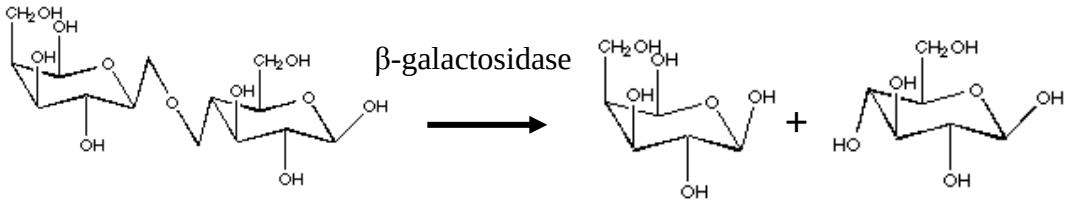


Figure 2.1 Mechanism of lactose hydrolyzing into glucose and galactose by β-galactosidase (adapted from: www.biochem.arizona.edu).

It is widely distributed in nature and many studies have been reported on the physiology and regulation of the enzyme from different sources, including plants, animals and various of microorganisms such as bacteria, yeasts, molds, and archaebacteria (Table 2.1) (Nagy *et al.*, 2001; Kang *et al.*, 2005; Lu *et al.*, 2007). The

application of the enzyme β-galactosidase in bioprocess has been achieved exclusively with microbial enzymes, which have long been used for the hydrolysis of lactose for increasing the digestibility of milk or for the improving the properties of dairy products. Although many β-galactosidase-producing microorganisms are known (Table 2.1), however, there are few microbial sources of β-galactosidases used in manufacture. Some of these microorganisms are used for the commercial enzyme preparations as shown in Table 2.2. Most of these are of fungal origin, such as *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Aspergillud niger*, *and Aspergillus oryzae*. These fungi were chosen mainly because they can inexpensively produce the β-galactosidase and are generally recognized as safe (GRAS) as food additive (Nakayama and Amachi, 1999). The major industrial β-Galactosidase from *Kluyveromyces lactis* is one of the most used enzymes for manufacturing milk and dairy products (Kim *et al.*, 2004b; Kim *et al.*, 2006; Klewicki, 2006). During the past decade, another potential application of the enzyme β-galactosidase has been developed especially for prebiotic production via the transgalactosylaion. β-Galactosidase is the most frequently used reporter for studying gene expression in bacteriology, molecular and cell biology (Di Lauro et al., 2008). Among its many advantages is the ability to function in a wide range of bacteria, the availability of substrates for enzymatic assays and genetic screens, and the variety of tools that have been developed for constructing fusions to the *lacZ* gene of the *lac* operon in *E. coli* (Goulian and van der Woude, 2006). The enzyme's function in the cell is to cleave lactose to glucose and galactose so that they can be used as carbon/energy sources. Although, the enzyme most studied is from *Escherichia coli* encoded by the *lacZ* gene, which serves as a model for understanding the catalytic mechanism of β-galactosidase action, nevertheless, it is no considered suitable for use in foods owing

Table 2.1 Some microorganisms produced β-galactosidase enzyme.

Type of Microorganisms	**Example**
Yeasts	*Candida pseudotropicalis*, *Brettanomyces anomalus*, *Cryptococcus laurentii*, *Kluyveromyces* (*Saccharomyces*) *lactis*, *K. fragilis*, *K. marxianus*, *Lipomyces* sp., *Wingea roberstsii*
Molds	*Alternaria altenata*,*Alternaria palmi*, *Aspergillus awamori*, *A. cellulosae*, *A foetidus*, *A. niger*, *A. flavus*, *A. oryzae*, *A phoenicis*, *A terreus*, *Curvularia inargualis*, *Chaetomium globosum*, *Fusarium* sp., *Geotricum candida*, *Mucor pucillus*, *Mucor miehei*, *Neurospora crassa*, *Penicillum* sp., *Rhizopus* sp., *Scopulariopsis* sp., *Sclerotium tuliparum*, *Thermomyces lanugimosus*, *Torula thermophila*, *Trichoderma viride*
Basidiomycetes	*Corticium rolfsii*, *Culvularia inaequalis*, *Pycnoporus cinnabarinus*, *Sporobolomyces singulalis*
Bacteria	**Gram-negative** *Aeromonas cavie*, *Agrobacterium rediobacter*, *Bacteroides polypragmatus*, *Enterobacter dysenteriae*, *Thermotoga maritima*, *Thermus* sp. *Treponema phagedenis*, *Xanthomonas campestris*, *X. manihotis* **Gram-positive** *Arthobacter* sp., *Bacillus acidocaldarius*, *B. coagulans*, *B. macerans*, *B. megaterium*, *B.subtilis*, *B. bifidum*, *B. sterothermophilus*, *Bifidobacterium* sp., *B. longum*, *Clostridium acetobutylicum*, *Corynebacterium murisepticum*, *Lactobacillus delbruckii* subsp. *bulgaricus*, *L. casei*, *L. helveticus*, *L. murinus*, *L. plantarum*, *L. sake*, *Lactococcus lactis*, *Leuconostoc citrovorum*, *L. lactis*. *Streptococcus salivarius* subsp. *thermophilus*, *S. thermophilus*, *Clostridium thermosulfurigenes* **Actinomycetes** *Actinomyces viscosus*, *Nocardia* sp., *Saccharopolyspora rectivigula*, *Streptomyces lividans*, *S. venezuelae*, *S. violaceus*
Archaebacteria	*Caldariella acidophila*, *Sulfolobus solfataricus*, *Pyrococcus woesei*, *Haloferax alicantei*

Adapted from Nakayama and Amachi (1999).

Table 2.2 Some commercial sources of β-galactosidase.

Microorganism	Trade name	Company
Bacteria		
Bacillus sp.	Novozyme 231	Novozymes A/S, Bagsvaerd, Denmark
Escherichia coli	β-galactosidase	Sigma-Aldrich, UK
	-	CF Boeringer GmbH, Mannheim, Germany.
		Worthington Biochemical Corp., Freehold,
	-	USA.
Yeast		
Kluyveromyces lactis	Maxilact	DSM Food Specialties, Delt, The Netherlands
	Lactase	SNAM Progetti, Italy
	β-galactosidase	Sigma-Aldrich, UK
Kluyveromyces fragilis	β-galactosidase	Sigma-Aldrich, UK
Kluyveromyces marxianus	Lactozyme	Novozymes A/S, Bagsvaerd, Denmark
Kluyveromyces sp.	Lactase NL	Enzyme Development Corp., NY, USA
Candida pseudotropicalis	Neutral lactase	Pfizer, Milwaukee, USA
Fungi		
Asppergillus niger	Sumylact	Sumitomo Xhemical. Japan
	Lactase	Valio Lavoratory, Finland
Aspergillus oryzae	Fungal lactase	Enzyme Development Corp., NY, USA
	Biolactase	Biocon (US) Inc., Lexington, USA
	Lactase 2214C	Rohm, Darmstadt, Germany
	β-galactosidase	Sigma-Aldrich, UK

(Gekas and Lopez-Leiva, 1985; Panesar *et al.*, 2006).

to toxicity problems associated with the host coliform (Mahoney, 1998). The enzymatic hydrolysis of lactose can be achieved either by free enzymes, usually in a batch fermentation process, or by immobilized enzymes, or even by immobilized whole cells containing enzyme (Gekas and Lopez-Leiva, 1985). The synthetic compound *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) is also recognized as a substrate and cleaved to yield galactose and *o*-nitrophenol which has a yellow color. When *o*NPG is in excess over the enzyme in a reaction, the production of *o*-nitrophenol per unit time is proportional to the concentration of β-galactosidase; thus, the production of yellow color can be used to determine enzyme concentration. β-Galactosidase is the most frequently used reporter for studying gene expression in bacteriology, molecular and cell biology (Di Lauro et al., 2008). Among its many advantages is the ability to function in a wide range of bacteria, the availability of substrates for enzymatic assays and genetic screens, and the variety of tools that have been developed for constructing fusions to the *lacZ* gene of the *lac* operon in *E. coli* (Goulian and van der Woude, 2006). The enzyme's function in the cell is to cleave lactose to glucose and galactose so that they can be used as carbon/energy sources. The synthetic compound *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) is also recognized as a substrate and cleaved to yield galactose and *o*-nitrophenol which has a yellow color. When *o*NPG is in excess over the enzyme in a reaction, the production of *o*-nitrophenol per unit time is proportional to the concentration of β-galactosidase; thus, the production of yellow color can be used to determine enzyme concentration. There are many advangetages from β-galactosidase-catalyzed reactions application such as (1) for lactose intelerance people, whose low digestibily of lactose in milk product because of lack of intestinal lactase (2) to reduce the high lactose content in nonfermented milk, often causes lactose crystallization during

preservation, normally, the product must not be greater than 10 µm in length, or they would make the product sandy (3) the whey and whey permeate, which are the lactose containing by-products from cheese manufacturing, have pose a serious problem as environmental pollutants, although these are potentially good carbohydrate sources for foods and animal feeds, consequently these problems can be circumvented by decomposing lactose in the products by the action of β-galactosidase (Gekas and Lopez-Leiva, 1985; Nakayama and Amachi, 1999).

2.2 β-Galactosidase structural classification

Glycoside hydrolases (GHs) are widely distributed Enzymes that hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Coutinho and Henrissat, 1999). The glycosyl hydrolases enzymes have been studied and classified including many microbial β-galactosidases. These enzymes also have been sequenced and have been classified on the basis of sequence similarities into at least four categories according to Henrissat's classification (Henrissat, 1991). At present, an inspection of the carbohydrate-active enzyme classification; CAZY (http://www.cazy.org/) shows that β-galactosidases belong to glycoside hydrolase (GH) families GH1, 2, 35, and 42; these families group enzymes which hydrolyze the substrate by following a retaining mechanism of hydrolysis in which the products have the same anomeric configuration as the substrate (Henrissat, 1991; Shipkowski and Brenchley, 2006; Di Lauro *et al.*, 2008).

2.2.1 Glycosyl hydrolase family 1

The family 1 β-galactosidases include the enzyme of an archaeon, *Sulfolobus solfataricus*. This enzyme is essentially a β-glycosidase that can efficiently hydrolyze

β-galactosidases as well as the other β-glycosides. The *S. solfataricus* enzyme, consisting of 489 amino acid residues, differs in subunit size from the family 2 of enzymes and exhibits no sequence similarity to that family. Interestingly, the lactase, a mammalian β-galactosidase playing a central role in lactose digestion in the small intestine, belongs to family 1. Thus, it might be possible that family 2 β-galactosidases evolved to the animal β-glucuronidases whereas family 1 enzyme evolved to the digestive lactase enzymes functioning in the mammalian intestines.

2.2.2 Glycosyl hydrolase family 2

β-Galactosidases belong to this glycosyl hydrolase family, typified by the *E. coli lacZ* β-galactosidase, consist of a large subunit protein of approximately 1,000 amino acids and show very high sequence similarities to the animal β-glucuronidases, suggesting that the β-galactosidases of family 2 have a close evolutionary relationship with the β-glucuronidases. Many β-galactosidases of this family require monovalent and divalent metal ions for maximum activity.

2.2.3 Glycosyl hydrolase family 35

GH Family 35 contains β-galactosidases (BGALs; EC 3.2.1.23), which catalyse the hydrolysis of terminal β-galactosyl residues from carbohydrates, galactolipids, and glycoproteins. These enzymes are widespread in bacteria and eukaryotes. Found throughout the higher plant body, they are believed to play key roles in medication of cell wall components during fruit ripening (Pressey, 1983; Carey *et al.*, 1995; Smith *et al.*, 1998), loosening of the cell wall during growth (Dopico *et al.*, 1989; Sekimata *et al.*, 1989). The completion of the *Arabidopsis thaliana* genome sequencing project has provided an ideal opportunity to examine

the gene structure and evolutionary patterns for all members of GH Family 35 hydrolases within are presentative (model) angiosperm (Ahn *et al.*, 2007). The bacteria, *Xanthomonas manihotis* which is a Gram-negative phytopathogenic bacterium, *Arthobacter* sp., *Bacillus circulans*, and *Aspergillus niger* produce β-galactosidases with strong sequence similarities to animal β-galactosidases belonging to family 35 of glycosyl hydrolases.

2.2.4 Glycosyl hydrolase family 42

The β-galactosidase from an extreme thermophile, *Thermus thermophilus* A4 (A4-β-Gal), is thermostable and belongs to the glycoside hydrolase family 42 (GH-42). As the first known structures of a GH-42 enzyme, Hidaka *et al.* (2008) determined the crystal structures of free and galactose-bound A4-β-Gal at 1.6^{o}A and 2.2^{o}A resolution. It was found that A4-β-Gal forms a homotrimeric structure resembling a flowerpot. The putative catalytic residues of A4-β-Gal (Glu141 and Glu312) superimpose well with the catalytic residues of *E. coli* β-galactosidase. The environment around the catalytic nucleophile (Glu312) is similar to that in the case of *E. coli* β-galactosidase, but the recognition mechanism for a substrate is different (Hidaka *et al.*, 2002). Most of β-galactosidases belong to glycosyl hydrolase family 2, whereas those from thermophilic, psychrophilic and halophilic microorganisms belong to GH gamily 42 (Ohtsu *et al.*, 1998; Sheridan and Brenchley, 2000; Shipkowski and Brenchley, 2006).

2.3 The hydrolytic reaction of β-galactosidase

During the normal hydrolytic reaction, β-galactosidase hydrolyze lactose and transfer galactose to the hydroxyl group of water, acceptor molecule, resulting in the

liberation of galactose and glucose (Alliet *et al*., 2007). A model with competitive product inhibition by galactose, assuming that the glucose molecule is the first to leave the active site of the enzyme, leave a covalent galactosyl-enzyme complex for further hydrolysis. The reaction mechanism for lactose hydrolysis can be described as follows, assuming that the process described by Equation (2) rapidly reaches its equilibrium state (Shang-Tian Yang, 1989):

$$E + lac \underset{K-1}{\overset{K1}{\rightleftharpoons}} E : lac \xrightarrow{K2} E\text{–}gal + glu \qquad (1)$$

$$E\text{–}gal \underset{K-3}{\overset{K3}{\rightleftharpoons}} E + gal \qquad (2)$$

where lac; gal; glu; E; E : lac, and E-gal are lactose, galactose, glucose, enzyme, noncovalent enzyme-lactose complex, and covalent galactosyl-enzyme complex, respectively; k1, k-1, k2, k3, and k-3 are primary reaction rate constants. The hydrolytic activity has been applied in the food industry for decades for reducing the lactose in milk products, which presents one possibility to decrease the problem of lactose intolerance, prevalent in more than half of the world population (Lu *et al.*, 2007).

2.4 Transgalactosylation of β-Galactosidase

It is known, however, that the enzymatic hydrolysis of lactose occurs at low lactose concentrations and that oligosaccharide production by the transgalactosylation reaction increases with increasing lactose concentration, low water content and high temperature (Chen *et al.*, 2003; Gaur *et al.*, 2006). β-galactosidases is able to transfer galactose to the hydroxyl groups of the galactose or the glucose moiety in lactose, resulting in the production of galacto-oligosaccharides (GOS) (Alliet *et al*., 2007).

Transgalactosylation is the method which comprises of 2 reactions, intramolecular and intermolecular reaction (Splechtna *et al*., 2006).

1. Intramolecular transgalactosylation or direct galactosyl transfer to D-glucose yields regioisomers of lactose. The glycosidic bond of lactose is cleaved and immediately formed again at a different position of the glucose molecule before it diffuses out of the active site.

2. Intermolecular transgalactosylation is the method which di-, tri-, tetra-, or pentrasaccharides and higher oligosaccharides are produced. The transgalacto-sylation reaction can be described as the following:

$$E + lac \underset{K-1}{\overset{K1}{\rightleftharpoons}} E : lac \xrightarrow{K2} E\text{–}gal + glu \quad (1)$$

$$E\text{–}gal \underset{K-3}{\overset{K3}{\rightleftharpoons}} E + gal \quad (2)$$

$$E\text{–}gal + glu \underset{K-4}{\overset{K4}{\rightleftharpoons}} E+D \quad (3)$$

$$E\text{–}gal + lac \underset{K-5}{\overset{K5}{\rightleftharpoons}} E + T \quad (4)$$

where lac, gal, glu, E, E : lac, E–gal, D and T are lactose, galactose, glucose, enzyme, noncovalent enzyme-lactose complex, covalent galactosyl-enzyme complex, galactosyl–glucose disaccharides and galactosyl–glucose trisaccharides, respectively, respectively; k1, k-1, k2, k3, k-3, k4, k-4, k5 and k-5 are reaction rate constants. In this model, galactose binds to the free enzyme to make the galactosyl-enzyme complex for further transgalactosylation reactions with glucose or lactose as the acceptors, but does not bind to the galactosyl-enzyme complex. Glucose, however, acts as a better acceptor for transgalactosylation reactions, reacting only with the

galactosyl-enzyme complex to form galactosyl–glucose disaccharides. Assuming that the reactions described by Equations (2)-(4) are rapidly equilibrated (Kim *et al.*, 2004b).

2.5 Purification and characterization of β-galactosidase

The intracellular β-galactosidase enzymes from wild type strains of microorganisms including recombinant microbes have been used the machines such as sonicator, French press and/or homogenizer to breakdown the cells to release β-galactosidases enzymes out of the cells. The enzyme purification requires one and/or more than one step to achieve the desire level of product purity. In addition, it needs the suitable condition to transfer the product to perform the next step. β-galactosidases has been isolated from the cell extracts of microbes using the purification method, for example, anion exchange chromatography, superose gel filtration chromatography, sephadex gel filtration chromatography, and hydrophobic interaction chromatography. The purification of β-galactosidase from *Lactobacillus reuteri* L103 and L461 was isolated using a purification protocol based on ammonium sulfate precipitation, hydrophobic interaction chromatography and affinity chromatography on *p*-aminobenzyl thiogalactoside agarose, the results of the two purified enzyme were 16- and 50-fold, respectively (Nguyen *et al.*, 2006). The *Talaromyces thermophilus* β-galactosidase was purified using DEAE sepharose columnand affinity chromatography column (Nakkharat and Haltrich, 2006). Intracellular β-galactosidase from *Penicillium chrysogenum* NCAIM 00237 was purified by procedures including precipitation with ammonium sulfate, ion-exchange chromatography on DEAE-sephadex, affinity chromatography, and chromatofocusing. These steps resulted a purification of 66-fold, a yield of about 8%, and a specific activity of 5.84 U/mg protein (Nagy *et al.*, 2001).

There are various properties of the β-galactosidase enzymes which are from the different sources of microorganisms. They are multimeric β-galactosidase enzyme of bacteria, dimeric in *Sterigmatomyces elviae*, *Enterobacter agglomerans* B1, and *Lactobacillus reuteri* and tetrameric in *E. coli*, *Bacillus macerans*, *Lactobacillus helveticus*, *Penicillium chrysogenum*, and *Bifidobacterium infantis* as well as in some fungi and ascomycete are monomeric such as *Aspergillus oryzae*, and *Talaromyces thermophilus*, respectively, (Kim *et al.*, 2004b; Nakkharat and Haltrich, 2006; Lu *et al.*, 2007). The pH and temperature optimum of β-galactosidase enzymes are also different from various microorganisms, for example, the pH and temperature optimum of the activity of β-galactosidase of *P. chrysogenum* were about 4.0 and 30°C, respectively (Nagy *et al.*, 2001) and the optimal pH and temperature for *o*NPGal hydrolysis of *Enterobacter agglomerans* B1 were 7.5-8.0 and 37-40^{o}C, respectively (Lu *et al.*, 2007). The molecular mass of the enzyme has been determined by SDS-PAGE, Native-PAGE, and active staining. The Km and Vmax values have been determined using *o*NPG and lactose as substrate and some kinetic parameters have been shown in Table 2.3. The end product inhibition, the different glucose and galactose concentration exhibit to inhibit the hydrolysis both of *o*NPG and lactose as substrate, for instance, galactose acted as an inhibitor at low concentrations of galactose and lactose, but did not inhibit the activity of β-galactosidase at high concentrations of galactose (above 50 mM) and lactose (above 100 mM). The addition of glucose at concentrations below 50 mM resulted in an increased reaction rate (Kim *et al.*, 2004b). The inhibition type, galactose and glucose, as well as constants of microbial β-galactosidase have been shown in the Table 2.4. Besides, the enzyme activity was stimulated by a lot of divalent and monovalent

Table 2.3 Kinetic parameters of microbial β-galactosidase.

Source of Microorganisms	**Substrate**	**K_m (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (s^{-1} mM^{-1})**	**Reference**
Aspergillus aculeatus	*p*NP-Gal Lactose	3.3 35	932 254	284 7.0	(van Casteren *et al.*, 2000)
Arthrobacter psychrolactophilus	*o*NP-Gal Lactose	2.7 42	13 42	4.7 0.1	(Nakagawa *et al.*, 2007)
Caldicellulosiruptor saccharolyticus	*p*NP-Gal *o*NP-Gal Lactose	0.1 1.2 30	3.0 32 149	296 123 1.4	(Park and Oh, 2009)
Lactobacillus acidophilus	*o*NP-Gal Lactose	0.7 4.0	632 50	865 13	(Nguyen *et al.*, 2007a)
Lactobacillus reuteri	*o*NP-Gal Lactose	1.0 31	338 58	345 1.9	(Nguyen *et al.*, 2006)
Saccharopolyspora rectivirgula	*p*NP-Gal Lactose	0.1 0.7	56 63	1,395 84	(Nakao *et al.*, 1994)
Sulfolobus solfataricus	*o*NP-Gal Lactose	0.2 13	95 1.9	47 0.3	(Pisani *et al.*, 1990)

Adapted from Park and Oh (2009).

Table 2.4 Inhibition type and constants of microbial β-galactosidase.

Source of enzyme	Substrate	Temp. (°C)	pH	Inhibitor	Inhibition Type	K_i (mM)
Arthrobacter sp.	*o*NPGal	20	7.2	Galactose	C	12 [(1)]
Kluyveromyces lactis	*o*NPGal	25	6.5	Galactose	C	45 [(2)]
	*o*NPGal	25	6.5	Glucose	N	758
	Lactose	37	7.0	Galactose	C	90 [(3)]
Lactobacillus reuteri	*o*NPGal	30	6.5	Galactose	C	115 [(4)]
	*o*NPGal	30	6.5	Glucose	C	683
	Lactose	30	6.5	Galactose	C	89
Thermus sp.	*o*NPGal	70	6.5	Galactose	C	3 [(5)]
	*o*NPGal	70	6.5	Glucose	N	50
	Lactose	80	6.5	Glucose	U	- [(6)]
Sulfolobus solfataricus	*o*NPGal	75	6.5	Glucose	C	96 [(7)]
Caldicellurosiruptor saccharolyticus	*p*NPGal	80	6.0	Galactose	U	12 [(8)]
	*p*NPGal	80	6.0	Glucose	U	1,170

Abbrev.: C = competive, I = incompetitive, U = uncompetitive.

[(1)] (Coker *et al.*, 2003), [(2)] (Mateo *et al.*, 2004), [(3)] (Kim *et al.*, 2004b), [(4)] (Nguyen *et al.*, 2006), [(5)] (Pessela *et al.*, 2003), [(6)] (Ladero *et al.*, 2002), [(7)] (Pisani *et al.*, 1990), [(8)] (Park and Oh, 2009). Adapted from Park and Oh (2009).

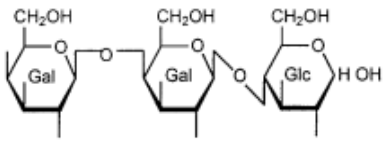
ions, such as Mg^{2+}, Mn^{2+}, Na^{+}, K^{+}, and so on. The requirement for Mg^{2+} or/and Mn^{2+} is well-known for a number of different β-galactosidases from such species as *E. coli*, *Bifidobacterium bifidum*, *Kluyveromyces lactis*, *Bacillus* sp. and *L. reuteri*. Na^{+} and K^{+} also activated the β-galactosidase activity, similar to that for β-galactosidases

from *Lactobacillus casei*, *B. bifidum*, *Streptococcus thermophilus*, and *L. reuteri*. Ca^{2+} slightly enhanced the β-galactosidase activity whereas it was a known inhibitor of other relevant enzymes (Nguyen *et al.*, 2006; Lu *et al.*, 2007).

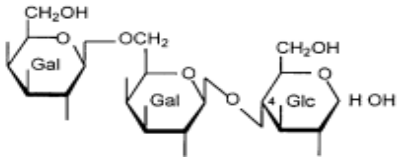
2.6 Galacto-oligosaccharide production

Galacto-oligosaccharides (GOSs) are produced from lactose or other structurally related galactosides as the substrate by glycosyl transfer of one or more D-galactosyl units onto D-galactose moiety of lactose $(galactosyl)_n$ lactose oligomer, where *n* may vary from 2 to 4 and catalyzed by β-galactosidase (or lactase, EC 3.2.1.23) through the transgalactosylation reaction (Prenosil *et al.*, 1987; Mahoney, 1998; Splechtna *et al.*, 2006). GOSs represent a mixture of various di-, tri-, tetra-, penta- or hexasaccharides of galactose and glucose with the molecular structure of $(Gal)_n$-Glu (Nakkharat and Haltrich, 2006; Zheng *et al.*, 2006; Splechtna *et al.*, 2007). Depending on the origin of the enzyme and reaction conditions, different between D-galactose units may be formed as well as structures and concentrations of GOSs are quite different, the oligosaccharides have predominantly β(1-4) and/or β (1-6) linkages, for example, the β-galactosidase of *Kluyveromyces lactis* produced predominantly β-(1-6) oligosaccharides (6'-galactosyl-lactose and β-D-Gal(1-6)D-Gluc), a β-galactosidase of *Sterigmatomyces elviae* produced predominantly 4'-galactosyl-lactose whereas *Bacillus circulans* β-galactosidase forms β-(1-2), β-(1-3), β-(1-4) or β-(1-6) linkages to produce a large variety of oligosaccharides (Zheng *et al.*, 2006; Gänzle *et al.*, 2008; Martinez-Villaluenga *et al.*, 2008). Therefore, the chemical structure and composition of GOS greatly depends on the enzyme source (Mahoney, 1998; Boon *et al.*, 2000). Besides, glucose, galactose, mannose, fructose,

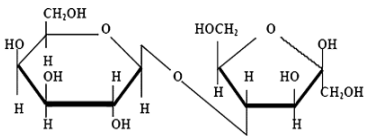
maltodextrins, N-acetylneuraminic acid, glucuronic acid and a number of aromatic compounds have been shown to act as galactose acceptor for β-galactosidase, providing a virtually unlimited variety of oligosaccharides (Lee *et al.*, 2004; Miyasato and Ajisaka, 2004; Bridiau *et al.*, 2006). They may also be produced synthetically from lactose syrup using β-galactosidase, mainly of bacterial origin (e.g. *Bacillus circulans*) (Tanaka and Matsumoto, 1998), and the resulting products are called (trans) galacto-oligosaccharides (TOS/GOS) (Stahl *et al.*, 2007). These oligosaccharides consist of a chain of galactose molecules, usually with a glucose molecule at the reducing terminus, varying in chain length (DP3-8) and linkages, for example, β1-6, β1-3, and β1-4 (Chen *et al.*, 2003; Gibson *et al.*, 2004; Matella *et al.*, 2006; Martinez-Villaluenga *et al.*, 2008).



Galβ1-4Gal β1-4Glc
(4'-galactosyllactose)



Galβ1-6Gal β1-4Glc
(6'-galactosyllactose)



Lactulose

Figure 2.2 Some chemical structures of galacto-oligosaccharide (GOS). Abbrev. Gal = galactose, Glc = glucose (Sako *et al.*, 1999; Hutkins, 2006; Aider and Halleux, 2007).

The chemical structure, an example, of galacto-oligosaccharide has been shown in Figure 2.2. Their chemical synthesis is tedious, hence enzymatic synthesis using β-galactosidase is advocated. The GOS yield and its composition change dramatically with reaction time, are very complex, and can hardly be predicted (Gaur *et al*., 2006). GOS yield depends on the enzyme and the conditions used in the process, and values ranging from 25% to 50% of total sugar have been reported (Mahoney, 1998). Some structures of galacto-oligosaccharide have been described as shown in Table 2.5. GOSs have a generally recognized as safe (GRAS) status due to the fact that they are componants of human milk and traditional yoghurt and they are produced from ingested lactose by resident intestinal bacteria which produce β-galactosidase (Sako *et al.*, 1999). Moreover, galactose-containing oligosaccharides are present in human and cows' milk (Stahl *et al.*, 2007), especially hey are constitute the major part of oligosaccharides of human milk, and are also recognized as prebiotics because of their non-digestibility (Sako *et al.*, 1999). Following the concept of prebiotic, it must be (1) neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract (GIT), (2) able to alter the colonic flora in favour of a healthier composition, (3) induce luminal or systemic effects that are beneficial to the host health (Kaur *et al.*, 2002). GOS are not broken down in the stomach or small intestine that means resistant to gastrointestinal digestive enzyme and thus, reach the colon largely intact to be readily utilized by bifidobacteria and lactobacilli, also acting as growth promoting substrates known as bifidus growth factor for desirable intestinal microflora in human intestine as well as already has been demonstrated for fructo-oligosaccharide (FOS) (Kunz and Rudloff, 1993; Sako *et al.*, 1999; Nakkharat and Haltrich, 2006; Stahl *et al.*, 2007). Most recently, it could be demonstrated that GOSs inhibit

adhesion of pathogenic *E. coli* to *in vitro* cell cultures, even better than FOS, inulin, raffinose, or lactulose (Shoaf *et al.*, 2006).

Table 2.5 Type of some galacto-oligosaccbarides formed during β-galactosidase action on lactose.

Type of saccharide	**Structure**	**Type of GOS**
Disacccharides	β-D-Gal (1-6)-D-Glc β-D-Gal (1-6)-D-Gal β-D-Gal (1-3)-D-Glc β-D-Gal (l-2)-D-Glc β-D-Gal (1-3)-D-Gal	allolactose galactobiose
Trisaccharides	β-D-Gal (1-6)-β-D-Gal (1-6)-D-Glc β-D-Gal (1-6)-β-D-Gal (l-4)-D-Glc β-D-Gal (1-6)-β-D-Gal (l-6)-D-Gal β-D-Gal (1-3)-β-D-Gal (1-4)-D-Glc β-D-Gal (1-4)-β-D-Gal (1-4)-D-Glc	6’ digalactosyl-glucose 6’ galactosyl-lactose 6’ galactotriose 3’ galactosyl-lactose 4’ galactosyl-lactose
Tetrasaccharides	β-D-Gal (1-6)-β-D-Gal (1-6)-β-D-Gal (1-4)-D-Glc β-D-Gal (1-6)-β-D-Gal (1-3)-β-D-Gal (1-4)-D-Glc β-D-Gal (l-3)-β-D-Gal (l-6)-β-D-Gal (l-4)-D-Glc	6’ digalactosyl-lactose
Pentasaccharide	β-D-Gal (1-6)-β-D-Gal (1-6)-β-D-Gal (1-6)-β-D-Gal (1-4)-D-Glc	6’ trigalactosyl-lactose

Gal, galactose; Glc, glucose. Structure from (Asp *et al.*, 1980; Toba *et al.*, 1985; Onishi *et al.*, 1995). Adapted from Mahoney (1998).

There are indications that GOSs also contribute to colonization resistance and thus reduction of pathogenic and infectious bacteria (Tanaka *et al.*, 1983; Ito *et al.*, 1993;

Chierici *et al.*, 2003; Hopkins and Macfarlane, 2003). As a result, GOSs are recognized as prebiotics because they can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the human intestine and decrease in the number of pathogen (Sheu *et al.*, 1998; Splechtna *et al.*, 2006; Martinez-Villaluenga *et al.*, 2008). Galacto-oligosaccharides are non-digestible oligosaccharides, which are not hydrolyzed by human small-intestinal β-galactosidase, and this makes them suitable for use as a low calorie sweetener and for consumption by individuals with diabetes (Karasová *et al*., 2000). Galacto-oligosaccharides, although fully fermented in the large intestine, did not beneficially affect putative risk markers of colon cancer or change significantly the composition of the intestinal microflora (Alles *et al*., 1999).

In recent years, GOSs have become established as functional components in beneficial physiological effects for human (Kunz and Rudloff, 1993). The prebiotic, GOS, have been used in human nutrition in significant quantities as active components or as side products of processed milk or milk products, and more side effects have been reported (Stahl *et al.*, 2007). Indeed, for a GOS administration up to 0.9 g per 100 mL (in combination with 0.1 g long chain, lc, FOS) to infants, no adverse effects of GOS are known (Boehm *et al.*, 2005). GOS are selectively utilized by bifidobacteria *in vitro*, and show a bifidogenic effect at a daily intake of more than 2.5 g in human studies, whereas at least 5 g are needed to have effects on stool frequency. It is suggested that the products of fermentation of GOS in the colon, mainly short chain fatty acids (SCFA), i.e. acetate, propionate and butyrate, have a role in the improvement of the colonic environment, energy supply to the colonic epithelium, lower gas production, and calcium and magnesium absorption (Fooks *et al.*, 1999; Sako *et al.*, 1999). Most of the SCFA formed by intestinal bacteria are absorbed, and systematically metabolized, thereby contributing towards host energy

gain (Cummings, 1995). The confirmed health claims of GOS have significantly increased the public demand for foods containing GOS. In addition, GOSs are water soluble and mildly sweet in comparison with the commonly used mono- and disaccharides. Their relatively low sweetness is useful in food production if enhancement of other food flavors is desirable. In food industry, GOSs are of great interest due to their possible health benefits for consumers. These galacto-oligosaccharides are not utilized by the mouth microflora (*Streptococcus mutants*), therefore, limit the formation of caries (Karasová *et al*., 2000). Due to the possible health benefits associated with the consumption of these compounds, their use as food ingredients has grown rapidly, particularly in Japan and Europe. The GOSs are considered prebiotics and important functional food ingredients linked to numerous health benefits, including reduced colon cancer risk and enhanced immunity. The functional food market is estimated to be $20 billion in the United States, $15 billion in Europe, and $12 billion in Japan, and is growing at an annual rate of 7.5% and is expected to be 10% of all food choices in 10 year. The prebiotic market alone is expected to reach $103.2 million in the United States by 2010. As a result, GOSs have high value in the United States and global markets and may be added to a variety of products, including breads, fermented dairy foods, and beverages (Matella *et al*., 2006). The following are the examples of the productions of GOS from microorganism: *E. coli* β-galactosidase produced the highest amount of GOS 29.7% allolactose and 14% other oligosaccharides at pH 6.5 (Chen *et al*., 2003). *Bullera singularis* ATCC 24193, yeast cell, β-galactosidase can produce continuously of 55% (w/w) oligosaccharides with a productivity of 4.4 g/(L-h) from 100 g/L lactose solution during a 15-day operation, and batch productivity was 6.5 g GOS/(L-h) from 300 g/L lactose (Shin *et al.*, 1998). The optimal condition for producing GOS,

trisaccharide and disaccharide, from *Kluyveromyces lactis* β-galactosidase was 40^{o}C, pH 7.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 120 min. As a result, 17% trisaccharide (6' galactosyl lactose) and 13% disaccharide (galactobiose and allolactose) yields (GOS) were obtained (Martinez-Villaluenga *et al.*, 2008). The enzyme β-galactosidase from many microorganisms has been investigated so far, including from psychrophiles, mesophiles, thermophiles, and hyperthermophiles as shown in Table 2.6. The maximum activity of β-galactosidase from the psychrophile *Arthrobacter psychrolactophilus* with transgalactosylation is founded to be at 10^{o}C (Nakagawa *et al.*, 2007) whereas the thermophiles from *Alicyclobacillus acidocaldarius* (Di Lauro *et al.*, 2008), *Bacillus stearothermophilus* (Chen *et al.*, 2008), and *Thermus sp.* (Ladero *et al.*, 2002) with transgalactosylation are higher than that of at 60^{o}C. Furthermore, the hyperthermophiles showed the maximum activity with the transgalactosylation above 70^{o}C. Human milk oligosaccharides represent the first nondigestible oligosaccharides a human being can receive (Stahl *et al.*, 2007). For situations in which breastfeeding is not possible, an infant formula has been developed containing 90% short-chain GOS and 10% lc FOS (scGOS/lcFOS) to mimic functions and molecular size distribution of neutral oligosaccharide in human milk (Boehm *et al.*, 2003). It has also been found that a controlled administration of GOS in 0.12 g/kg body weight for male Japanese adults may help to restrain the growth of pathogenic bacteria, to retard disorders caused by imbalanced fermentation in colon and to avoid intestinal disorders such as constipation, inflammatory bowel disease (Qiang *et al.*, 2009). Besides, GOSs have many useful health benefits such as the suppression of serum phenol and p-cresol levels, prevention of diarrhea and constipation, reduction of serum cholesterol and blood pressure and prevention of colon cancer (Sako *et al.*, 1999; Kawakami *et al.*, 2005).

Table 2.6 Some properties of microbial β-galactosidase with transgalactosylation activities.

Type of β-galactosidase	**Type of Mo.**	**Temp. (^oC)**	**pH**	**Half-life (min)**	**Reference**
Psychrophiles	*Arthrobacter psychrolactophilus*	10	8.0	60 (30^oC)	(Nakagawa *et al.*, 2007)
Mesophiles	*Aspergillus oryzae*	30	4.8	10 (70^oC)	(Todorova-Balvay *et al.*, 2006)
	Bacillus circulans	50	5.0-6.0	-	(Fujimoto *et al.*, 1998)
	Bacillus megaterium	40	6.0-9.0	-	(Li *et al.*, 2009b)
	Bifidobacterium aldolescentis	50	6.0	10 (50^oC)	(Hinz *et al.*, 2004)
	Bifidobacterium infantis	50-60	7.5	120 (60^oC)	(Hung and Lee, 2002)
	Escherichia coli	37-45	6.5	-	(Chen *et al.*, 2003)
	Kluveromyces lactis	37-40	6.6-7.0	-	(Kim *et al.*, 2003)
	Lactobacillus reuteri	50	6.0-8.0	-	(Nguyen *et al.*, 2006)
Thermophiles	*Alicyclobacillus acidocaldarius*	65	5.5	6 (80^oC)	(Di Lauro *et al.*, 2008)
	Bacillus stearothermophilus	70	7.0	540 (70^oC)	(Chen *et al.*, 2008)
	Thermus sp.	70	6.5	1200 (70^oC)	(Ladero *et al.*, 2002)
Hyperthermo-philus	*Thermus aquaticus*	80	5.5	-	(Berger *et al.*, 1997)
	Sulfolobus solfataricus	75	6.5	180 (85^oC)	(Pisani *et al.*, 1990)
	Thermotoga maritima	80-85	6.5	16 (90^oC)	(Kim *et al.*, 2004a)

Adapted from (Park and Oh, 2009).

Currently, various commercial products with GOSs are available in the market, and they usually contain a mixture of tetrasaccharide, trisaccharide, lactose, glucose, and galactose (Shin and Yang, 1998). Among the oligosaccharides, galacto-oligosaccharide is one of the top prebiotics produced commercially (Sako et al., 1999) and the estimated yearly (in year 2007) of the GOS production from lactose by enzymatic synthesis is approximately 15,000 tons (Stahl *et al.*, 2007). GOSs have many beneficial effects such as improving lactose tolerance and digestibility of milk products; preventing pathogenic, autogenic diarrhea and constipation; increasing absorptions of different minerals in the intestine; reducing toxic metabolites, undesirable enzymes and serum cholesterol; depressing blood pressure, etc. (Morishita *et al.*, 2002). Therefore, their comprehensive applications as a food additive for health purposes have led to an increased demand for commercial GOSs (Zheng *et al.*, 2006).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains

Various strains of bacteria were obtained from research collaborator at Kasetsart University such as *Pediococcus acidilactici* KUB-M6, *P. acidilactici* KUB-M14, *P. acidilactici* KUB-M15 and *Lactobacillus pentosus* KUB-ST10-1. In addition, *L. bulgaricus* TISTR 451 and *L. sake* TISTR 911 were obtained from the Thailand Institute of Scientific and Technological Research (TISTR; Bangkok, Thailand) as well as *L. delbrueckii* subsp. *bulgaricus* DSM 20081 and *Bacillus licheniformis* DSM 13 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen or the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). *Escherichia coli* TOP10 and *E. coli* BL21 (DE3) competent cells were purchased from Invitrogen and Novagen, respectively.

3.1.2 Plasmid and sequences of the primers

The plasmid pOJBlilacA2 containing the complete gene *lacA* of β-galactosidase from *B. licheniformis* DSM 13 was constructed. The oligonucleotides used for PCR amplification of the *B. licheniformis* DSM 13 were designed by the sequence comparison of β-galactosidase *lacA* gene. The primers were used in this study as shown in Table 3.1.

Table 3.1 The nucleotide sequences of the primers used in this study.

Primer	Nucleotide sequence 5' - 3'	Sites	Reference sequence accession no.
lacA_F1	CTGTGCCTCGAGATGCCAAAAATTTATACG ACCCAAGCAAGATACATG	*Xho*I	AE017333
lacA_R1	CTGTGCGGTACCCTCTTTTGCTTTTACCGCT ATTCTGGCCTC	*Kpn*I	AE017333
lacA_F2	GCAAGCTTCGCTCCATATGCCA	*Nde*I	AE017333
lacA_R2	GTGGTCGACAGATCTCTCGAGCTC TTTTG	*Xho*I	AE017333
lacA_R2_S	GTGGTCGACAGATCT CTCGAGTCATTTTGC	*Xho*I	AE017333

F: denotes forward primers; R: denotes reverse primers.

Restriction sites are underlined.

lacA stands for β-galactosidase gene from *B. licheniformis* DSM 13.

3.1.3 Enzymes

Enzyme for determination of D-glucose: Glucose oxidase (GOD) from *A. niger* (lyophilized 211 U/mg enzyme preparations) was purchased from Fluka (Buchs, Switzerland). Horseradish peroxidase (POD) (lyophilized 181 U/mg enzyme preparations) was purchased from Sigma-Aldrich Corp. (St. Louis, Missouri; MO., USA).

Restriction enzymes: *Xho*I, *Kpn*I, *Nde*I, and *Dpn*I were purchased from New England Biolabs (Beverly, Massachusetts; MA., USA), while Phusion DNA polymerase was purchased from Finnzymes Oy (Espoo, Finland).

Enzyme for ligation: T4 DNA ligase was purchased from Fermentus (Vilnius, Lithuania).

3.1.4 Chemicals

o-Nitrophenyl *β*-D-galactopyranoside (*o*NPG), boric acid, 4-amino anti-pyrine phenol, phenol, *o*-nitrophenol (*o*NP), sodium dodesyl sulphate (SDS), tris-HCl, Bromphenol blue, 4-methylumbelliferyl-*β*-D-galactopyranoside (4MUGal), and sodium chloride were purchased from Sigma-Aldrich Corp. (St. Louis, Missouri; MO., USA). Isopropylthio-β-D-galactoside (IPTG), glucose, ampicillin, glycerol, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), hydrochloric acid (HCl), 1,4-dithiothreitol (DTT), and agar were purchased from Roth (Karlsruhe, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from BioRad (California, USA). Acetone, toluene, imidazole, sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH), mercaptoethanol, urea, acetic acid, *o*-phosphoric acid 85%, potassium dihydrogen phosphate (KH_2PO_4), lactose, peptone from casein enzymatic digest, yeast extract, sodium bicarbonate ($CHNaO_3$), sodium carbonate (Na_2CO_3), phenylmethansulfonylfluoride 99% (PMSF), magnesium-chloride hexahydrate ($MgCl_2.6H_2O$), and meat extract were purchased from Fluka (Buchs, Zwitzerland) and the test kit for the determination of the amount of glucose as well as lactose and D-galactose kit were from Megazyme (Wicklow, Ireland).

All other chemicals were of the highest analytical grade available and were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich Corp. (St. Louis, Missouri; MO., USA).

3.2 Methods

3.2.1 Culture conditions for bacterial culture collection.

Pediococcus spp. and *Lactobacillus* spp. were grown in Man Rogosa Sharp (MRS) broth (10 g/L proteose peptone, 10 g/L beef extract, 5 g/L yeast extract, 20 g/L dextrose, 1 g/L polysorbate 80, 2 g/L ammonium citrate, 5 g/L sodium acetate, 0.1 g/L magnesium sulphate, 0.05 g/L manganese sulphate, 2 g/L dipotassium phosphate) adapted from (Nguyen *et al.*, 2006), and incubated under facultative anaerobe condition at 37^{o}C for 24 h, while *Bacillus* sp. were grown in nutrient broth (NB) (5 g/L peptone, 3 g/L meat extract) under aerobic condition.

All strains were stored in sterilized 1.5 mL eppendorf tubes at -70^{o}C in MRS broth medium containing 15% (v/v) glycerol whereas *Bacillus* strain was kept in NB medium containing 15% (v/v) glycerol until used.

3.2.2 Primary screening on agar plate

MRS agar plates were prepared for culturing *Lactobacillus* spp. and *Pediococcus* spp., while Nutrient Agar (NA) plates were prepared for *Bacillus* sp. Added 40 μL of 2% X-gal and 60 μL of 100 mM IPTG were pipetted onto the center of agar plates and the solution was spreaded over the entire surface by using sterilized spreader. The plates were incubated at 37^{o}C until all of the fluid had disappeared.

The bacterial strains were diluted to proper dilution. Then, 100 μL of appropriate dilutions were inoculated and spreaded over the entire surface of the plates. The plates were incubated in an inverted position for 24 h at 37^{o}C. After incubation, the plates were removed from the incubator and stored at 4^{o}C for several hours. The blue colonies were appeared (Sambrook and Russell, 2001) if the bacteria had β-galactosidase activity. The strains were collected when the blue colonies appeared on the agar plate.

3.2.3 Partial characterization of the crude enzyme

3.2.3.1 Permeabilization of the strains

The strains of bacteria, *Pediococcus* spp. and *Lactobacillus* spp., from frozen stock at -70^{o}C were used to spread on the plate. The single colony were inoculated into a 5 mL culture MRS broth added 0.1 M lactose and *Bacillus* sp. were also cultivated in NB containing 0.1 M lactose as well, and incubated at 37^{o}C for 16 h. One mL of cell suspension from the overnight cultures was harvested by centrifugation at 10,000 rpm at 4^{o}C for 10 min with aseptic condition and carefully poured off prior to discard the supernatant by decanting the tube. Cell pellets were washed twice with 50 mM sodium phosphate buffer (pH 7), and draining the tube on sterilized paper towel. Cell pellets were resuspended in 500 μl Z-buffer (see in appendix K) and kept in crushed ice until used. Then, 50 μl of acetone : toluene (9 : 1) were added, vortexed for 5 sec, incubated at 37^{o}C for 20 min, and determined the β-galactosidase activity (Somkuti *et al.*, 1998). The method for β-galactosidase assay is described in the next section.

3.2.3.2 β-Galactosidase assay for partial characterization of the crude enzyme

All of permeabilized cells (500 μL) were added into 200 μl of 4 mg/mL *o*NPG in 0.1 M sodium phosphate buffer (pH 7), and incubated at 37^{o}C for 10 min. Then, the reaction was stopped by using 500 μL of 1 M Na_2CO_3 and the cells were precipitated by centrifugation at 7,000 rpm for 5 min. The released of *o*NP was assessed by determining the absorbance at 420 nm. Cells dried weight were measured to calculate the unit of enzyme, (Unit/mg cell dried weight). One unit of enzyme activity was defined as 1 μmol of *o*-nitrophenol formed per min under the condition as described above.

3.2.4 Cloning of *lacA* gene form *B. licheniformis* DSM 13

3.2.4.1 DNA preparation

A single colony of *B. licheniformis* DSM 13 was picked with the sterilized toothpick or a sterile tip from the agar plate and then suspended in 100 μL autoclaved distilled water in the tightly closed lid microfuge tube. The mixture was heated at 95^{o}C in heating box for 5 minutes, and then transferred the mixture into the ice bath for 1 min. The mixture was then vigorously mixed with the vortex. The supernatant was obtained by centrifugation at 13,000 rpm and 2 min. The clear supernatant was transferred into a new microfuge tube, and taken 20 μL of the supernatant as template in a 50 μL PCR reaction. Plasmid p10HisFLAG from *E. coli* was purified using GenElute Plasmid Miniprep Kit (Sigma Aldrich St. Louis, MO., USA).

3.2.4.2 DNA amplification

The degenerated oligonucleotides lacA_F1 and lacA_R1 (Table 6) for forward and reverse primers for PCR amplification of the *B. licheniformis lacA* gene were designed based on a reference sequence of *B. licheniformis* DSM 13 from GenBank accession number AE017333 (compatible with the cloning sites of the p10HisFLAG expression vectors which has the original backbone from pFLAG-CTS). The primers were obtained from VBC-Biotech (Vienna, Austria). The amplifications were performed using a T3 Thermocycle (Biometra; Goettingen, Germany) in a total volume of 50 µl of reaction mixtures containing 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 5 µL of 10xPfu buffer (final concentration of $MgCl_2$ was 1.5 mM), 1.5 U of Pfu DNA polymerase, and 20 µL of DNA prepared as described above. The initial denaturation step at 95^{o}C for 5 min was followed by 35 cycles of denaturation at 95^{o}C for 45 s, annealing at 55^{o}C for 30 s, and extension at 72^{o}C for 4.5 min. The final cycle was followed by additional 10 min elongation at 72^{o}C. The amplified products were visualized by gel electrophoresis at 7 V/cm in 0.8% agarose gel (containing 0.2 µg/mL of ethidium bromide) in 1xTAE (Tris–Acetate) electrophoresis buffer (Tris Base 4.8 g/L, acetic acid 1.2 g/L, 1 mM EDTA pH 8.0). The amplified products were purified from the agarose gel using the Wizard® SV gel and PCR Clean-Up system (Promega).

3.2.4.3 Gene cloning

PCR-amplified products were ligated into *Xho*I and *Kpn*I stites of the p10HisFLAG expression vector. The constructed was transformed (see appendix B) into *E. coli* strain TOP10 resulting in the plasmid pOJBlilacA1. The transformant

were selected on LB agar containing 100 μg/mL ampicillin. The plasmids were extracted using Sigma miniprep kit.

3.2.4.4 Screening of *E. coli* harboring recombinant β-galactosidase gene

The colonies of *E. coli* TOP10 harboring variant β-galactosidase gene were grown on LB agar containing 100 μg/mL ampicillin, and 40 μL of 2% X-gal and 60 μL of 100 mM IPTG on the surface of LB agar. The plates were incubated at 37^{o}C for overnight (Sambrook and Russell, 2001). The *E. coli* cells which carry empty vector were used as a negative control, while the positive control was *E. coli* TOP10 harboring recombinant expression vector of β-galactosidase gene. After incubation step, the plates were removed from the incubator and stored at 4^{o}C for several hours. The blue colonies were appeared in *E. coli* harboring recombinant expression vector of β-galactosidase gene, on the other hand, the white colonies were appeared when *E. coli* had no recombinant expression vector.

3.2.4.5 Construction of *lacA* into expression vector

The plasmid pFLAG-CTS from Sigma-Aldrich Corp. was modified with the addition of 10 histidine tag downstream of multiple cloning sites which was modified by MY Group in School of Biotechnology, Suranary University of Technology, Thailand and upstream of FLAG tag, and with the removal of *ompA*, a signal sequence for secretion of C-terminal FLAG fusion proteins to periplasmic space, resulting in a linearalized vector with *Nde*I and *Xho*I sites. Upstream and downstream primers lacA_F2, lacA_R2 and lacA_R2_S (Table 3.1) were used to amplify the fragment containing *lacA* gene from pOJBlilacA1. These primers created a

restriction site at each end of the gene fragment, *Nde*I and *Xho*I, respectively. The PCR amplified fragment was digested with *Nde*I and *Xho*I and inserted into the respective sites of the modified vector pFLAG-CTS (as described above) resulting in the expression plasmid pOJBlilacA2 contains the complete genes (*lacA*) of β-galactosidase from *B. licheniformis* DSM 13. The expressed protein carries a C-terminal His-Tag.

All PCR products were purified from the agarose gel by using Promega kit and ligated into the p10HisFLAG without *ompA* and pET-21a(+) expression vectors as appropriate. All ligated plasmids from pOJBlilacA2 and pET-21a(+) were transformed into *E. coli* TOP10 and *E. coli* BL21 (DE3) electrocompetent cells, respectively. The transformants were selected on LB agar containing 100 µg/mL ampicillin according to describe in the previous section. The plasmids were extracted by Sigma miniprep kit.

3.2.5 Nucleotide sequencing and sequence analysis

The sequence of the inserts was verified by DNA sequencing. The nucleotide sequence was determined by VBC-Biotech (Vienna, Austria). Assembly and analysis of DNA sequences were done by using Vector NTI. The basis local alignment tool (BLAST) from the National Center for Biotechnology Information (NCBI) BLAST website was used for database searches.

3.2.6 Comparison of amino acid sequence alignment of β-galactosidase genes from *B. licheniformis* DSM 13 and some other bacteria

The comparison of amino acid sequence alignment of β-galactosidase from *B. licheniformis* DSM 13 with homologous proteins from 8 other strains of bacteria

which belong to glycosyl hydrolase family 42 such as *Thermus thermophilus*, BGAL_THETH; *Thermotoga maritime*, BGAL_THEMA; *T. neapolitana*, BGAL_ THENE; *Clostridium perfringens*, BGAL_CLOPE; *Haloferax* sp., BGAL_HALSQ; *B. subtilis*, BGAL_BACSU; *B. circulans*, BGAL_BACCI; and *B. stearothermophilus*, BGAL_BACST was carried out using the CLUSTAL W program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) and assigned the secondary structure using Espript program that was available on the internet.

3.2.7 Expression of recombinant β-galactosidase gene in *E. coli*

3.2.7.1 The optimization of cell density for expression of recombinant β-galactosidase gene in *E. coli*

The *E. coli* harboring appropriate recombinant plasmids were inoculated into 5 mL of LB broth containing 100 μg/mL of ampicillin at 37^{o}C in the shaking incubator with 150 rpm for 16 h. and then 1 mL of overnight culture was inoculated into 200 mL of LB broth containing 100 μg/mL ampicillin and grown at 37^{o}C until the OD_{600} reached to approximately 0.4, 0.6, 0.8 1.0, 1.5 and 2.0, respectively. Then, IPTG was added into the culture broth to a final concentration of 1 mM. The culture was then incubated at 25^{o}C, 120 rpm for 4 h. Fifty milliliters of the culture was then collected and chilled in an ice box for 5 min and centrifuged at 14000 rpm for 2 min at 4^{o}C to collected cells and poured out supernatant. One hundred μl of gel loading buffer (or 3x protein dye) was added into the pellet and then vigorously vortexed. The mixture was then centrifuged at 14000 rpm for 1 min after that the mixture was put into the heating box at 100^{o}C for 5-10 min. then centrifuged at 14000 rpm for 5 min. To compare the different cell density for expression of recombinant β-

galactosidase gene in *E. coli*, the samples were analysed by SDS-PAGE (see appendix C).

3.2.7.2 The optimization of induction time for expression of recombinant β-galactosidase gene in *E. coli*

To cultivate the recombinant cells as above, for expression, the induction times were used at 4h, 6h, 8h, and overnight and then the samples were analysed by SDS-PAGE (see appendix C).

3.2.7.3 Optimization of cell concentration and time induction for expression of recombinant β-galactosidase gene in *E. coli*

The recombinant cells was cultivated the same as in 3.2.7.1. For induction, IPTG was added to a final concentration of 1 mM into the culture broth with appropriate of cell concentration and time induction. The culture was then incubated at 25^{o}C 120 rpm for 4 h. The culture was then collected and chilled in an ice box for 5 min and centrifuged at 14000 rpm for 2 min at 4^{o}C to collected cells. The precipitated cells from the previous step were washed once with 10 mL lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM PMSF, and 1 mg/mL lysozyme), combined cells into one tube and again resuspended with 10 mL of lysis buffer and vigorously vortexed. After that, the cell suspension was sonicated (Ultrasonic Processor; 60 amplitude, pulser 1 s, for 2 min and on-off 4 times) always on ice (see appendix H). The cell debris was then spun down at 17,000 rpm for 45 min and the supernatant was collected as the cell lysate. The cell lysate was kept in the freezer (-20^{o}C) for the next manually purification using bead Ni-NTA agarose column (see appendix D) and analysed by SDS-PAGE (see appendix C).

3.2.7.4 Expression of recombinant β-galactosidase gene in *E. coli* TOP10 and BL21 (DE3)

E. coli TOP10 and *E. coli* BL21 (DE3) harboring plasmids with *lacA* gene were cultivated in LB medium containing 100 µg/mL ampicillin overnight at 37^{o}C with 150 rpm shaking. The overnight cultures were used 2% as seed inocula cultures into the TB medium containing 100 µg/mL ampicillin, and incubated at 37^{o}C with 110 rpm shaking to an OD_{600} of 1.0. The culture was then induced with 0.01 mM IPTG and incubated at 18^{o}C with 110 rpm shaking. Cells were harvested at 45 hr after induction by centrifugation at 6000 rpm for 20 min at 4^{o}C and washed once with 50 mM sodium phosphate buffer, pH 6.5. To extract the crude extract, precipitated cells were sonicated or homoginised (see appendix H). The cell debris was then spun down at 13200 rpm for 30 min and the supernatant was collected as the crude extract. The crude extract was kept in the freezer (-20^{o}C) for the next purification using affinity chromatography and analysed by SDS-PAGE (see in the next section; 3.2.8 and 3.2.9, respectively).

3.2.8 Purification of recombinant enzyme (Nguyen *et al.*, 2006)

3.2.8.1 Recombinant enzyme preparation

After the growth step of the cells, the cell cultures were harvested by centrifugation at 10000 rpm for 10 min and 4^{o}C. Approximately 50 g wet weight of biomass was resuspended in 100 mL of 50 mM sodium phosphate buffer, pH 6.5. The cells were disrupted by using French press, and debris was removed by using centrifugation at 25000 g for 15 min and 4^{o}C to obtain the crude extract. Consequently, the crude enzyme extract was purified to the next step.

3.2.8.2 Affinity chromatography

The crude extract was loaded onto a 15 mL Ni Sepharose 6 fast flow column (GE Healthcare Bio Science AB, Uppsala, Sweden) that was pre-equilibrated with buffer A (50 mM sodium phosphate buffer, 0.2 M NaCl, 20 mM imidazol, pH 6.5). The enzyme was eluted by using buffer B (50 mM sodium phosphate buffer, 0.2 M NaCl, 1 M imidazol, pH 6.5) with the rate 1.5 mL/min as well as 60 mL of linear gradient from 0 to 100%. The active fractions were pooled, desalted, and concentrated by using a membrane (Amicon Ultra Centrifugal filter tubes; 10 kDa cutoff; Millipore, Beverly, MA). The purified enzyme was stored in 50 mM sodium phosphate buffer pH 6.5 at 4^{o}C.

3.2.9 Characterization of recombinant enzyme

3.2.9.1 Enzyme assay

β-galactosidase activity was determined by using *o*-nitrophenyl- *β*-D-galactopyranoside (*o*NPG) and lactose as the substrates. When using chromogenic *o*NPG as the substrate, the reaction was initiated by adding 20 µl of enzyme solution to 480 µl of 22 mM *o*NPG in 50 mM sodium phosphate buffer, pH 6.5. The reactions were incubated at 30^{o}C by shaking at 600 rpm and stopped reaction after 10 min by adding 0.4 M Na_2CO_3. The releasing of *o*-nitrophenol (*o*NP) was measured by determining at absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions.

When using lactose as the substrate, the reaction was initiated by adding 20 µL of enzyme solution to 480 µL of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. The reactions were incubated at 30^{o}C by shaking at 600

rpm and stopped reaction after 10 min by heating the reactions at 99^{o}C for 5 min. After the reaction mixtures were cooled to the room temperature, the releasing of glucose was determined by using GOD/POD assay. One unit of lactase activity was defined as amount of enzyme releasing 1 μmol of D-glucose per minute under the described condition.

3.2.9.2 Protein determination

The protein concentration was determined with the Bio-Rad Coomassie Blue reagent based on the method of Bradford using bovine serum albumin (BSA) as the standard (Bradford, 1976) (see in appendix E).

3.2.9.3 Determination of molecular mass

SDS-PAGE, Native-PAGE and active staining were used to determine the band of the lacA. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The enzyme was preincubated with SDS buffer (47 mM Tris-HCl, pH 6.8) containing 34 mg/mL SDS, 0.1 mg/mL bromophenol blue, 5% (v/v) mercaptoethanol, and 15% (v/v) glycerol, at 60^{o}C for 5 min. Coomassie blue staining was used for the visualization of the protein band. The native-PAGE was performed without SDS. Active staining for the visualization of the bands with β-galactosidase activity was carried out by applying filter paper soaked with the stain solution (50 mM sodium phosphate buffer, pH 6.5 and 3.5 mg/mL 4-methyl-umbelliferyl β-D-galactoside) onto the gel, and incubated at 37^{o}C for 10 min. After application of 1 M carbonate-bicarbonate buffer (pH 10) onto the gel using a filter paper, the protein bands displaying enzyme activity was visualized under UV light, detecting the release of 4-methylumbelliferone (Nguyen *et al.*, 2006).

3.2.9.4 Determination of the isoelectric point

Isoelectric focusing was performed in the range of pH 3-9 using PhastGel IEF media from Phamacia. PhastGel IEF media are precast homogeneous (5% T, 3% C) polyacrylamide gels containing 2-6% Pharmalyte™ as carrier ampholyte. Pharmalyte generates stable, linear pH gradients with uniform conductivity across the entire pH range, allowing field strengths >500 V/cm for high-resolution separations. PhastGel IEF media were run without buffer strips. They were stored at 4°C in the toxic cupboard until used.

Protein samples were diluted with distilled water to the concentration of 0.5 mg protein/mL. Samples were applied to the gel with PhastGel sample applicator, which had a series of capillary wells. The choice of sample applicator depended on the number and volume of the samples. For example, the applicator 8/1 would apply eight samples each 1 µL to the gel. Samples were soaked into the capillaries and held until the applicator is lowered onto the gel (adapted from Inez, people communication). Coomassie staining method was used for staining PhastGel IEF media as shown in Table 3.2.

Table 3.2 Coomassie staining method for PhastGel IEF media (Phamacia).

Step	Solution	Time (min)	Temperature (^{o}C)
1	Fixed[1]	5	20
2	Washed/Destained[2]	2	20
3	Stained[3]	10	50
4	Washed/Destained[2]	10	50

PhastGel electrophoresis media: A volume of 80 mL each solutions were required to fill the chamber and tubing:

[1] Fixed: 20% trichloroacetic acid

[2] Washed/destain: 30% methanol and 10% acetic acid in distilled water (3 : 1 : 6) (prepare at least 300 mL of this solution; enough to fill the chamber three times).

[3] Stained: 0.1% PhastGel Blue R solution in 30% ethanol and 10% acetic acid in distilled water (stock solution: dissolve 1 tablet of PhastGel Blue R in 80 mL of distilled water and stir for 5-10 min. Add 120 mL of methanol and stir for 2-3 min. This makes a 0.2% solution; final solution: mix 1 part of filtered stock solution with 1 part of 20% acetic acid in distilled water, prepare this solution fresh the day you plan to use it and do not recycle this solution).

3.2.9.5 Steady-state kinetic measurements

All steady-state kinetic measurements were obtained at 30^{o}C using *o*NPG and lactose as the substrates in 50 mM sodium phosphate buffer (pH 6.5) with concentrations ranging from 0.1 to 22 mM for *o*NPG and from 1 to 600 mM for lactose. The inhibition of *o*NPG hydrolysis by D-galactose and D-glucose as well as that of lactose hydrolysis by D-galactose was investigated. The kinetic parameters and inhibition constants were calculated by nonlinear regression, and the observed data were fit to the Henri-Michaelis Menten equation (Sigma Plot, SPSSc Inc., Chicago, IL).

3.2.9.6 The optimum pH and temperature

The Britton-Robinson buffer (containing 20 mM each of phosphoric acid, acetic acid, and boric acid adjusted to pH over the range of 4-9 with NaOH) was used for measuring the pH optimum of enzyme activity. The temperature optimum of enzyme activity was measured by assaying the enzyme samples over the range of 20-

70^{o}C for 10 min using 22 mM *o*NPG and 600 mM lactose in sodium phosphate buffer, pH 6.5 as substrate.

3.2.9.7 The pH and temperature stability

When determine the pH stability, the enzyme sample was incubated at various pH over ranges of 4-9 at temperature 37^{o}C. The remaining enzyme activity was measured at the time intervals by using *o*NPG as the substrate under the condition described above.

The thermal stability of the enzyme was conducted by incubating the enzyme samples in 50 mM sodium phosphate buffer, pH 6.5 at 4, 30, 37, 42, 50, 55, 60, 65, 70^{o}C, and room temperature. The samples were withdrawn at the certain time and the residual activity was measured by using *o*NPG as the substrate under the condition described above.

3.2.9.8 Effect of various reagent

The enzyme samples were assayed with 22 mM *o*NPG solution (in 50 mM sodium phosphate buffer, pH 6.5) in the presence of 1 and 10 mM DTT, EDTA, 2-mercaptoethanol, urea, and 0.1, 0.5 and 1 mM PMSF, individually, at 30^{o}C for 10 minutes. Enzyme activity measured without added reagents was used as a control.

3.2.9.9 Effect of various cations

To study the effect of various cations on the release of *o*NP from *o*NPG, the enzyme samples were assayed with 22 mM *o*NPG (in 10 mM Bis-Tris buffer, pH 6.5). The presence of various mono- and di-valent metal cations with final concentrations of 1.0, 10 and 100 mM (chloride or sulfate form) at 30^{o}C for 10 min

(see appendix F). The measured activities were compared with the activity of the enzyme solution without added cations under the same conditions.

3.2.10 Hydrolysis of lactose and GOS production

Hydrolysis of lactose was carried out in the discontinuous mode using recombinant purified β-galactosidase from *B. licheniformis* DSM 13 expressed in *E. coli* (eight lactase units per mL of reaction mixture). Reaction conditions were 50 and 200 g/L initial lactose concentration in 50 mM sodium phosphate buffer (pH 6.5), varying process temperatures (37, 55 and 60^{o}C), and constant agitation was applied 300 rpm with a thermomixer (Eppendorf, Hamburg, Germany). Samples were withdrawn for 200 μL at interval times. One hundred μL of sample was detected the rest of the activity by performing assays with 22 mM *o*NPG in 50 mM sodium phosphate buffer pH 6.5 and another 100 μL of the sample was stopped the enzyme activity by heating in the heating block at 99^{o}C for 5 min and then cool on an ice box. The samples were kept at -20^{o}C until used.

3.2.11 GOS analysis

The compositions of the sugar mixtures of the sample were analyzed by thin layer chromatography (TLC), capillary electrophoresis (CE) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

3.2.11.1 Thin layer chromatography (TLC)

TLC was carried out using high performance TLC silica plates (HPTLC-Alufolien plates) (Kieselgel 60 F245, Merck). All sample were diluted with distilled water to the final concentration of 20 g/L each samples (1 : 10 and 1 : 2.5 for 200 g/L

and 50 g/L lactose as initial substrate, respectively). To prepare TLC silica plate, drew a base line with pencil 1 cm from the lower edge of the TLC silica plates and divided the line 1 cm from each other. The surface of the TLC silica plate should not be touched by hand. To prepare 100 mL of mobile phase, the solution were mixed (n-butanol : n-propanol : ethanol : distilled water; 2 : 3 : 3 : 2). Two μL of the samples were applied onto the dot of the bottom of the surface of the TLC silica plate and then dried with hair dryer. The plate was then put into the solvent bath and covered the upper bath with glass. The samples were run by the solvent of the mobile phase until around 2 h or the mobile phase reach to the upper of 0.5 cm from the edge. The TLC silica plate was taken out of the bath and dried with the hair dryer. The TLC silica plate was then put again into the solvent bath. When the solvent run up to the upper TLC silica plate, the plate was taken out and stained in the next step. To stain, the TLC silica plate was dip into the thin tank containing the staining solution (0.5 g thymol in 95 mL ethanol and 5 mL H_2SO_4 conc. and then taken it out. The TLC silica plate was dried with the hair dryer for a short time and then put into the hot air oven at 135^{o}C for 5 min (or until it shows the color on the paper).

3.2.11.2 Capillary electrophoresis (CE)

To prepare the sample by derivatization method with 2-aminopyridine, the samples were diluted with deionized water. Ten μL if a sample was dried under vacuum for 1 h at 60^{o}C using the SPD SpeedVac system (Thermo Savant). Twenty μL of an aminopyridine solution (1 g of 2-aminopyridine in 470 μl of acetic acid and 600 μl of methano) was added to the dried sample. The mixure was incubated on a thermo-block at 90^{o}C for 15 min. After the incubation time, the sample was placed under vacuum in a SPD SpeedVac system for 30 min at 60^{o}C for evaporating the

excess of the reagents. The mixture was added with 25 μl sodium cyanoborohydride solution (59 mg/mL sodium cyanoborohydride in 30% acetic acid), the mixture was then incubated at 90^{o}C for 30 min in still condition. The sample was dried under vacuum in a SPD SpeedVac system at 60^{o}C for 2 h or until it dried and then resuspened in 200 μl deionized water. The samples were kept at -20^{o}C until used.

To determine the sugar in the samples by a capillary electrophoresis system, a capillary electrophoresis system with a UV-DAD detector (Agilent Technologies, Palo Alto, CA) together with a fused silica capillary column (internal diameter of 25 μm) equipped with a bubble cell detection window (bubble factor of 5) was used for carbohydrate analysis. The capillary had a total length of 64.5 cm and an effective length of 56 cm. The capillary was preconditioned before each run by flushing with 50 mM phosphorous buffer for 4 min followed by flushing with running buffer (100 mM phosphorous buffer, H_3PO_3, titrated with 1 M sodium hydroxide to pH 2.5) for 7 min. The sample was injected into the capillary at the anodic end by a positive pressure of 50 mbar for 5 s. The positive polarity mode and an operating temperature of 30^{o}C were employed. A current of 20 μA was applied after sample injection and kept constant during the running. The resulting voltage was approximately 23 kV. The detection wavelength was set at 240 nm with a bandwidth of 10 nm (adapted from Splechtna et al., 2006).

3.2.11.3 High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

HPAEC-PAD was set up on a Dionex DX-500 system consisting of a GP50 gradient, an ED 40 electrochemical detector with a gold working electrode and an

Ag/AgCl reference electrode, and Chromeleon version 6.5 software (Dionex Corp., Sunnyvale, CA). For eluents preparation, Milli Q water, NaOH (50% w/v) and NaOAc (Fluka, Germany) were used. All eluents were degassed by flushing with helium for 30 min. Separations were performed at room temperature on a CarboPac PA-1 column (4 mm x 250 mm) connected to a CarboPac PA-1 (50x4 mm) guard column (Dionex). Four eluents were used for effective GOS separation. Eluent A (100 mM NaOH), eluent B (water), eluent C (100 mM NaOH and 1 M NaOAc), and eluent D (100 mM NaOH and 50 mM NaOAc) were mixed to form the following gradient: gradient 1, 100% A from 0 to 20 min and from 0 to 100% D from 20 to 70 min; and gradient 2, 15% A and 85% B from 0 to 70 min. After each run, the column was washed for 10 min with 100% C and re-equilibrated for 15 min with the starting conditions of the employed gradient. All eluent solutions were kept under helium gas to avoid contamination of the alkaline solutions with atmospheric carbondioxide because the separation method is extremely sensitive to even traces of carbonate in the mobile phase (Pfaff *et al.*, 1999). Twenty μL of samples were injected via a Spark basic marathon autosampler, and separations were performed at a rate of 1 mL/min. Quantification of sugar was performed by external calibration using glucose galactose and lactose as standard (adapted from Splechtna et al., 2006).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of β-galactosidase-producing bacteria

The enzyme β-galactosidase has been used to hydrolyzed lactose in milk to glucose and galactose (Yang and Tang, 1988; Mahoney, 1998) of which benefit for people who are lactose intolerant. This has led to be many reports studied about the characterization of β-galactosidase enzyme from various microorganisms; *Kluveromyces marxianus* (Rajakala and Karthigai, 2006), *Bacillus subtilis* (Rahim and Lee, 1991), *Lactobacillus plantarum* and *L. sakei* (Halbmayr *et al.*, 2008b), *L. reuteri* (Nguyen *et al.*, 2006) etc. in order to improve processes for dairy product from this enzyme. Thus, to screen the appropriate bacteria producing β-galactosidase enzyme were carried out. Eight strains of bacteria obtained from various sources were screened for their β-galactosidase activity. The result shows that all of them showed the blue colonies on the agar plate (Figure 4.1) containing 2% X-gal and 100 mM IPTG. For this reason, all 8 strains of bacteria were judged to be the β-galactosidase-producing bacteria on the basis of the ability of which produced β-galactosidase enzyme.

4.2 Partial characterization of bacterial β-galactosidase

Permeabilization of bacterial cultures with solvents (toluene) solvent mixtures (acetone-toluene) generally has been restricted to increasing the measurable level of intracellular β-galactosidase activity (Somkuti *et al.*, 1996). Although, this organic

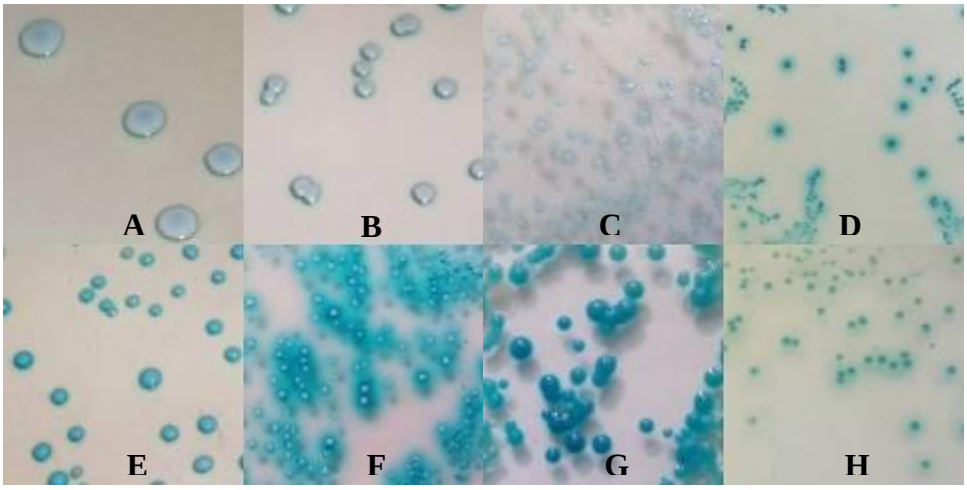


Figure 4.1 The blue colonies from various bacteria cultivate on agar plates containing 2%X-gal and 100 mM IPTG. (A) *Pediococcus acidilactici* KUB-M6; (B) *P. acidilactici* KUB-M14; (C) *P. acidilactici* KUB-M15; (D) *Lactobacillus pentosus* KUB-ST10-1; (E) *L. bulgaricus* TISTR 451; (F) *L. sakei* TISTR 911; (G) *L. delbrueckii* subsp. *bulgaricus* DSM 20081; (H) *Bacillus licheniformis* DSM 13.

solvents are excellent permeabilizing agents, their residues would be objectionable in dairy foods (Somkuti *et al.*, 1998), hence, in this study, acetone-toluene had been used to select the best one of β-galactosidase-producing bacteria. The partial characterization of the crude enzyme from eight strains of bacteria was investigated by determining the activity of β-galactosidase enzyme based on per mg cell dried weight using permeabilization technique. Three bacterial strains produced the highest enzyme activity were *B. licheniformis* DSM 13, *L. delbrueckii* subsp. *bulgaricus* DSM 20081 and *L. pentosus* KUB-ST10-1, respectively. The activities of these 3 strains were found to be 73, 58.3 and 26.7 U/mg cell dried weight, respectively. Interestingly, the activity from *B. licheniformis* DSM 13 was the highest when compared with the other 7 strains of bacteria (Figure 4.2). Consequently, *B.*

licheniformis DSM 13, *L. delbrueckii* subsp. *bulgaricus* DSM 20081 and *L. pentosus* KUB-ST10-1 were used for further partial characterization such as optimum pH and temperature. The optimum pH and temperature were performed using crude extract from these 3 strains of bacteria. It was found that the pH and temperature optimum were at pH 7, 7.5 and 6.5 as well as 55, 45, and 50^{o}C for *B. licheniformis* DSM 13 (Figure 4.3), *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (Figure 4.4), and *L. pentosus* KUB-ST10-1 (Figure 4.5), respectively, hence, *B. licheniformis* DSM 13 which gave the highest enzyme activity was selected for further studies.

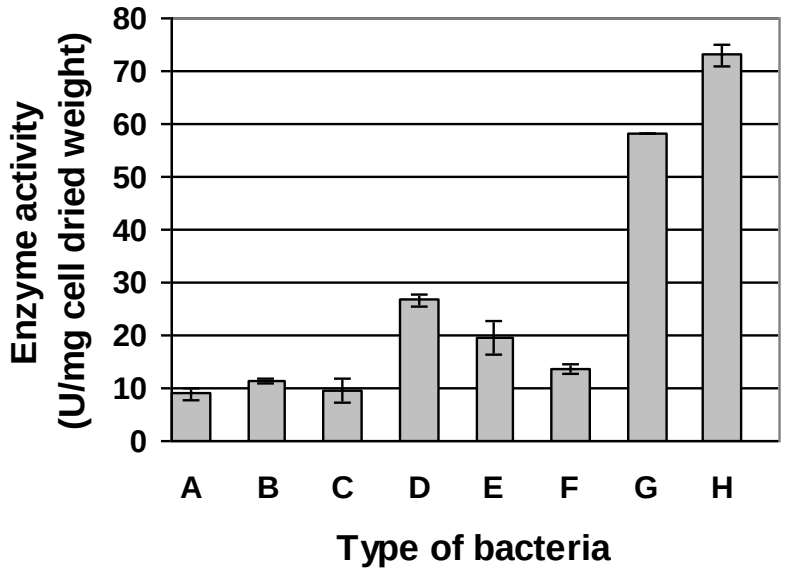


Figure 4.2 Comparison of the β-galactosidase activity from eight strains of bacteria based on per mg of cell dried weight. (A) *Pediococcus acidilactici* KUB-M6; (B) *P. acidilactici* KUB-M14; (C) *P. acidilactici* KUB-M15; (D) *Lactobacillus pentosus* KUB-ST10-1; (E) *L. bulgaricus* TISTR 451; (F) *L. sakei* TISTR 911; (G) *L. delbrueckii* subsp. *bulgaricus* DSM 20081; (H) *Bacillus licheniformis* DSM 13.

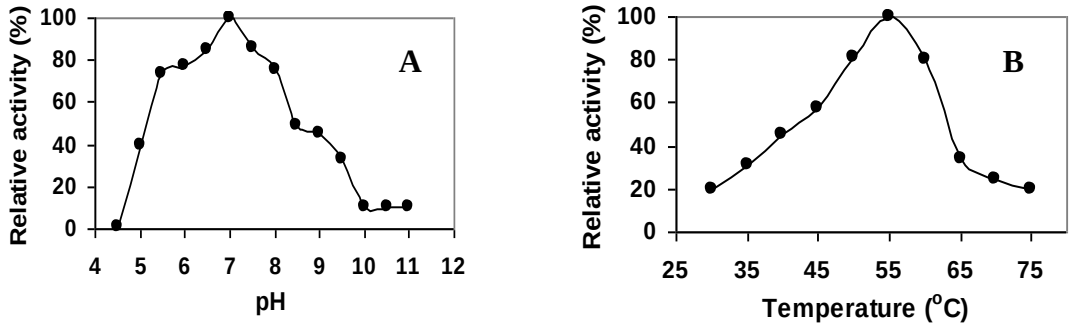


Figure 4.3 Optimum pH (A) and temperature (B) of crude extract from *B. licheniformis* DSM 13 using 20 g/L lactose as substrate in NB.

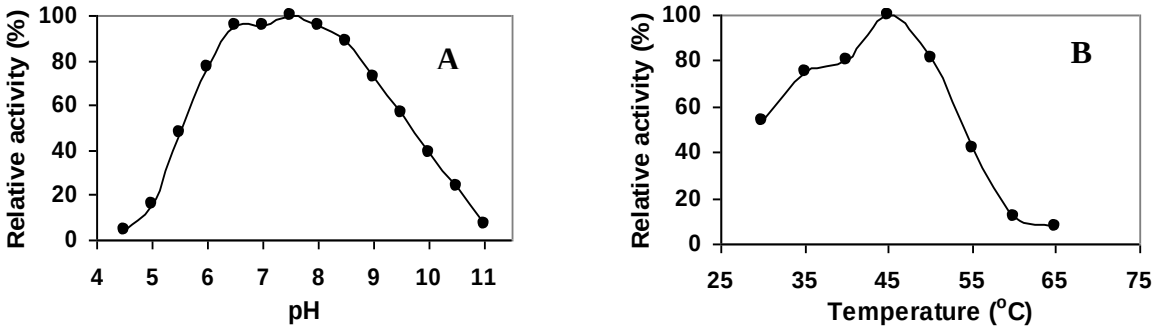


Figure 4.4 Optimum pH (A) and temperature (B) of crude extract from *L. delbruskii* subsp. *bulgaricus* DSM 20081 using 20 g/L lactose as substrate in MRS.

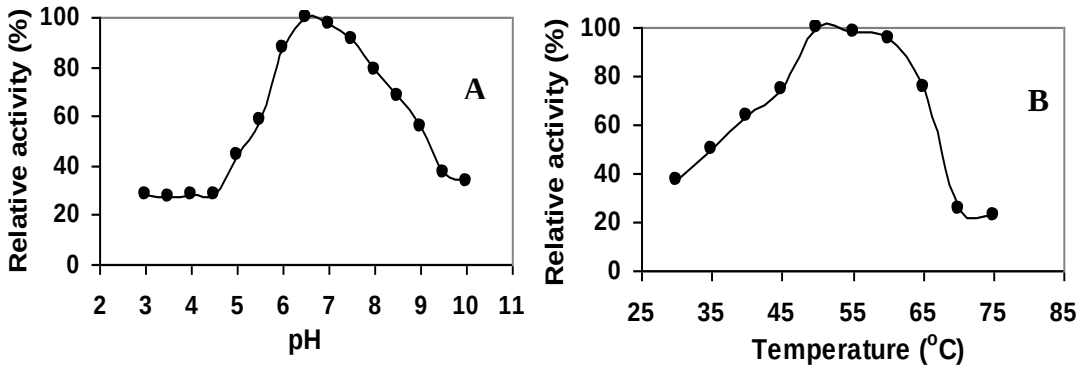


Figure 4.5 Optimum pH (A) and temperature (B) of crude extract from *L. pentosus* KUB ST10-1 using 20 g/L lactose as substrate in MRS.

To compare cell growth and enzyme activity, *B. licheniformis* DSM 13 was cultivated in NB medium containing 20 g/L lactose as substrate at 37^{o}C under the aerobic condition and 150 rpm constant shaking in the incubator shaker. The result shows that the cells had grown rapidly from time 5 h to 10 h and gradually increased until at 24 h (Figure 4.6) whereas the enzyme activity was slowly increased at the initial time and rapidly increased from 10 h to 24 h. It shows the highest activity of 0.35 U/mL at 24 h as well. We can summarized that the amount of the enzyme activity of *B. licheniformis* DSM 13 was simutaneously produced along with the cell growth.

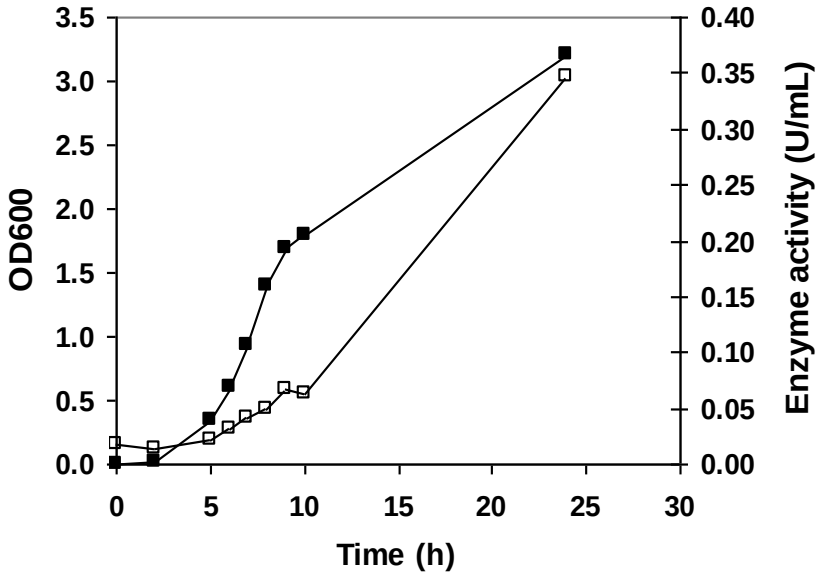


Figure 4.6 Comparison of cell growth (■) and enzyme activity (□) from whole cell extract of *B. licheniformis* DSM 13 in NB medium containing 20 g/L lactose and 150 rpm constant shaking.

Besides, it was found that the enzyme activity of *B. licheniformis* DSM 13 was higher in the whole cell extract than in broth of the cultivation (Figure 4.7) this indicated that the β-galactosidase enzyme from *B. lichemiformis* DSM 13 could be an intracellular enzyme as other bacteria such as *Lactobacillus reuteri* (Nguyen *et*

al., 2006), *Bacillus circulans* (Fujimoto *et al*., 1998), and *B. stearothermophilus* (Chen *et al*., 2008).

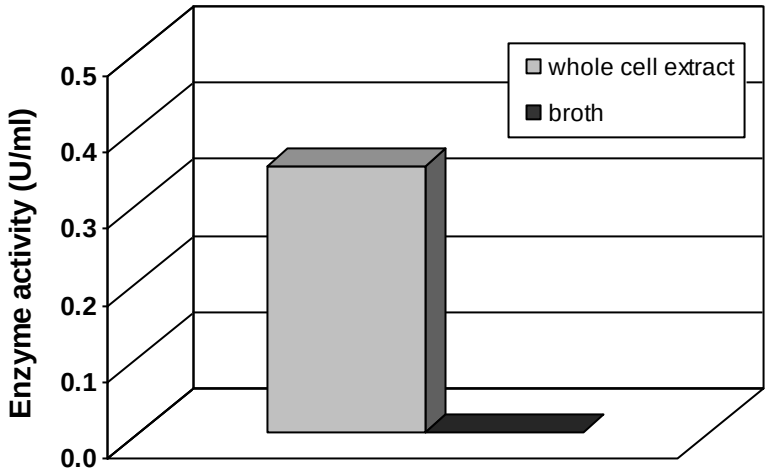


Figure 4.7 Comparison of enzyme activity from whole cell extract and broth of *B. licheniformis* DSM 13 cultivated in NB medium using 20 g/L lactose as substrate for 24 h.

4.3 Cloning of β-galactosidase gene from *B. licheniformis* DSM 13

The β-galactosidase genes or *lacA* genes were amplified by PCR using oligonucleotides as the primer which designed base on the sequencing data of *Bacillus licheniformis* DSM 13 from the Genbank an accession no. AE017333. The 2,055 nucleic acids of *lacA* gene were successfully amplified and ligated into p10HisFLAG vector, then transformed into the *E. coli* TOP10 and DH5α electro-competent cells. To select the cloned, the colonies were then spot onto the agar plate containing 2% X-gal as substrate and 100 mM IPTG as an inducer and amplicilin as the resistant antibiotic. The blue colonies were occurred after incubate at 37^{o}C overnight (Figure 4.8) and the agarose electrophoresis gel (Figure 4.9) showed that 4 clones from the transformation including p10HisFLAG and the gene of interest were

done double digestion with the two restriction enzymes, *Xho*I and *Kpn*I. Consequently, two band of DNA occurred, one band at approximately 2 kb was the gene of interest and the other one at 5 kb was the gene from p10HisFLAG vector because of the size of vector p10HisFLAG from a single digestion. This means that *E. coli* was harboring the recombinant plasmid of β-galactosidase from *B. licheniformis* DSM 13 resulting in pOJBlilacA1. Initially, the expression vector, pFLAG-CTS (Sigma) containing signal peptide (*ompA*) was used for the expression of *lacA* gene from *B. licheniformis* DSM 13 (data not shown). However, no enzyme activity was detected for neither intracellular nor extracellular enzyme. It suggested that the enzyme is probably too large in size for secretion.

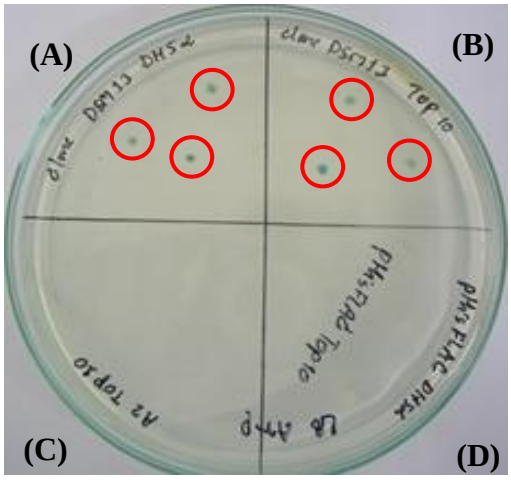


Figure 4.8 Blue colonies of recombinant β-galactosidase from *B. licheniformis* DSM 13 in *E. coli* DH5α (A) and TOP10 (B) compare with recombinant chitinase in *E. coli* TOP10 (C) and empty vector p10HisFLAG in both of *E. coli* TOP10 and DH5α (D) on agar plate containing ampicillin, 2% X-gal, and 100 mM IPTG.

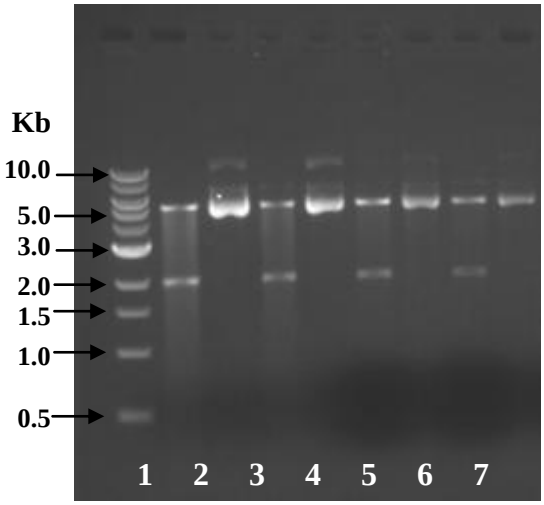


Figure 4.9 Agarose electrophoresis gel of recombinant plasmid from *lacA* gene of *B. licheniformis* DSM 13. Lane 1, DNA marker (BioLabs) ; lane 2, 4, 6 and 8, double digestions of recombinant DNA; lane 3, 5, 7 and 9, single digestion of p10HisFLAG vector.

However, intracellular enzyme activity was also very low for detection. Subsequently, this expression vector was modified by removing *ompA* and the addition of ten histidine-tag at the carboxyl terminus resulting in the expression plasmid pOJBlilacA2, which yielded high expression of the enzyme as shown the direction of the construction from the original backbone plasmid, pFLAG-CTS, to pOJBlilacA2 (Figure 4.10) as well as the details of pOJBlilacA2 constuction has been described in Figure 4.11.

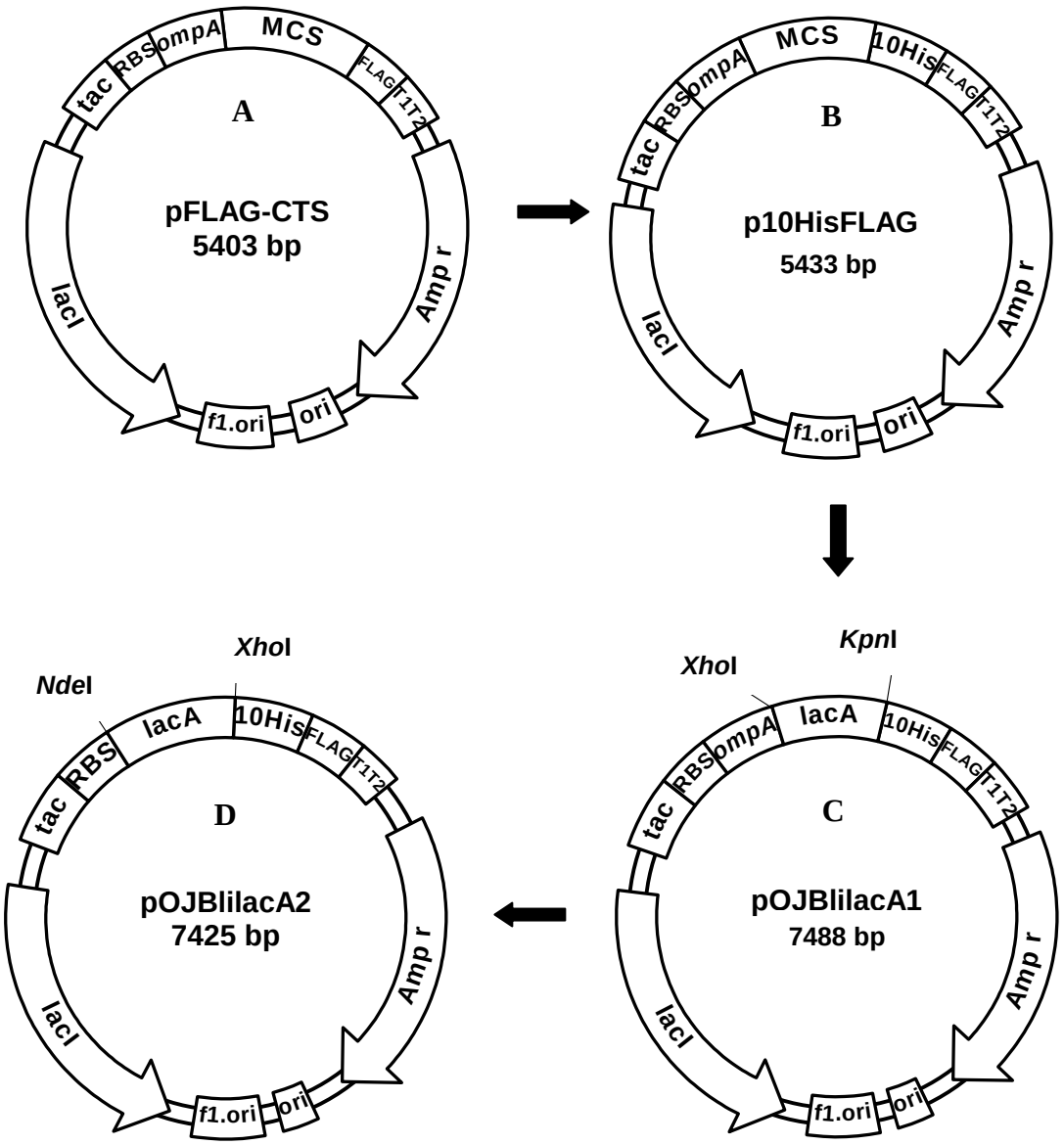


Figure 4.10 Schematic overview of pOJBlilacA2 construction. (A) pFLAG-CTS, purchased from Sigma Aldrich Corp; (B) p10HisFLAG, modified from pFLAG-CTS by adding 10 histidine after MCS region (MY's Group was constructed this plasmid); (C) pOJBlilacA1, inserted *lacA* gene from *B. licheniformis* DSM 13 into p10HisFLAG; (D) pOJBlilacA2, the plasmid containing *lacA* gene without *ompA.*

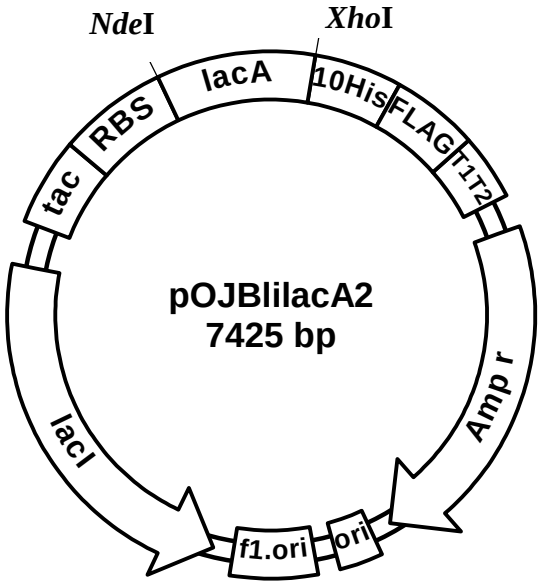


Figure 4.11 Schematic overview of the pOJBlilacA2 plasmid used in this study (based on pFLAG-CTS vector, Sigma, St. louis, Mo, USA). tac promoter; RBS, Shine-Dalgarno ribosome binding site; structural gene *lacA*; His-Tag; FLAG, octapeptide for binding of Anti-FLAG M2 Monoclonal antibody; T_1T_2, ribosomal RNA operon compound terminator; Amp r, ampicillin resistance marker; ori (pBR322 ori), double strand replication of pFLAG-CTS; f1•ori, single strand replication of positive strand of pFLAG-CTS via M13 K07 Helper Phage; *lacI*, repression of tac promoter.

4.4 Amino acid sequence alignment of lacA of the β-galactosidases from *B. licheniformis* DSM 13

Figure 4.12 shows the amino acid sequence alignment of β-galactosidases from *B. licheniformis* DSM 13 with β-galactosidases sequences from other 8 strains of bacteria which are belonging to glycosyl hydrolase family 42, GH-42, which

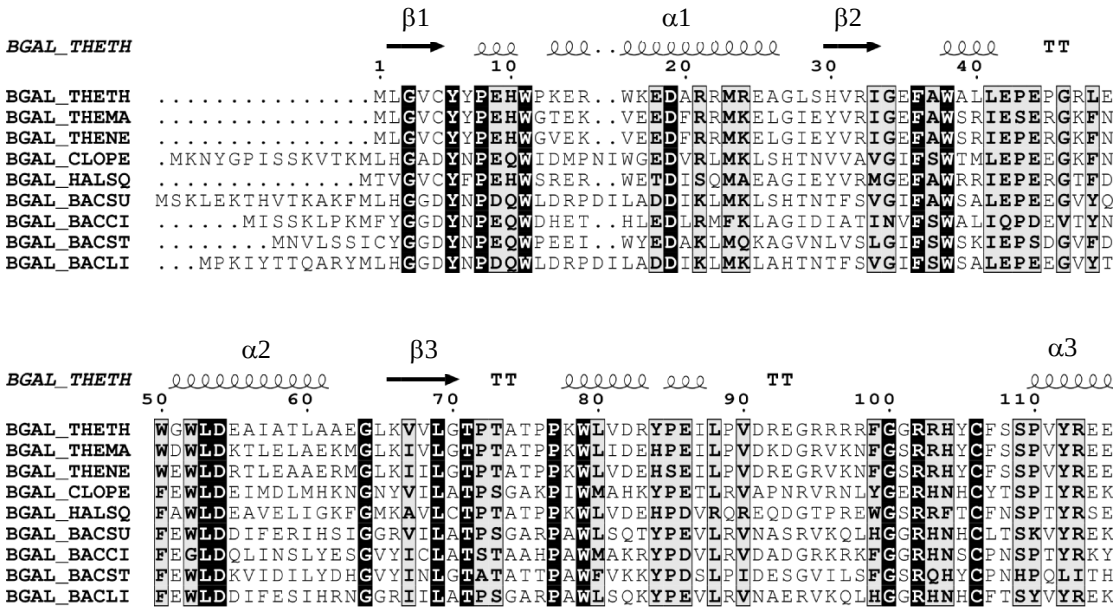


Figure 4.12 Amino acid sequence alignment of lacA of the β-galactosidases from *Bacillus licheniformis* DSM 13, BGAL_BACLI; and other β-galactosidases from 8 strains of bacteria in glycosyl hydrolase family 42; *Thermus thermophilus* A4, BGAL_THETH; *Thermotoga maritime*, BGAL_THEMA; *T. neapolitana*, BGAL_THENE; *Clostridium perfringens*, BGAL_CLOPE; *Haloferax* sp., BGAL_HALSQ; *Bacillus subtilis*, BGAL_BACSU; *B. circulans*, BGAL_BACCI; and *B. stearothermophilus*, BGAL_BACST. β-strands forming the core of the TIM-barrel are referred as β1 to β8, α-helices are referred as α1 to α8. A white character in a box indicates strict identity, while a black character in a frame indicates similarity across a group.The white down arrow and the black down arrow indicate the positions of acid-base catalyst and nucleophilic recognition site, respectively, of *T. thermophilus* A4 β-galactosidase.

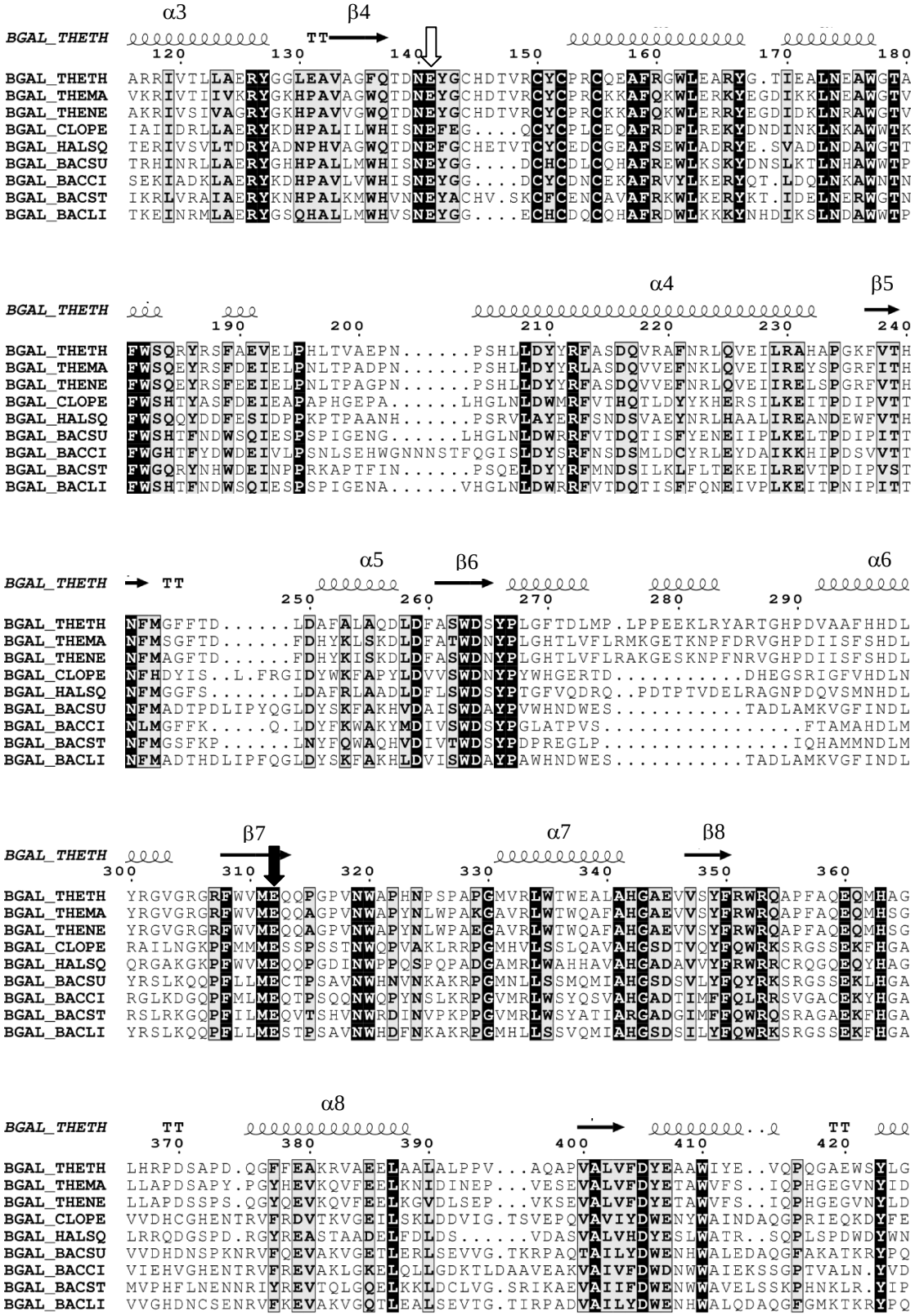


Figure 4.12 (Continued).

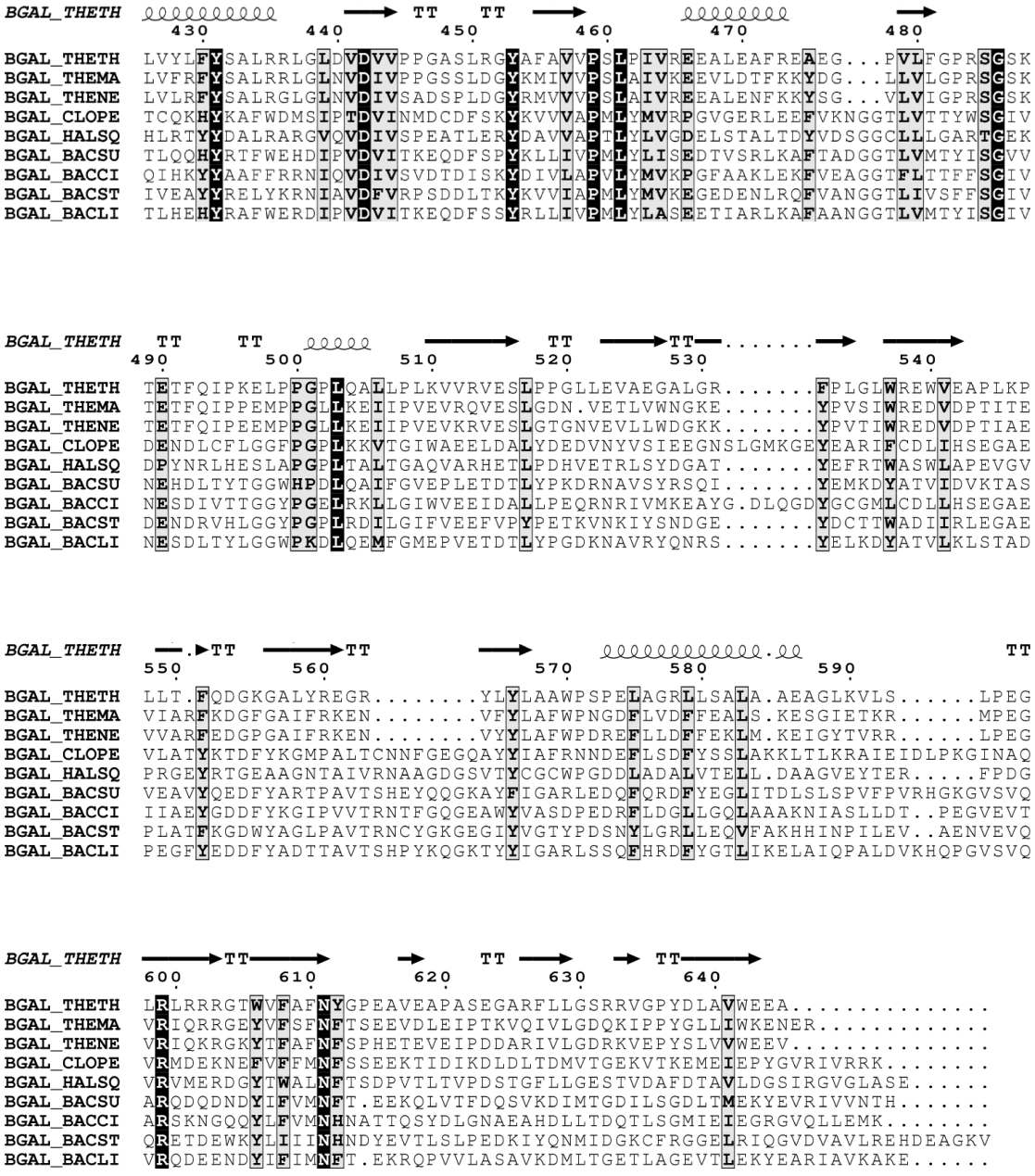


Figure 4.12 (Continued).

submitted to Swiss-PROT. The alignment of *B. licheniformis* DSM 13 β-galactosidases and similar β-galactosidases from GH-42 in the relative conserved regions compared to the secondary structure of *Thermus sp*. A4 β-galactosidase. Amino acid sequence similarities of *B. licheniformis* DSM 13 β-galactosidases (GenBank accession number AAU43090) (Veith *et al.*, 2004) and the reported

sequences of β-galactosidases from other strains of GH-42 range between 76.6 for *B. subtilis* β-galactosidase (GenBank accession number ABQM01000009) (Srivatsan *et al.*, 2008), 47.9 for *Clostridium perfringens* β-galactosidase (GenBank accession number BAB79873) (Shimizu *et al.*, 2002), 36.9 for *B. circulans* β-galactosidase (GenBank accession number AAA22258) (Nelms and Fotheringham, 1993), 35.6 for *B. stearothermophilus* β-galactosidase (GenBank accession number P19668) (Hirata *et al.*, 1986), 26.9 for *Thermotoga neapolitana* β-galactosidase (GenBank accession number AAC24217) (Swiatek *et al.*, 1998), 26.8 for *Thermus thermophilus* β-galactosidase (GenBank accession number BAA28362) (Ohtsu, 1996), 25.3 *T. maritime* β-galactosidase (GenBank accession number AAD36270) (Nelson *et al.*, 1999) and 24.9 for *Haloferax* sp. (strain Aa 2.2) β-galactosidase (GenBank accession number AAB40123) (Holmes and Dyall-Smith, 2000). The amino acid sequence of β-galactosidase from *B. licheniformis* DSM13 shows the highest similarity to β-galactosidase from *B. subtilis* when the sequences were aligned with CLUSTAL W and the secondary structure of A4-β-Gal of *Thermus thermophilus* A4 was assigned with Espript 2.2 that was available on the internet. The amino acid sequence alignment of β-galactosidases from *B. licheniformis* DSM 13 with β-galactosidases sequences from other 8 strains of bacteria (all from glycosyl hydrolase family 42, GH-42) revealed that the sequence of this enzyme conserved with Glu141 and Glu312, respectively. It was suggested that Glu141 is a general acid-base catalyst and Glu312 is a nucleophile in the active site of β-galactosidases from *T. thermophilus* A4, a member of GH-42 (Hidaka *et al.*, 2002). As judged from the sequences of the *lacA* gene from *B. licheniformis* DSM 13 and the conservation of the catalytic residues, it possibly belongs to the glycosyl hydrolase family 42. Besides, we found that the *lacA* gene had the highest sequence similarity to *B.*

subtilis β-galactosidases gene within the comparison sequences. Thus, it confirms that this enzyme should belong to the glycosyl hydrolase family 42. Interestingly, each of the organisms that are source of enzymes in family 42 can survive extreme circumstances, as these organisms are psychrotrophic (*Arthrobacter* sp.), thermophilic (*B. stearothermophilus*, *Thermotoga* sp., *Thermus* sp.), halophilic (*Haloferax* sp.), or spore-forming (*Bacucillus* sp. and *Clostridium* sp.) (Ohtsu *et al.*, 1998).

4.5 Overexpression of β-galactosidase gene from *B. licheniformis* DSM 13

To select the suitable medium for an overexpression of β-galactosidase enzyme, the *E. coli* harboring recombinant β-galactosidase gene were cultivated in LB, TB, and YT medium for 24 h. The highest of the growth levels were found in the TB medium. Indicated that TB medium was more suitable to cultivate these bacteria than that of the other media, however, it was no significance among the media (Figure 4.13). To optimize the optical density of the crude extract from recombinant β-galactosidase from *B. licheniformis* DSM 13, it was found that the optical density at OD_{600} 1.0 or 1.5 expressed the band of the protein of interest was to be clear than that of the other bands (Figure 4.14) and the result shows that the induction time was suitable at 4 h (Figure 4.15), although, it shows no difference among the other media. The partial characterization was investigated using Ni-NTA bead column, the crude extract was loaded onto the colume (by manully). The band of the protein of interest occurred at the right position approximately 78 kDa (Figure 4.16).

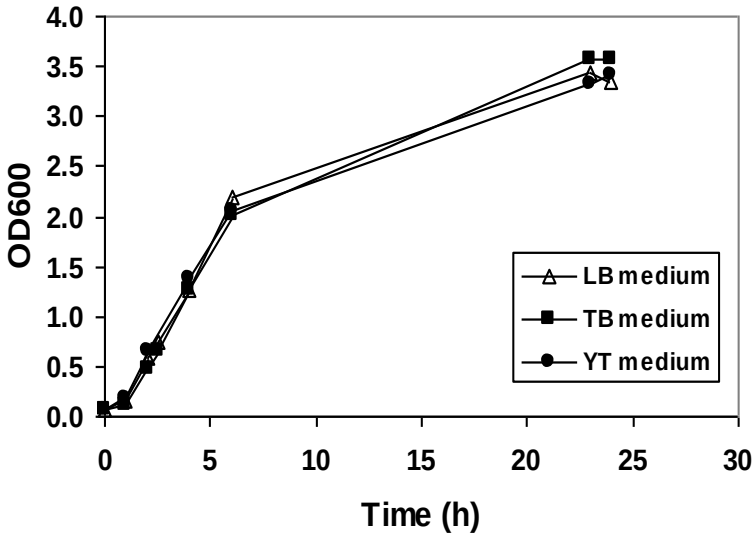


Figure 4.13 The growth curve of *E. coli* TOP10 harboring recombinant of *lacA* gene into p10HisFLAG in various medium.

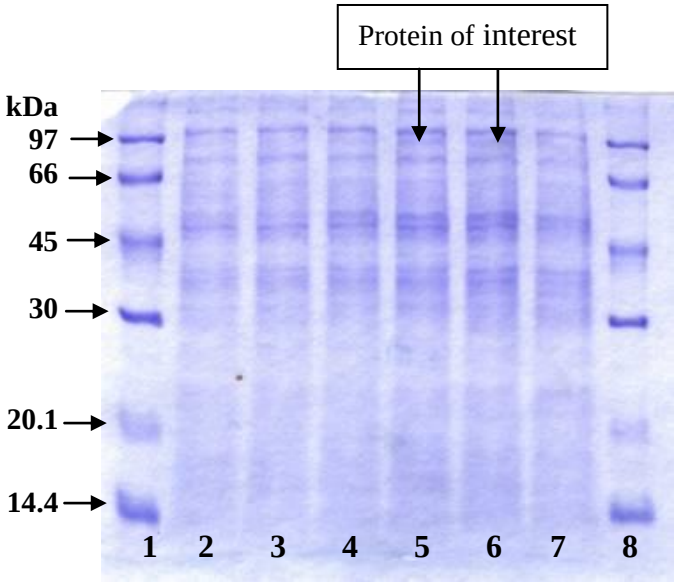


Figure 4.14 SDS-PAGE analysis of crude extract of recombinant β-galactosidase from *B. licheniformis* DSM 13 at different optical density. Lane 1 and 8, protein ladder (Amersham); lane 2-7, crude extract from various optical densitys at OD_{600}: 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0, respectively.

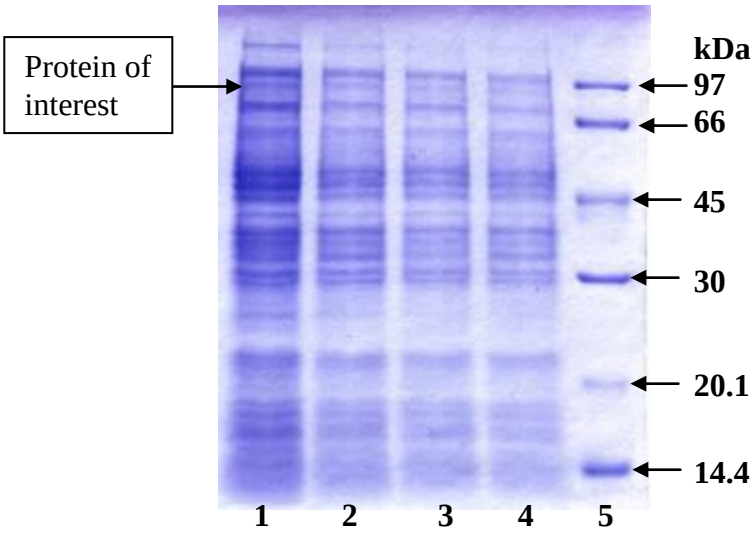


Figure 4.15 SDS-PAGE analysis of crude extract of recombinant β-galactosidase from *B. licheniformis* DSM 13 during various time inductions. Land 1, at time 4 h; lane 2, at time 6 h; lane 3, at time 8 h; lane 4, overnight, lane 5, protein ladder (Amersham).

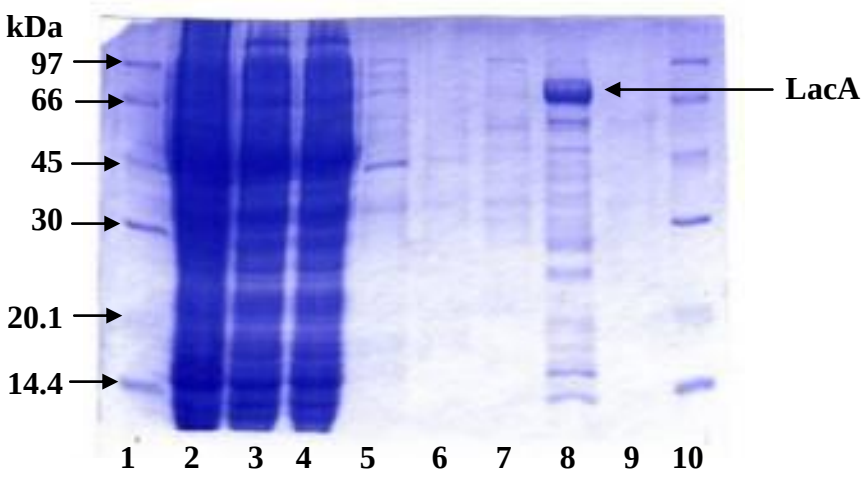


Figure 4.16 SDS-PAGE analysis of purified protein using Ni-NTA bead column of affinity chromatography. Lane 1 and 10, protein ladder (Amersham); lane 2, crude supernate; lane 3, flow-through fraction; lane 4, 1st washed fraction (washed 1.1); lane 5, 2nd washed fraction

(washed 1.2); lane 6, 3rd washed fraction (washed 2); lane 7, 1st elution fraction; lane 8, 2nd elution fraction; lane 9, 3rd elution fraction.

The cloned of β-galactosidase from *B. licheniformis* DSM 13 was cultivated in TB medium. The result shows that the cell of *E. coli* TOP10 haboring the recombinant β-galactosidase shows no different between with and without IPTG induction, noticed that the enzyme activity in medium with IPTG induction was slightly higher than that of no IPTG induction (Figure 4.17 and 4.18) when compared of both cultivations in term of specific activity. It presents approximately 10 times of the cultivation with IPTG induction higher than that of without IPTG (Figure 4.19). For this reason IPTG induction had the influence of β-galactosidase activity of *B. licheniformis* DSM 13.

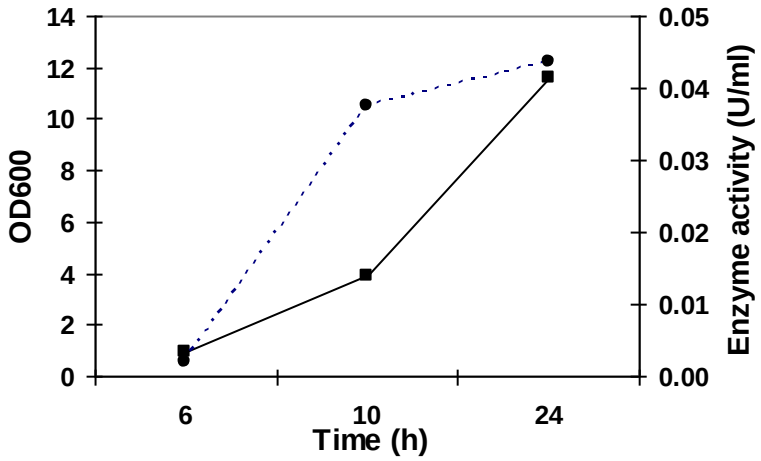


Figure 4.17 Comparison of cell growth (■) and enzyme activity (●) of *E. coli* TOP10 harboring p10HisFLAG (with *ompA*) and *lacA* gene from *B. licheniformis* DSM 13 in TB medium without IPTG.

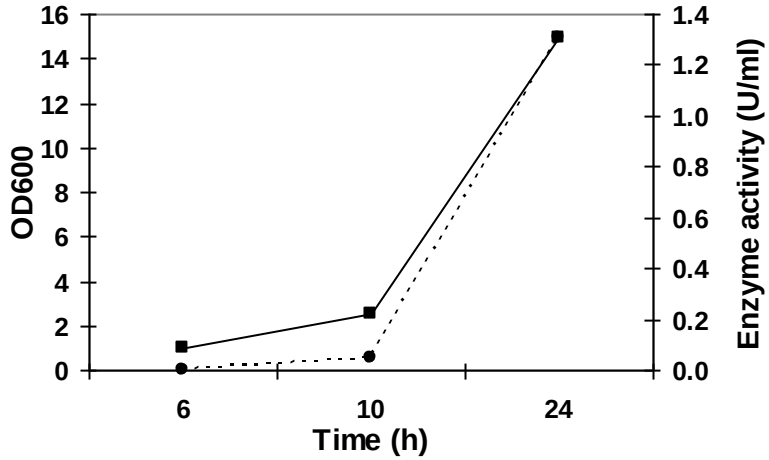


Figure 4.18 Comparison of cell growth (■) and enzyme activity (●) of *E. coli* TOP10 harboring p10HisFLAG (with ompA) and *lacA* gene from *B. licheniformis* DSM 13 in TB medium with 1 mM IPTG.

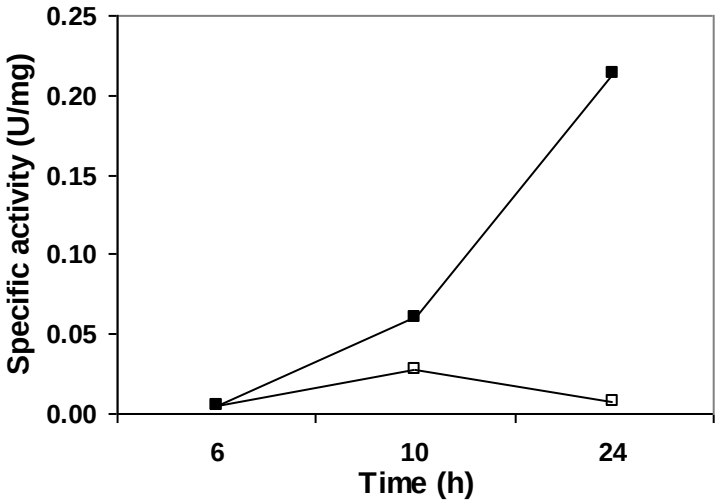


Figure 4.19 Specific activity of recombinant enzyme in *E. coli* TOP10 harboring p10HisFLAG (with *ompA*) and *lacA* gene from *B. licheniformis* DSM 13 in TB medium without (□) and with (■) 1 mM IPTG.

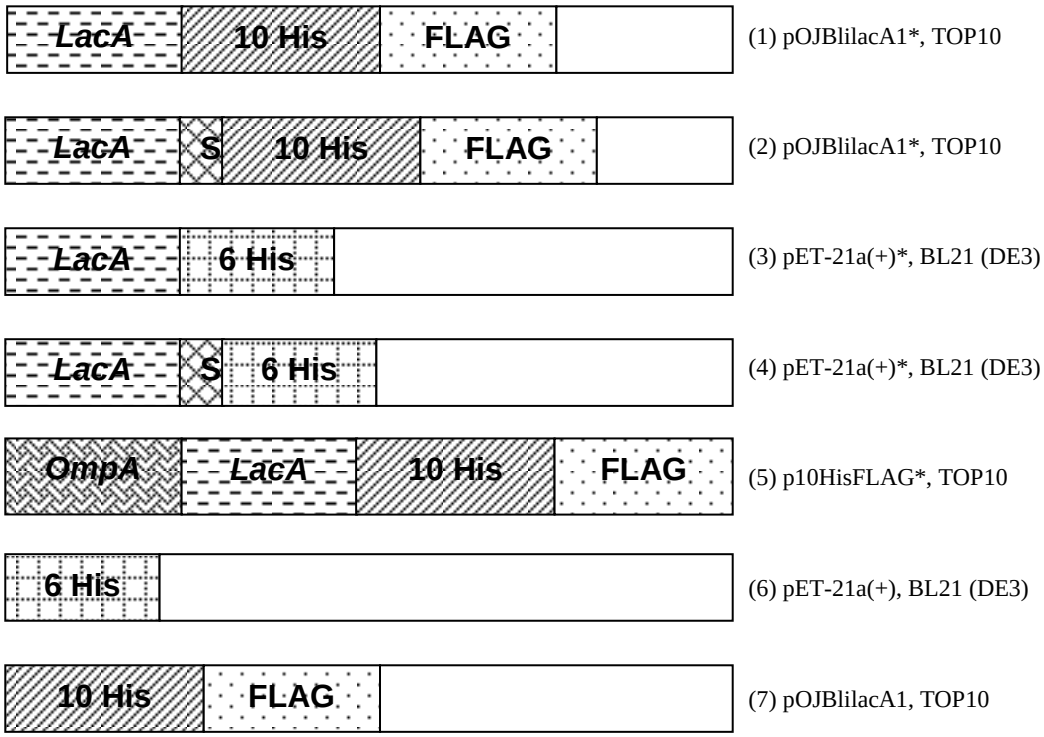


Figure 4.20 Schematic overview of plasmids construction (1-5) compared with backbone plasmid (6 and 7). Line (1) and (2), plasmid construction from p10HisFLAG without *ompA*; line (3) and (4), plasmid construction from pET-21a(+); line (5), plasmid construction from p10HisFLAG with *ompA*; line (6), pET-21a(+) backbone vector; line (7), p10HisFLAG backbone vector. Abbrev., S = stop codon, His = histidine. Remarkable symbol, black star (*) means the original plasmid before modified.

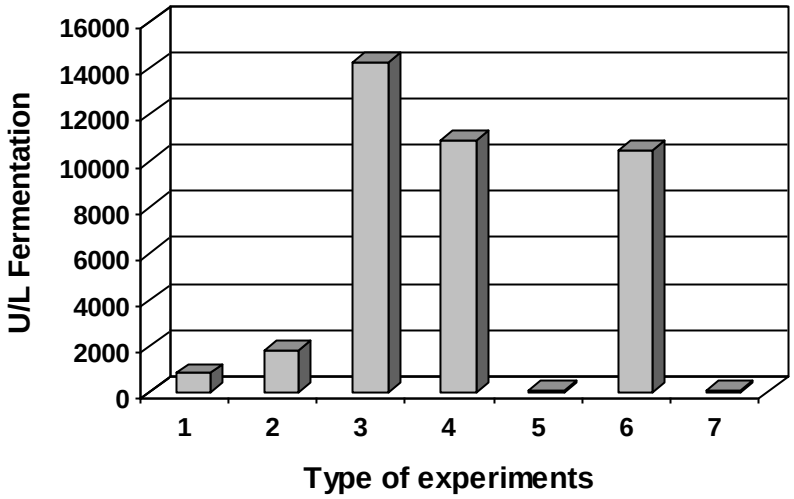


Figure 4.21 Comparison the specific activities of recombinant strains of bacteria in TB medium with 1 mM IPTG induction at 25°C. Lane 1, *lacA* insert into pOJBlilacA1 in *E. coli* TOP10; lane 2, *lacA* and stop codon insert into pOJBlilacA1 in *E. coli* TOP10; lane 3, *lacA* insert into pET-21a(+) in *E. coli* BL21 (DE3); lane 4, *lacA* and stop codon insert into pET-21a(+) in *E. coli* BL21 (DE3); lane 5, *ompA*, *lacA* and stop codon insert into p10HisFLAG in *E. coli* TOP10; lane 6, pET-21a(+) in *E. coli* BL21 (DE3); lane 7, p10HisFLAG in *E. coli* TOP10.

The recombinant β-galactosidase from pOJBlilacA2 was cultivated in both of inducers, IPTG and lactose. To induce at 25°C and 120 rpm, it was found that no induction, 1 g/L lactose and 0.01 mM IPTG gave the enzyme activity higher than that of the other cultivattions approximately 60, 50 and 40 kU/L fermentation, respectively (Figure 4.22). When cultivated the recombinant bacteria at 18 °C and 120 rpm, the result shows in Figure 4.23. The highest activity was obtained at 45 h induced at 18°C with 0.1 mM IPTG in TB medium, whereas the activity of the cultivation with 0.5 mM IPTG was reduced. This result was the same as using

lactose as the inducer. When the concentration of lactose was increased, the activity of β-galactosidase was reduced. In addition, the proper medium was influenced the overexpression. This result supported that the TB medium was suitable for *E. coli* TOP10 harboring *lacA* gene for the overexpression. The specific activity of β-galactosidase enzyme with 0.01 mM IPTG in TB medium was obtained approximately 76 kU/L of fermentation broth.

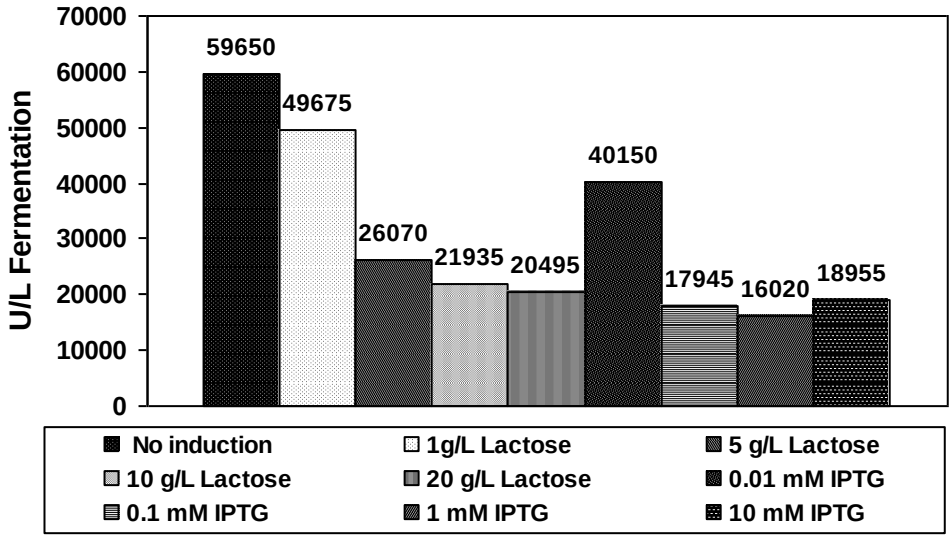


Figure 4.22 Comparision of the activity of crude extract from recombinant β-galactosidase (pOJBlilacA2) from *B. licheniformis* DSM 13 expressed in *E. coli* TOP10 in TB medium with and without IPTG and lactose at various concentrations at 25^{o}C with 120 rpm induction.

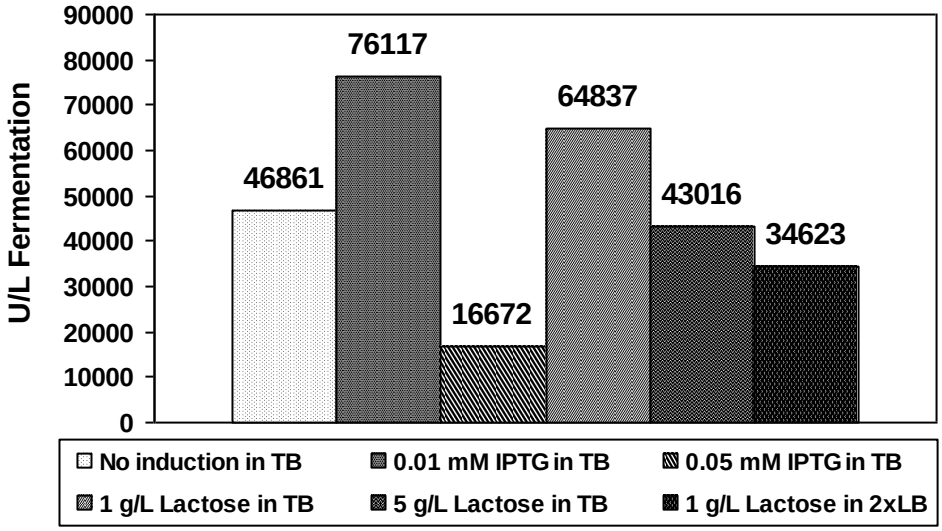


Figure 4.23 The activity of crude extract from recombinant β-galactosidase (pOJBlilacA2) from *B. licheniformis* DSM 13 in TB medium with and without IPTG and lactose at various concentrations at 18^{o}C, 120 rpm induction.

4.6 Purification of recombinant β-galactosidase from *B. licheniformis* DSM 13

Homologous expression of the tagged protein resulted in the production of ~76 kU of β-galactosidase activity per liter of fermentation broth, which could be efficiently purified by metal affinity chromatography in one single step. Due to the fact that the β-galactosidase gene was engaged with the pOJBlilacA1, the enzyme was purified in one single-step to apparent homogeineity using the Ni Sepharose 6 fast flow column. The various conditions for enzyme purification were verified as shown in Table 4.1. The specific activities from four purification conditions were to be 159, 137, 166 and 271 U/mL, respectively. After elution with buffer B, the purified enzyme was obtained after 100% gradient as shown in Figure 4.24-4.27. The

affinity chromatography for recombinant β-galactosidase from *B. licheniformis* DSM 13 operated with 0.5 mL/min initial flow rate using buffer A (50 mM sodium phosphate buffer, pH 6.5 and 0.2 M NaCl, 20 mM imidazol) and then the elution step using buffer B (50 mM sodium phosphate buffer, pH 6.5, 0.2 M NaCl and 1 M imidazol) was found to be appropriated condition for *B. licheniformis* DSM 13 β-galactosidase purification (Figure 4.27). The results revealed that it was corresponding to a single purification of the recombinant β-galactosidase from *L. reuteri* and more efficient than the three-step-purification that needed to be used for the wild type β-galactosidase, moreover, the activity of recombinant β-galactosidase enzyme from *L. reuiteri* L103 strain was to be 9-folds higher than that of the wild type strain (Halbmayr *et al.*, 2008a). Besides, Halbmayr indicated that the properties of the recombinant enzyme were found to be essentially the same as the enzyme produced from the wild type strain and it was easier to purify the recombinant enzyme than the wild type enzyme corresponding to the physiochemical and catalytic activity of both recombinant and wild type enzyme appear to be very similar (Torres and Lee, 1995). Therefore, only the properties of recombinant enzyme were studied in this section.

Table 4.1 Protein purification treatment of recombinant β-galactosidase from *B. licheniformis* DSM 13 using the Ni Sepharose 6 fast flow column of affinity chromatography.

Treatment	**Buffer A**	**Buffer B**	**Specific activity* (U/mg)**
1	50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl (1.5 ml/min)	50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl, 1 M imidazol (1.5 ml/min)	159
2	50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl (0.5 ml/min)	50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl, 1 M imidazol (1.5 ml/min)	137
3	50 mM Sodium phosphate buffer pH 6.5 and 0.5 M NaCl 20 mM imidazol (0.5 ml/min)	50 mM Sodium phosphate buffer, pH 6.5, 0.5 M NaCl and 500 mM imidazol (1.5 ml/min)	166
4	50 mM Sodium phosphate buffer, pH 6.5 and 0.2 M NaCl, 20 mM imidazol (0.5 ml/min)	50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl and 1 M imidazol (1.5 ml/min)	271

Remarkable symbol (*) means specific activity was derived after enzyme was passed through ultramembrane purification step.

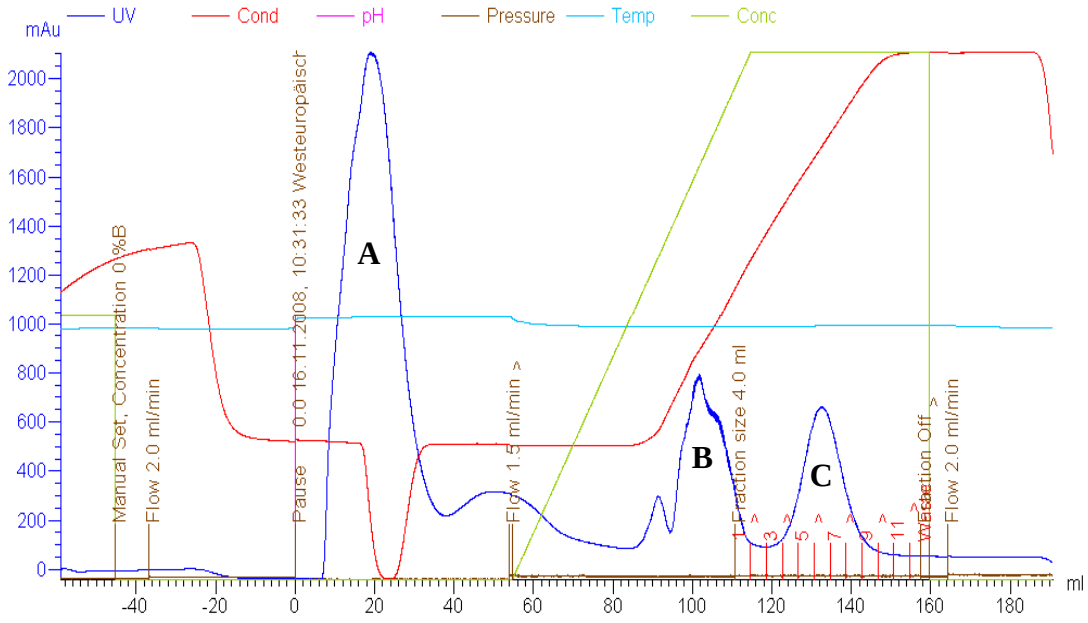


Figure 4.24 Chromatogram of affinity chromatography of recombinant β-galactosidase from *B. licheniformis* DSM 13 with 1.5 mL/min initial flow rate, 1.5 mL/min elution, buffer A : 50 mM Sodium phosphate buffer, pH 6.5 and 0.2 M NaCl, buffer B : 50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl and 1 M imidazol. Peak (A) and (B), flowthrough fraction; peak (C) purified enzyme fraction.

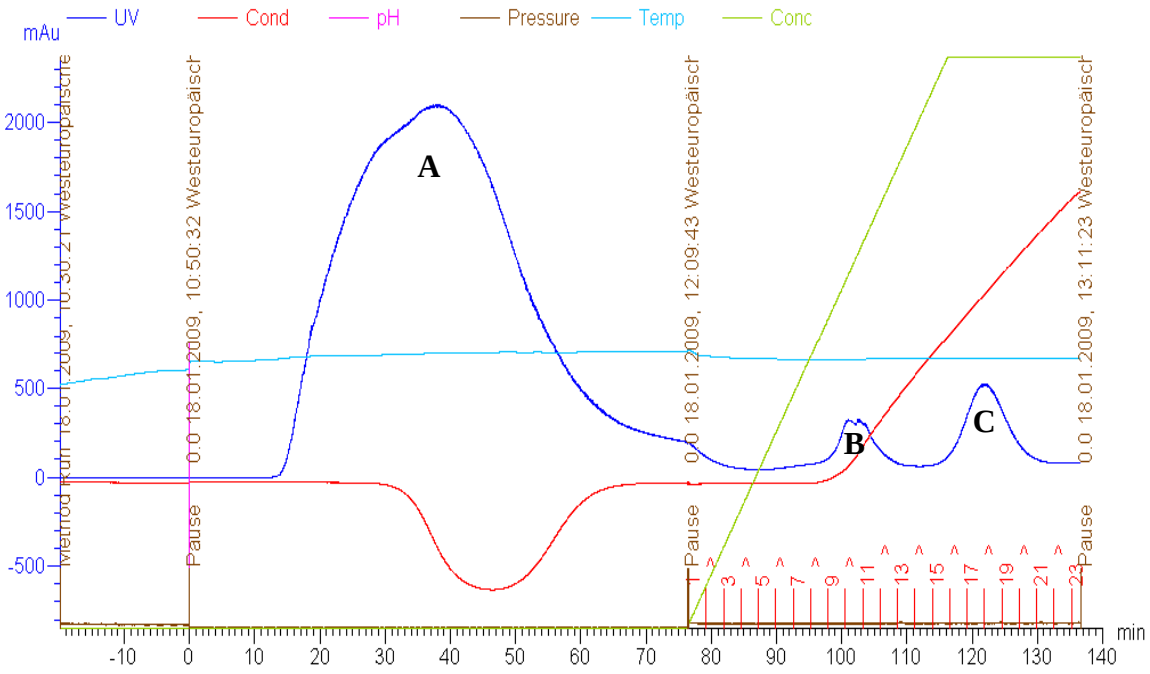


Figure 4.25 Chromatogram of affinity chromatography of recombinant β-galactosidase from *B. licheniformis* DSM 13 with 0.5 mL/min initial flow rate, 1.5 mL/min elution, buffer A : 50 mM Sodium phosphate buffer, pH 6.5 and 0.2 M NaCl, buffer B : 50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl and 1 M imidazol. Peak (A) and (B), flowthrough fraction; peak (C) purified enzyme fraction.

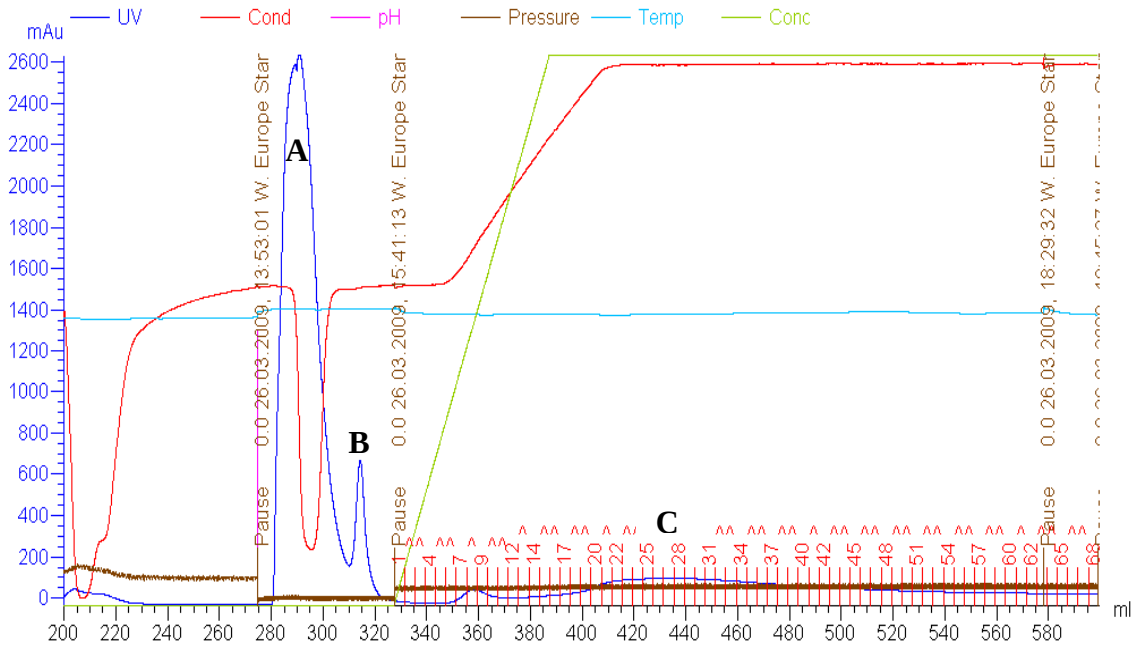


Figure 4.26 Chromatogram of affinity chromatography of recombinant β-galactosidase from *B. licheniformis* DSM 13 with 0.5 mL/min initial flow rate, 1.5 mL/min elution, buffer A : 50 mM Sodium phosphate buffer pH 6.5 and 0.5 M NaCl 20 mM imidazol, buffer B : 50 mM Sodium phosphate buffer, pH 6.5, 0.5 M NaCl and 500 mM imidazol. Peak (A) and (B), flowthrough fraction; peak (C) purified β-galactosidase enzyme fraction.

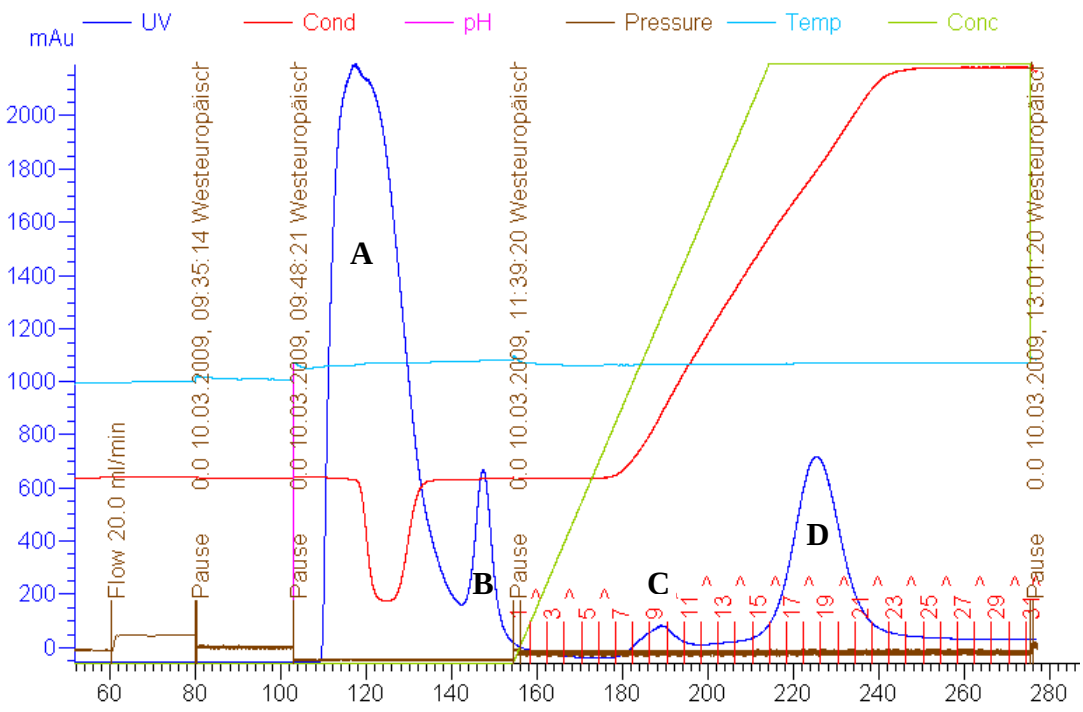


Figure 4.27 Chromatogram of affinity chromatography of β-galactosidase from *B. licheniformis* DSM 13 with 0.5 mL/min initial flow rate, 1.5 mL/min elution, buffer A : 50 mM Sodium phosphate buffer, pH 6.5 and 0.2 M NaCl, 20 mM imidazol, buffer B : 50 mM Sodium phosphate buffer, pH 6.5, 0.2 M NaCl and 1 M imidazol. Peak (A), (B), and (C), flowthrough fraction; peak (C) purified β-galactosidase enzyme fraction.

Table 4.2 Purification of recombinant β-galactosidase from *B. licheniformis* DSM 13 over-expressed in *E. coli* TOP10.

Purification step	**Total enzymatic activity (Units)**	**Total proteins (mg)**	**Specific activity (Units/mg)**	**Purification folds**	**Recovery (%)**
Crude enzyme extract	6000	117.7	51.0	1.0	100
Affinity chromatography (Ni Sepharose fast flow)	4396	16.2	271.3	5.3	73

Purified recombinant enzyme with a specific activity of 271 U/mg proteins using *o*NPG as substrate was obtained with overall 73% recovery from crude enzyme (Table 4.2). Beside, the enzyme was test with other substrates, as 4-nitrophenyl-α-D-galactopyranoside and 4-nitrophenyl-β-D-manopyranoside, it was no any activity (data not shown). The specific enzyme activity of the purify enzyme obtained was less than that from the cloned *Pyrococcus woesei* gene coding thermostable β-galactosidase 20 fold (Dąbrowski *et al.*, 1998) but higher than that from the recombinant β-galactosidase from *Thermotoga maritima* which cloned into pET-28a (+) was found to be 12 folds (Li *et al.*, 2009a). The specific activity of purified β-galactosidase was compared with some other enzyme sources (Table 4.3). The

purified β-galactosidase from *B. licheniformis* DSM 13 had the highest specific activity when compared with the others.

Table 4.3 Comparison between the specific activities of purified β-galactosidase from *B. licheniformis* DSM 13 with the other enzyme sources.

Enzyme source	**Enzyme**	**Substrate**	**Specific activity (substrate) U/mg**	**Purification Steps**
Arthobacter strain F2	bglA	*o*NPG	33.3 (20 mM)	$(NH_4)_2SO_4$, DEAE cellulose [1]
B. subtilis	lacA	*o*NPG	125 (10 mM)	Heat treatment, $(NH_4)_2SO_4$, DEAE, gel filtration (sephacyl S-100) [2]
Kluyveromyces marxianus (or *fragilis*)	ebgA	*o*NPG	790 (5 mM)	Ion exchange, Gel filtration and ultrafiltration, Hydroxylapatite [3]
Thermotoga maritima	bglA	*p*NPGlc	11 (5 mM)	Heat treatment, Ni-NTA, Q-sepharose Mono Q [4]
Thermus sp. A4	A4-β-Gal	*o*NPG	48.6 (2.8 mM)	Q-sepharose, Hydroxy apatite, Mono Q, Gel filtration, Phenyl-superose [5]
Thermus sp. IB21	BgaA	*p*NPGal *p*NPGlc	88.6 (5 mM) 101 (5 mM)	Heat precipitation, Ni-affinity [6]
B. licheniformis DSM 13	LacA	*o*NPG	271.3 (22 mM)	Ni-sepharose, (This study)

Remark; [1](Nakagawa *et al.*, 2006), [2](Chen *et al.*, 2008), [3] (O'Connell and Walsh, 2007), [4](Goyal *et al.*, 2001), [5](Ohtsu *et al.*, 1998), [6](Kang *et al.*, 2005).

Although, *Kluyveromyces marxianus* β-galactosidase gave the highest specific activity, it was purified more than one purification step whereas β-galactosidase from *B. licheniformis* DSM 13 used a single step purification by affinity chromatography. This shows the great potential for the large scale of this enzyme production.

4.7 Characterization of β-galactosidase enzyme

4.7.1 Molecular weight of purified recombinant β-galactosidase

The recombinant β-galactosidase overexpressed in *E. coli* TOP10 when using affinity to purify the enzyme. The molecular mass of *B. licheniformis* DSM 13 β-galactosidase was approximately of 78 KDa using SDS-PAGE (Figure 4.28 A). For the SDS-PAGE, samples were performed by heating at 60^{o}C and active staining using 4-methylumbelliferyl *β*-D-galactopyranoside as the substrate, however, the single band showed on SDS-PAGE staining with coomassie blue staining but there was no any activity with the active staining (data not shown). For the native-PAGE, purified β-galactosidase enzyme showed a single band on both native coomassie blue staining and active staining (the active staining was done at 37^{o}C for 10 min) (Figure 4.28 B). The result indicated that the active staining showed activity for the band corresponding to a polypeptide of the homodimer of β-galactosidase from *B. licheniformis* DSM 13 as the band of native staining.

The homodimeric β-galactosidase from *B. licheniformis* DSM 13 is encoded by *lacA* gene, which consists of an open reading frame of 2055 bp encoding 687 amino acid residues with calculated molecular mass of 78 kDa where as Trân *et a.l* (1998) found that *B. licheniformis* ATCC 9800 β-galactosidase encoding *lacBl* had 771 bp encoding with 149 amino acid (Trân *et al.*, 1998). The recombinant β-galactosidase overexpressed in *E. coli* TOP10 had a molecular mass of

approximately 160 kDa and consisted of two indentical subunits of ~ 78 kDa subunit as shown on native PAGE and SDS-PAGE. The β-galactosidase gene have been reported from many species of *Bacillus*: *mbgA* gene from *B. megaterium* ATCC 14581 (Shaw *et al.*, 1998), *lacA* gene from *B. subtilis* SG68 (Daniel *et al.*, 1997), *bgaA and bgaB* gene from *B. stearothermophilus* ATCC 8005 (Hirata *et al.*, 1984; Chen *et al.*, 2008).

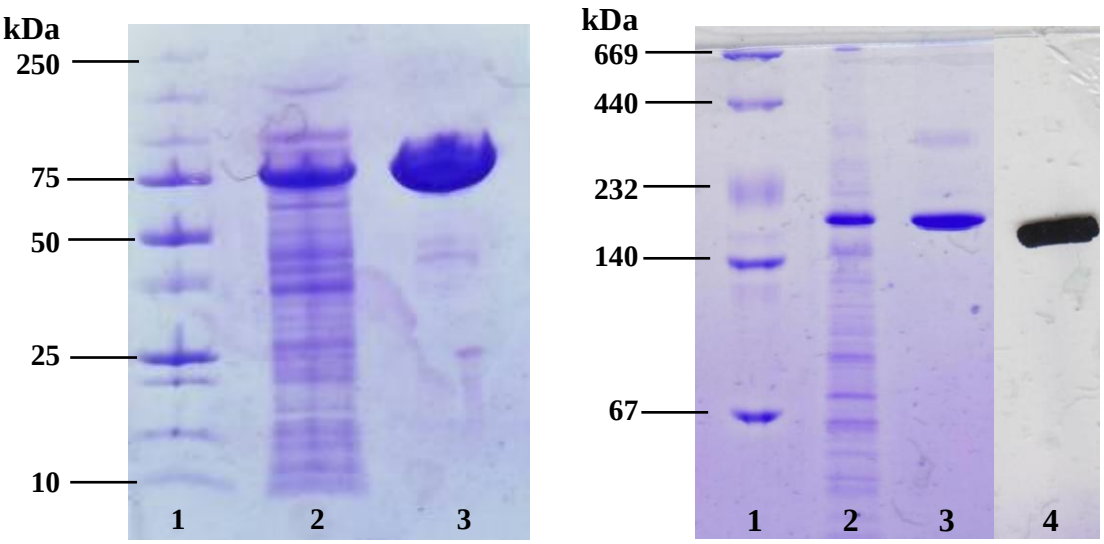


Figure 4.28 SDS-PAGE (A) and native PAGE (B) of β-galactosidase (*lacA*) from *B. licheniformis* DSM 13 over-expressed in *E. coli* TOP10. (A) Lane 1, recombinant molecular mass markers (Bio-Rad); lanes 2 and 3, Coomassie blue staining of crude extract (lane 2) and purified recombinant β-galactosidase (lane 3). (B) Lane 1, high molecular weight markers (GE Healthcare); lanes 2 and 3, Coomassie blue staining of crude extract (lane 2) and purified recombinant β-

galactosidase (lane 3); lane 4 activity staining with 4-methyl-umbelliferyl β-D-galactopyranoside of purified recombinant β-galactosidase.

4.7.2 Isoelectric focusing of purified recombinant β-galactosidase

The isoelectric point of β-galactosidase from *B. licheniformis* DSM 13 was determined by isoelectric focusing technique. The result shows that the isoelectric point of this enzyme was found to be in the range of 5.5-5.8 (Figure 4.29) according to the theoretical calculation value of 5.75 and similar to pI of *B. stearothermophillus* (5.7 of theoretical pI value) (Chen *et al.*, 2008). In contrast, the pI values of *L. reuteri* L461 and L103 were in the range of 3.8-4.0 and 4.6-4.8 (Nguyen *et al.*, 2006).

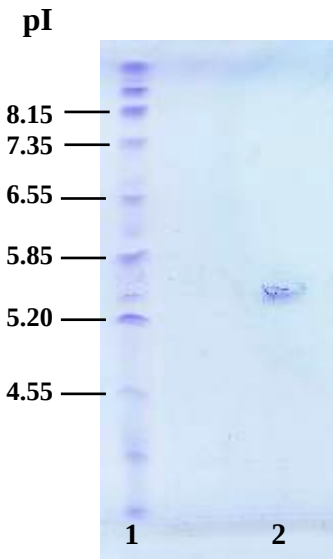


Figure 4.29 Isoelectric focusing of purified recombinant β-galactosidase with pI marker protein kit (GE Healthcare).

4.7.3 pH and temperature profile and stability

The optimum pH of purified β-galactosidase enzyme was performed using 22 mM *o*NPG and 600 mM lactose as substrate. The optimum pH was at 6.5 for both of *o*NPG and lactose as substrate (Figure 4.30) which the same optimum pH of β-galactosidase of *Thermotoga maritima* (Kim *et al.*, 2004a) and near the range of the optimum pH from the purified β-galactosidase of *B. sterothermophilus* (Griffiths and Muir, 1978). This enzyme had very low activity below pH 4 and higher than pH 9. However, it was different from the pH optimum (pH 5.5) of the purified β-galactosidase from *B. licheniformis* ATCC 9800 (Trân *et al.*, 1998). This property of the purified β-galactosidase led to this enzyme suitable for hydrolyzing lactose in dairy products. The purified β-galactosidase from *B. licheniformis* DSM 13 is stable at the pH range between pH 5 and pH 9 (Figure 4.32). These pH optimum and stability range were also found for β-galactosidases from *B. coagulans* RCS3 (Navneet *et al.*, 2002) and *B. circulans* (Fujimoto *et al.*, 1998; Vetere and Paoletti, 1998). The optimum temperature of recombinant β-galactosidase was 50^{o}C for both lactose and *o*NPG as substrate (Figure 4.31).

The purified β-galactosidase enzyme was the most stable at pH 6.5, when incubated at 37^{o}C (Figure 4.33). The result shows the remaining of activity approximately 80% after 3 weeks. The activity was not stable at pH 4 and pH 4.5 after 30 min and 10 days, respectively. However, the activities of the rest of other pH were still remaining more than 50% after 3 week. When compared the activity after 24 h and 30 days, the activity quite stable at pH 5 to pH 9, but it shown slightly low at pH 7.5.

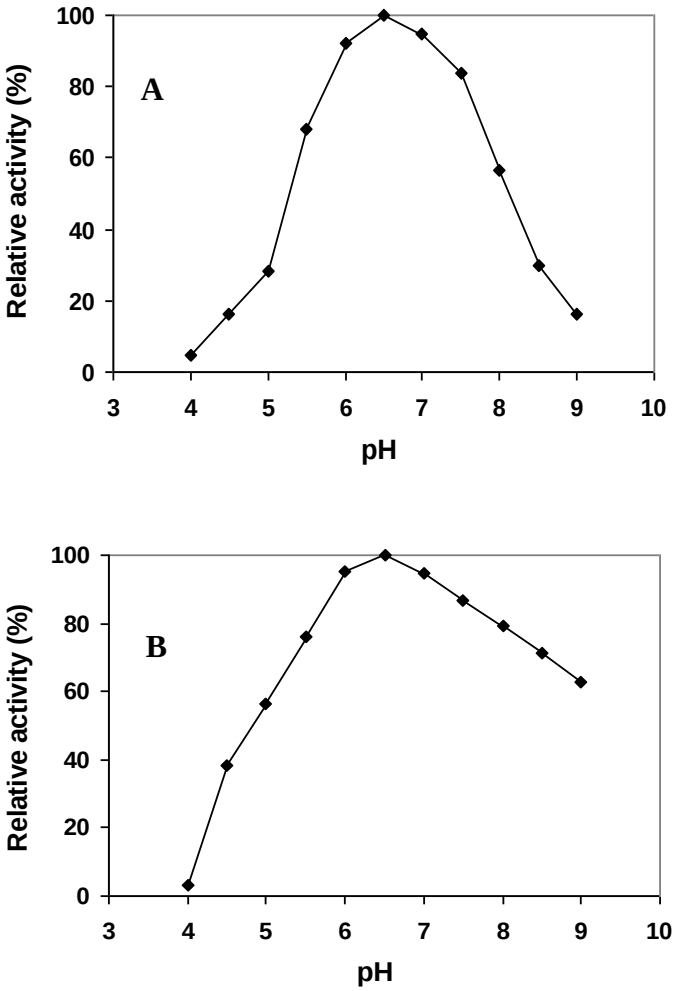


Figure 4.30 pH optimum of purified recombinant β-galactosidase from *B. licheniformis* DSM 13 using *o*NPG (A) and lactose (B) as substrates.

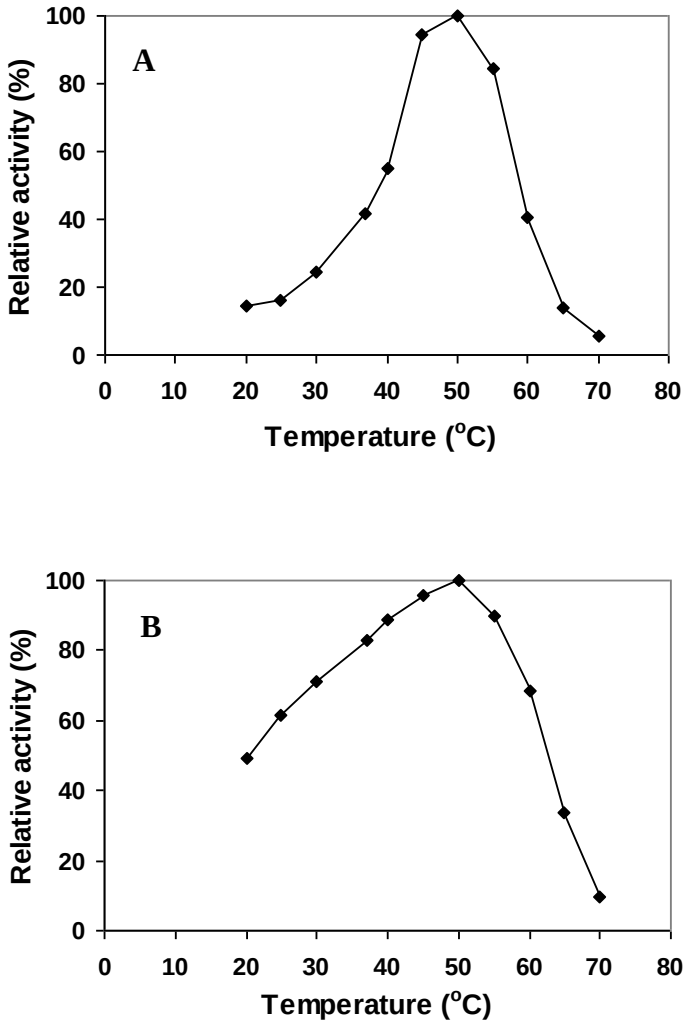


Figure 4.31 Temperature optimum of purified recombinant *β*-galactosidase from *B. licheniformis* DSM 13 using *o*NPG (A) and lactose (B) as substrates.

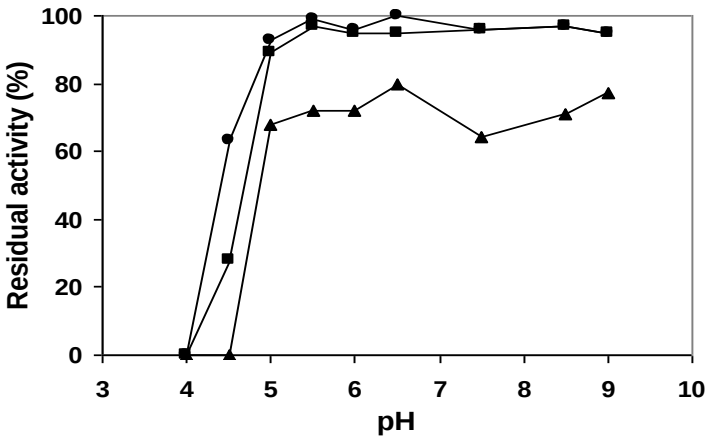


Figure 4.32 The residual activity of pH stability of purified β-galactosidase from *B. licheniformis* DSM 13 incubated at 37°C in 50 mM sodium phosphate buffer (pH 4-9) after 5 h (●), 24 h (■), and 1 month (▲).

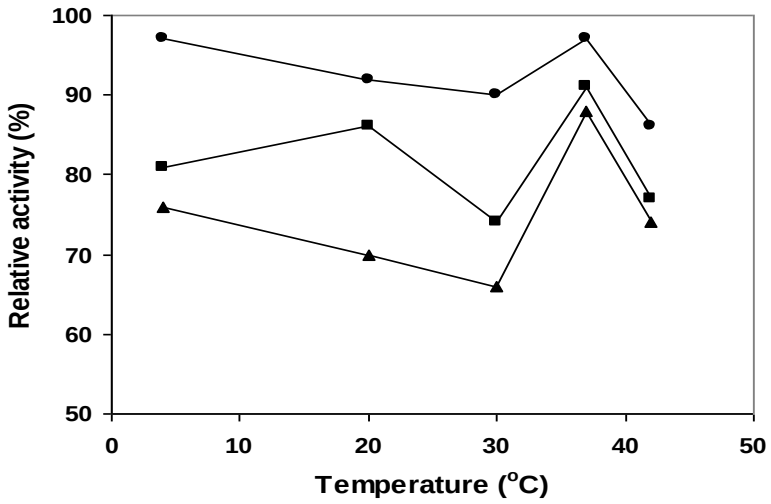


Figure 4.33 The relative activity of temperature stability of purified β-galactosidase from *B. licheniformis* DSM 13 incubated at 4°C, room temperature (20°C), 37°C, and 42°C in 50 mM sodium phosphate buffer, pH 6.5 after 24 h (●), 5 days (■), and 1 month (▲).

The recombinant β-galactosidase enzyme from *B. licheniformis* DSM 13 showed its remained activity more than 70% after 24 h and 1 month at 42^{o}C and the remaining activity was more than 80% at 37^{o}C after 3 weeks (Figure 4.33). Interestingly, this recombinant enzyme was stable at the moderate temperature (37^{o}C). Consequently, the recombinant β-galactosidase enzyme can be kept at 37^{o}C at least 1 month with the residual activity more than 85% and it was found that $MgCl_2$ was no effect to β-galactosidase enzyme from *B. licheniformis* DSM 13 as shown in Figure 4.34. In contrast, $MgCl_2$ had an effect to the enzyme acticity at 42^{o}C, but it was no significant between 1 and 10 mM $MgCl_2$ (Figure 4.35).

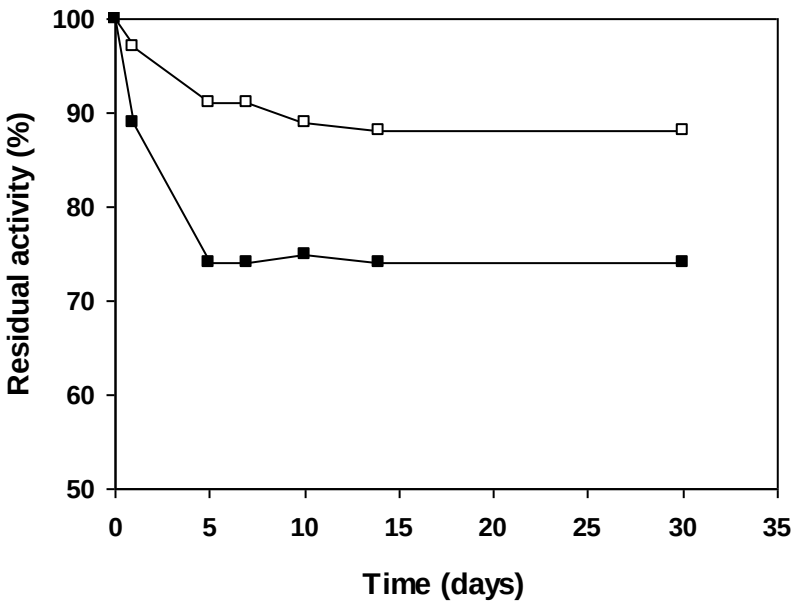


Figure 4.34 Residual activity of purified β-galactosidase enzyme from *B. licheniformis* DSM 13 after incubation without (▫) and with (■) 1 mM $MgCl_2$ at 37^{o}C for 1 month.

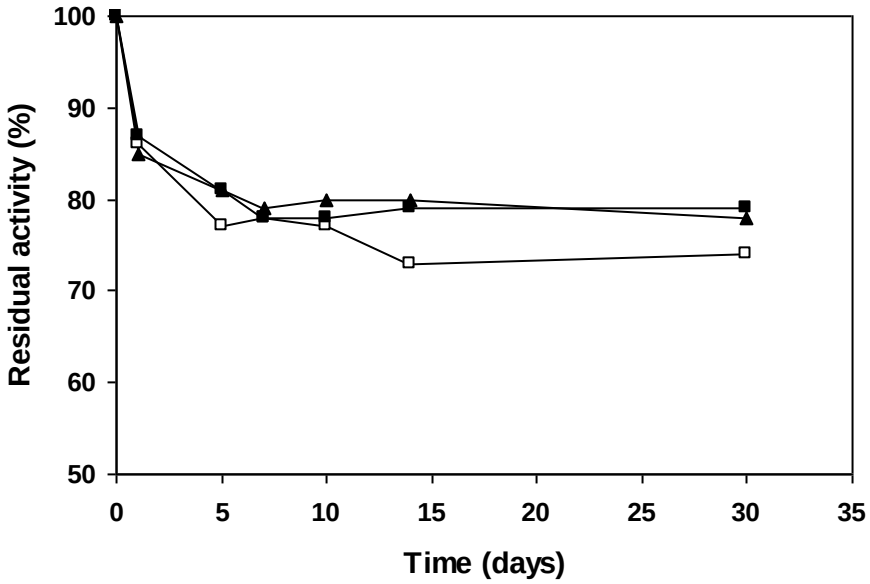


Figure 4.35 Residual activity of purified β-galactosidase enzyme from *B. licheniformis* DSM 13 after incubation without (▫), with 1 mM (■), and 10 mM (▲) $MgCl_2$ at 42°C for 1 month.

The relative activity of β-galactosidase without and with 1 mM $MgCl_2$ at 50 and 55°C revealed that the β-galactosidase from *B. licheniformis* DSM 13 had the half-life time approximately 3 and 5 days, respectively, whereas the half-life time of this enzyme was approximately more than 2 months at 42°C (data not shown). Furthermore, the relative activity was more than 60% at 55°C for 3 day and it was found that 1 mM $MgCl_2$ was no influence of the enzyme at this temperature. The relative activity was nealy 1% at 60°C for 24 h and it was no activity at 65°C (Table 4.4) compared with β-galactosidase from *B. subtilis* was restricted to 120 and 15 min at 60 and 70°C, respectively (Konsoula and Liakopoulou-Kyriakides, 2005). The results indicated that the relative activity of β-galactosidase from *B. licheniformis* DSM 13 reducred when increased temerature and $MgCl_2$ was no influence at temperature higher than 55°C. In conclusion, when incubated the β-galactosidase

from *B. licheniformis* DSM 13 with 1 mM $MgCl_2$, the half-life times were approximately 2 months, 4 days, 4 days, 2.5 h and 30 min at 42, 50, 55, 60 and 65^oC, respectively, yet the recombinant enzyme still had an activity from 50 to 65^oC. It was interesting with regard to use this enzyme for lactose hydrolysis in milk pasteurization process.

Table 4.4 The effect of high temperature to activity of purified β-galactosidase from *B. licheniformis* DSM 13.

Temperatre (^oC)	**1 mM $MgCl_2$**	**Relative activity (%)**			
		1 day	**2 days**	**3 days**	**5 days**
50	–	100	71.27	55.50	45.18
	+	100	77.15	60.35	47.78
55	–	100	72.97	62.96	59.39
	+	100	71.78	68.09	41.82
60	–	1.57	0	0	0
	+	1.94	0	0	0
65	–	0	0	0	0
	+	0	0	0	0

(+) means presence 1 mM $MgCl_2$ and (–) means no $MgCl_2$.

4.7.4 Kinetic parameter

Kinetic constants, the maximum reaction velocity (V_{max}) and kinetic constant (K_m) of enzyme were determined for the artificial and natural substrates, lactose and

*o*NPG, respectively, and calculated based on the Michaelis-Menten equation (Table 4.5). The Michaelis-Menten plots of activity from recombinant β-galactosidase enzyme of *B. licheniformis* DSM 13 were shown in Figure 4.36 and 4.37 for *o*NPG and lactose as substrate, respectively. The Michaelis constant for the chromogenic model substrate *o*NPG is significantly lower than the value determined for the natural substrate lactose at 30^{o}C, which is in accordance with β-galactosidases from a number of different sources (Samoshina and Samoshin, 2005; Nguyen *et al.*, 2006). This result shows that the purified β-galactosidases from *B. licheniformis* DSM 13 has a weak affinity for lactose contrast with the *Alicyclobacillus acidocaldarius* subsp. *rittmannii* β-galactosidases (Gul-Guven *et al.*, 2007). The K_m value for *o*NPG was found to be 13.7 mM higher than that of *Bacillus sp.* MCTT 3088 (6.34 mM) K_m value of 169 mM determined for lactose was found to be quite high whereas K_m values for lactose from the other members of glycosyl hydrolase family 42 were low, for instance, 3 isoforms of β-galactosidase from *Bacillus circulans* were 3.7, 2.94, and 2.71 mM, respectively (Vetere and Paoletti, 1998) and 6.18 mM from *Bacillus sp.* MTCC 3088 (Chakraborti *et al.*, 2000). K_m from *Thermus sp.* A4 and *Thermus sp.* IB-21 were 19 and 42 mM, respectively (Ohtsu *et al.*, 1998; Kang *et al.*, 2005). However it is comparable to the values reported for fungal and yeast enzymes that are commonly employed in biotechnological applications (36-180 mM for *Aspergillus oryzae*, 54-99 mM for *Aspergillus niger*) (De Roos, 2004). The V_{max} was found to be 13 and 299 μmol/min.mg for lactose and *o*NPG as substrate, respectively. In contrast, the V_{max} of β-galactosidases of *T. maritima* for both lactose and *o*NPG were 63.3 and 79.6, respectively (Kim *et al.*, 2004a).

Table 4.5 Kinetic parameters of recombinant β-galactosidase from *B. licheniformis* DSM 13 over-expressed in *E. coli* for the hydrolysis of lactose and *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG).

Substrate	**Method for determination of enzyme activity**	**Kinetic parameter**	**Recombinant β-gal**
Lactose	release of D-Glu	$V_{max,Glu}$ (μmol $min^{-1}mg^{-1}$)	13
		$K_{m,Lac}$ (mM)	169
		k_{cat} (s^{-1})	34.5
		k_{cat}/K_m ($mM^{-1}s^{-1}$)	0.20
		$K_{i,Gal}$ (mM)	0.93
*o*NPG	release of *o*NP	$V_{max,oNP}$ (μmol $min^{-1}mg^{-1}$)	299
		$K_{m,oNPG}$ (mM)	13.7
		k_{cat} (s^{-1})	785
		k_{cat}/K_m ($mM^{-1}s^{-1}$)	57.3
		$K_{i,Glu}$ (mM)	83.2
		$K_{i,Gal}$ (mM)	0.95

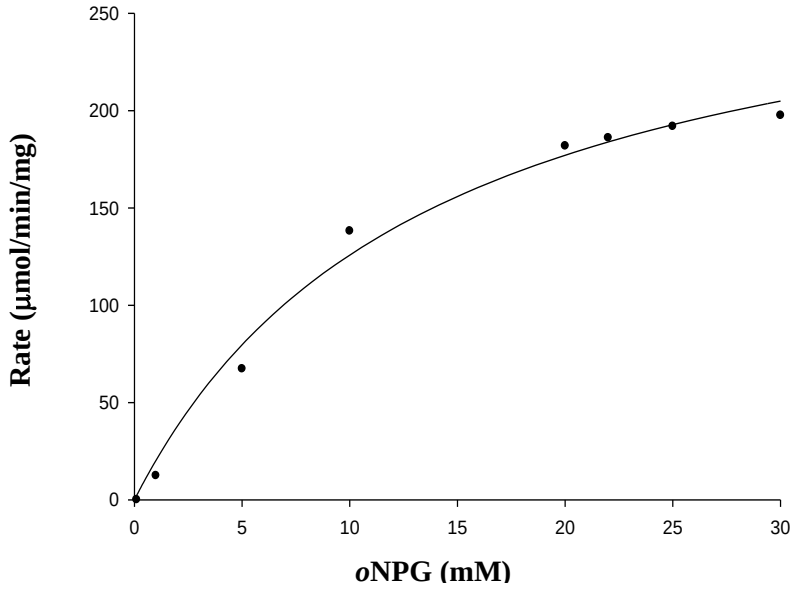


Figure 4.36 Michaelis-Menten plot of activity from recombinant β-galactosidase enzyme of *B. licheniformis* DSM 13 using *o*NPG as substrate.

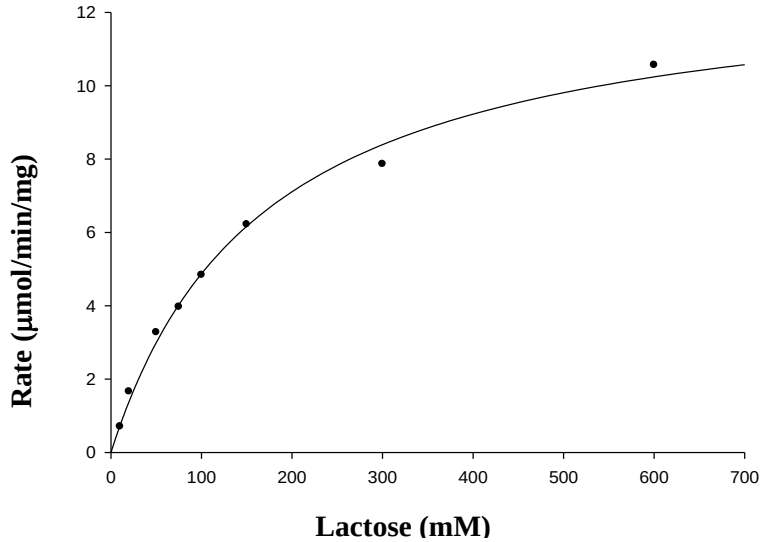


Figure 4.37 Michaelis-Menten plot of activity from recombinant β-galactosidase enzyme of *B. licheniformis* DSM 13 using lactose as substrate.

4.7.5 The effect of end product inhibition

The inhibition reaction from the end product of glucose and galactose was obtained when measured the activity of the recombinant β-galactosidase enzyme by using *o*NPG and lactose as substrate. The end product inhibition was conducted using lactose at various concentrations from 0-600 mM as substrate. As a result, the specific activity was continuely decreased when increased galactose concentration and the specific activity was lower at low lactose concentration than that of at high lactose concentration (Figure 4.38). The end product, galactose, reduced the activity immediately at 50 mM galactose concentration and its activity completely inactivated at 200 mM galactose concentration. Furthermore, the end producr, D-galactose, was found to competitively inhibitit the hydrolysis for lactose and *o*NPG as substrates similar to that of β-galactosidase from *L. reuteri* (Nguyen *et al.*, 2006) and the inhibition constants were found to be 0.93 and 0.95 mM for lactose and *o*NPG as substrates, respectively. These inhibitions were strong as reveal from the ratio of the inhibition constant for D-galactose and the Michaelis constant for lactose and *o*NPG which calculated for both substrates; $K_{i,Gal}$ /$K_{m,Lac}$ = $5.5x10^{-3}$, $K_{i,Gal}$ /$K_{m,oNPG}$ = $0.69x10^{-2}$. The end product, D-glucose, was found to be a non-competitive inhibitor of *o*NPG as the ratio of $K_{i,Glu}$ /$K_{m,oNPG}$ = 6.07. The result indicated that the end product galactose was the strongest inhibitor for both substrates lactose and *o*NPG.

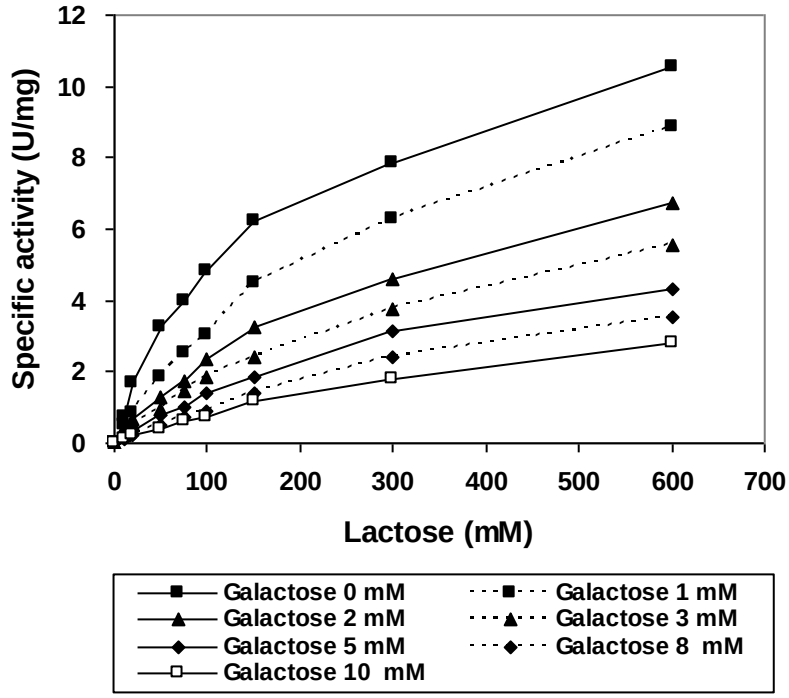


Figure 4.38 Galactose inhibition of lactose hydrolysis catalyzed by β-galactosidase enzyme from *B. licheniformis* DSM 13.

Accordingly, at various *o*NPG concentrations from 0-30 mM as substrate, the specific activity was continuely decreased when increased glucose concentration, the specific activity was lower at low *o*NPG concentration than that at high *o*NPG concentration (Figure 4.39). The activity of recombinant β-galactosidase enzyme was decreased to lower than 60% at 200 mM glucose concentration when using chromogenic as substrate, *o*NPG. The galactose had a competive inhibitor effect against *o*NPG and lactose; especially galactose was the highest competitive inhibitor (Figure 4.40). In contrast, glucose was the non-competitive inhibitor whereas it was the competitive inhibitor of β-galactosidase from *B. stearothermophilus* and *L. reuteri* (Nguyen *et al.*, 2006; Chen *et al.*, 2008).

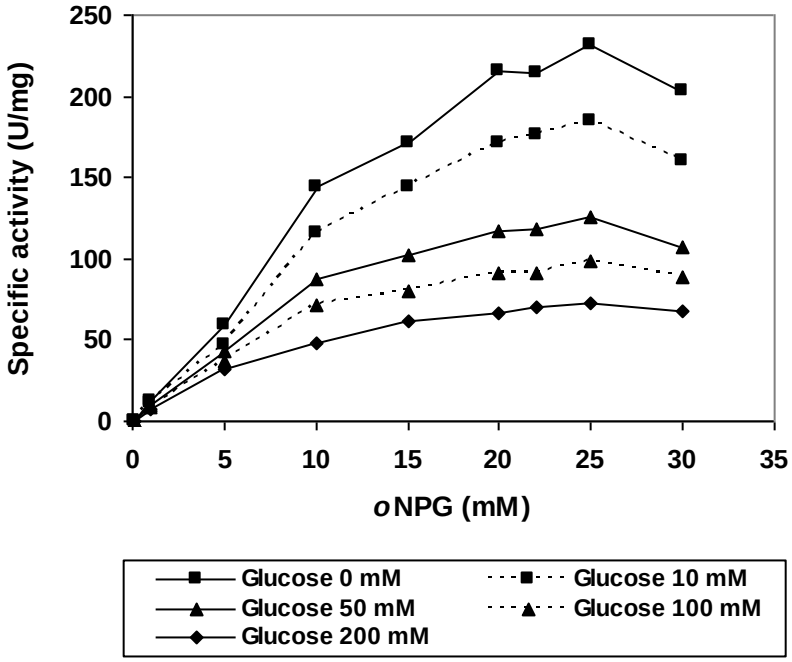


Figure 4.39 Glucose inhibition of *o*NPG hydrolysis catalyzed by β-galactosidase enzyme from *B. licheniformis* DSM 13.

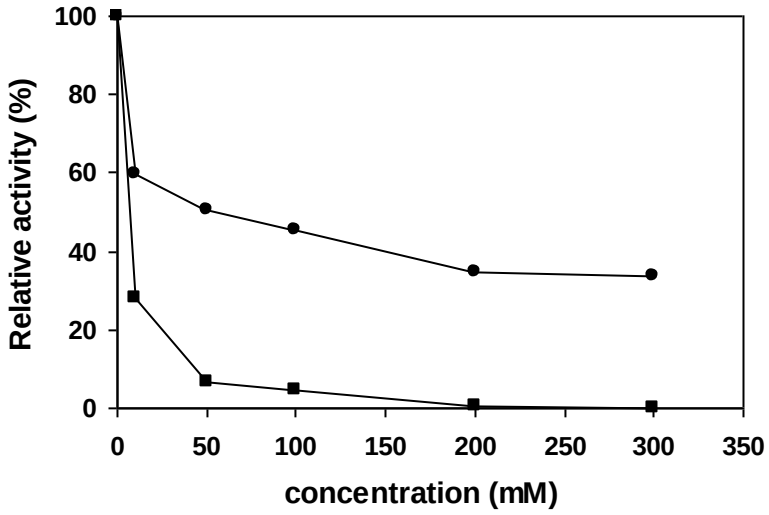


Figure 4.40 End product inhibition of purified β-galactosidase enzyme from *B. licheniformis* DSM 13 with 22 mM *o*NPG and 600 mM lactose hydrolysis using glucose (●) and galactose (■) as inhibitor.

4.7.6 The effect of metal cations and reagents

The hydrolysis of *o*NPG by β-galactosidases from *B. licheniformis* DSM 13 was slightly activated by monovalent ions Na^+ and K^+ (Table 4.6) as well as for the synergistic effect by 1 mM Mg^{2+}, Mn^{2+}, and Ca^{2+} in presence of 10 mM Na^+ (Table 4.7). The enzyme β-galactosidases from *B. subtilis* KL88 was activated by most of the alkaline earth metal, Na^+ and K^+ (Rahim and Lee, 1991) the same as in this research. The rate of the activation was not the same (Kim *et al.*, 1997). Although it is known that Mg^{2+} is required for enzyme activity (Kim *et al.*, 2004a; Lu *et al.*, 2007), this enzyme is not much necessary of this metal cation for activate the activity of this enzyme. This observation is in agreement with the reports on the requirements for mono- and divalent metal ions for optimal activity and stability for a number of different β-galactosidases (Nakayama and Amachi, 1999; Nguyen *et al.*, 2006; Nguyen *et al.*, 2007b). However, β-galactosidases from *L. reuteri* (Nguyen *et al.*, 2006) and *Bacillus* sp. MTCC 3088 (Chakraborti *et al.*, 2000) were more strongly activated by monovalent ions Na^+ and K^+ and by 1 mM Mg^{2+}, Mn^{2+} than that of β-galactosidases from *B. licheniformis* DSM 13. Kim *et al.* (2004) was found that Mn^{2+} is the most effective for *T. maritima* β-galactosidase, producing 17- and 12-fold activity increases with *o*NPG and lactose as a substrate, respectively (Kim *et al.*, 2004a). Ca^{2+} is a known inhibitor of β-galactosidases (Smart *et al.*, 1985; Garman *et al.*, 1996), but interestingly, it slightly activates β-galactosidase from *B. licheniformis* DSM 13 even at higher concentration (10 mM). This is an advantage for the application of this enzyme in lactose conversion processes of fluid milk and lactose-rich substrate with high level of free Ca^{2+} in solution. In addition, divalent metal Cu^{2+} and Zn^{2+} inactivated the enzyme activity even at the lower concentration according to that of *K. lactis* β-galactosidase (Kim *et al.*, 1997) and psychrotrophic *B. subtilis*

KL88 (Rahim and Lee, 1991). Besides, Mn^{2+} and Fe^{2+} revealed inhibited the enzyme activity at high concentration (10 mM) whereas these cations at low concentration (1 mM) activated the enzyme activity.

Table 4.6 Effect of Na^+ and K^+ on the activity of recombinant β-galactosidase from *B. licheniformis* DSM 13 over-expressed in *E. coli* TOP10.

Cation	**Relative activity (%)**[a]		
	1 mM	**10 mM**	**100 mM**
Na^+	128	126	131
K^+	118	122	130

[a] The relative activity of no added cation was 100%.

Table 4.7 Synergistic effect of different cations on the activity of recombinant β-galactosidase from *B. licheniformis* DSM 13 overexpressed in *E. coli* TOP10.

Relative activity (%)[a]						
10 mM Na^+ 1 mM K^+	10 mM Na^+ 10 mM K^+	10 mM Na^+ 1 mM Mn^{2+}	10 mM Na^+ 10 mM Mn^{2+}	10 mM Na^+ 1 mM Mg^{2+}	10 mM Na^+ 10 mM Mg^{2+}	10 mM Na^+ 1 mM Fe^{2+}
123	126	136	78	134	137	123
10 mM Na^+ 10 mM Fe^{2+}	10 mM Na^+ 1 mM Ca^{2+}	10 mM Na^+ 10 mM Ca^{2+}	10 mM Na^+ 1 mM Cu^{2+}	10 mM Na^+ 10 mM Cu^{2+}	10 mM Na^+ 1 mM Zn^{2+}	10 mM Na^+ 10 mM Zn^{2+}
42	135	136	28	0	35	8

[a] The relative activity of no added cation was 100%

Besides the methods for investigation on β-galactosidases have been employed with various concentration of buffer for assay the activity using *o*NPG as substrate and various amounts and types of metal ions to improve lactase performance (Kim *et al.*, 1997). Various reagents were examined with respect to inhibitory or stimulating effect on the enzyme activity of β-galactosidases from *B. licheniformis* DSM 13. This enzyme was slightly activated by EDTA and no significance between 0.1 and 1 mM. In contrast, EDTA was found to strongly inactivate both enzyme β-galactosidases from *L. reuteri* L461 and L103 (Nguyen *et al.*, 2006) and *Bacillus* sp. MTCC 3088 (Chakraborti *et al.*, 2000). Dithiothreitol (DTT), 2-mercaptoethanol, PMSF and urea were not influence to activate the enzyme activity (Table 4.8). Accordingly, the thiol reagents 2-mercaptoethanol and DTT had no distinct promotion effect on β-galactosidases activity from *B. stearothermophilus*, that means the intact disulfide groups are not important for enzyme activity (Chen *et al.*, 2008).

Table 4.8 Effect of various reagents on the stability of the activity of β-galactosidase from *B. licheniformis* DSM13 after 1 month at 4^{o}C.

Reagent	**Relative activity (%)**			
	0.1 mM	**0.5 mM**	**1mM**	**10mM**
None	100	100	100	100
DTT	-	-	102	101
EDTA*	121	-	121	115
2-mercaptoethanol	-	-	100	102
PMSF	96	97	94	-
Urea*	-	-	91	82

* Presence significance compared with none reagent.

4.8 Lactose hydrolysis and GOS production

Lactose hydrolysis was performed by *B. licheniformis* DSM 13 β-galactodidas in a 2 ml final volume of batch fermentation using 50 and 200 g/L (or 135 and 600 mM, respectively) lactose as substrate in 50 mM sodium phosphate buffer (pH 6.5) at 37, 55, and 60^{o}C. Low levels of galacto-oligosaccharides (GOS) formed for all lactose concentrations. The formation of GOS at 37^{o}C was the lowest for three lactose concentrations and its residual enzyme activity rapidly reduced from the initial time to 5 h. It was found that the enzyme activity was more than 85% from 5 h to 36 h and quite stable (Figure 4.41). In contrast, the residual activity was continuely reduced to approximately 20 and 0% at 55 and 60^{o}C, respectively. Moreover, the residual activity was more reduced at 60^{o}C than that of at 55^{o}C (Figure 4.42 and 4.43).

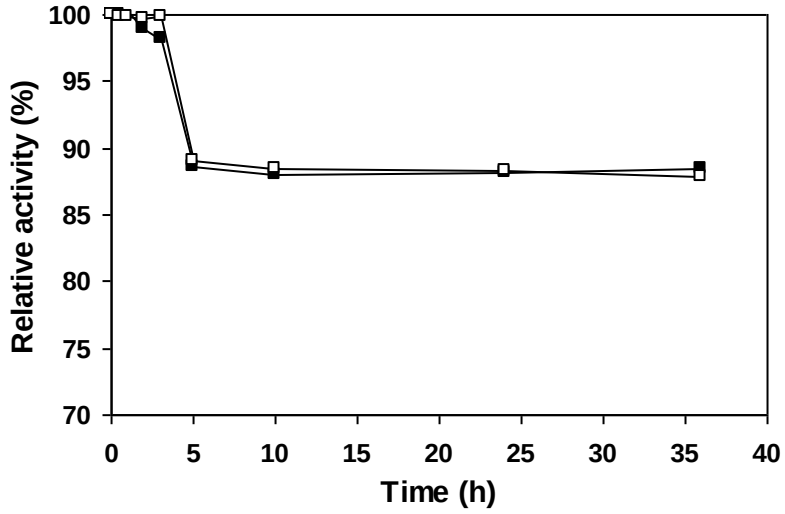


Figure 4.41 Residual activity of lactose hydrolysis at 37^{o}C and 300 rpm constant agitation using 50 g/L lactose (■) and 200 g/L lactose (▫) as substrate.

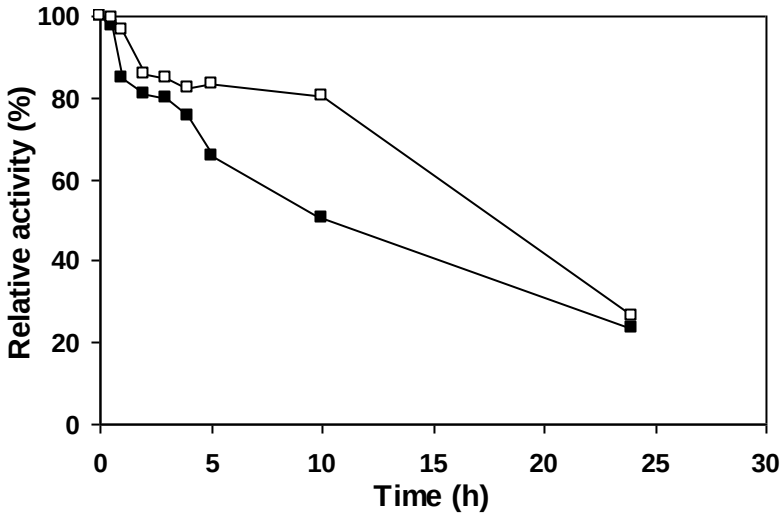


Figure 4.42 Residual activity of lactose hydrolysis at 55°C and 300 rpm using 50 g/L lactose (■) and 200 g/L lactose (▫) as substrate.

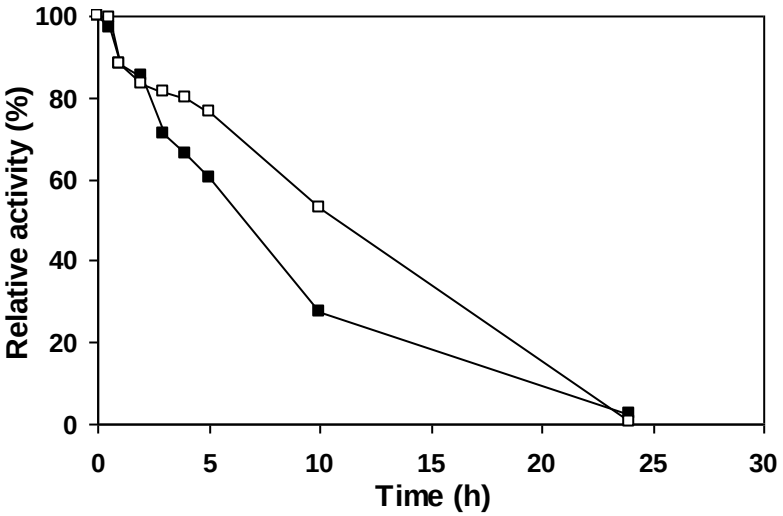


Figure 4.43 Residual activity of lactose hydrolysis at 60°C and 300 rpm constant agitation using 50 g/L lactose (■) and 200 g/L lactose (▫) as substrate.

Lactose hydrolysis products were collected at various time and analyzed by TLC. Figure 4.44 shows the results of lactose hydrolysis at 37°C which GOS production is very low compared with the standard Elix'or as well as at temperture 55 and 60°C as shown in Figure 4.45 and 4.46, respectively. However, GOS production at high

lactose concentration (200 g/L) was slightly higher than that of 50 g/L lactose concentration corresponding to the transglycosylation reaction of *E. coli* β-galactosidase is favored at high lactose concentrations (Huber *et al.*, 1976).

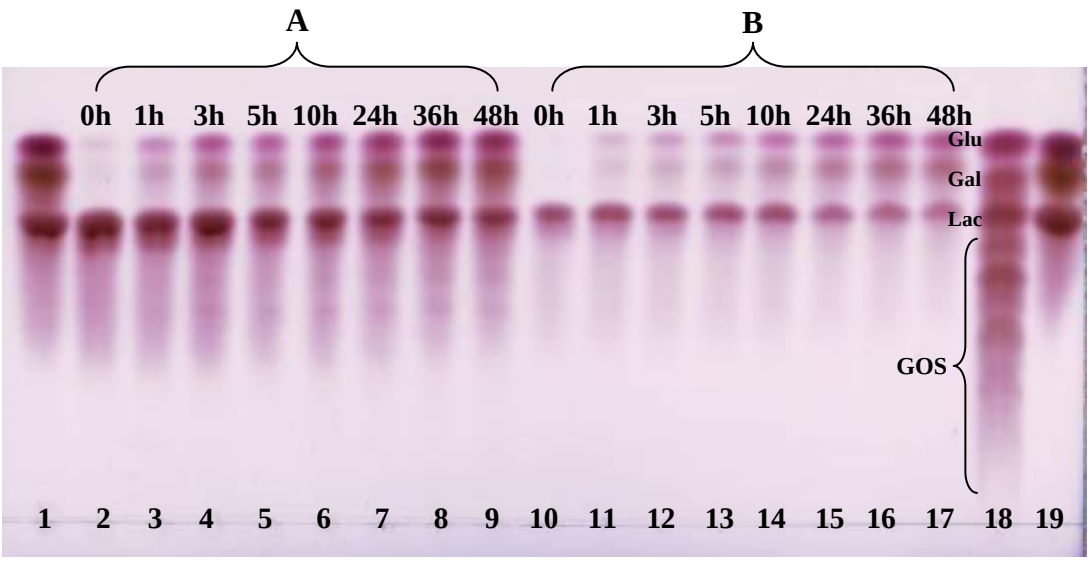


Figure 4.44 TLC of lactose hydrolysis by purified β-galactosidase from *B. licheniformis* DSM 13 at 37^{o}C and 300 rpm constant agitation using 200 g/L (A) and 50 g/L (B) lactose as substrate. Lane1 and 19, standard LGG (lactose, glucose and galactose), 10 g/L each; lane 2-9 (A), GOS production at various interval times; lane 10-17 (B), GOS production at various interval times; lane 18, standard Elix'or, 30 g/L. Abrev.; Glu, glucose; Gal, galactose; Lac, Lactose.

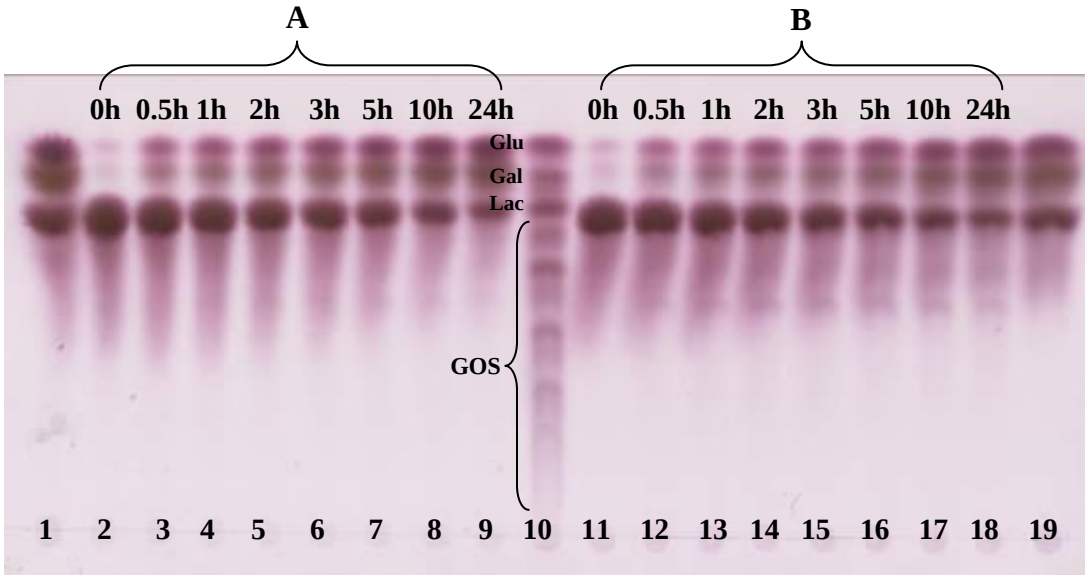


Figure 4.45 TLC of lactose hydrolysis by purified β-galactosidase from *B. licheniformis* DSM 13 at 55^{o}C and 300 rpm constant agitation using 50 g/L (A) and 200 g/L (B) lactose as substrate. Lane1 and 19, standard LGG (lactose, glucose and galactose), 10 g/L each; lane 2-9 (A), GOS production at various interval times; lane 10, standard Elix'or, 30 g/L; lane 11-18 (B), GOS production at various interval times. Abrev.; Glu, glucose; Gal, galactose; Lac, Lactose.

Capillary electrophoresis (CE) is a high-resolution technique for the separation of complex biological mixtures. Analysis of oligosaccharides by capillary electrophoresis was accomplished by derivatization of the sugars with 2-aminopyridine according to a mechanism of reductive amination because this compound lack suitable chromophores for UV detection and ionized functional groups so that pre-column derivatization is the method of choice to add a chromophore and/or an ionic functionality to the carbohydrate molecule. The derivatized products were carried out at pH 2.5 where the electroosmotic flow is almost

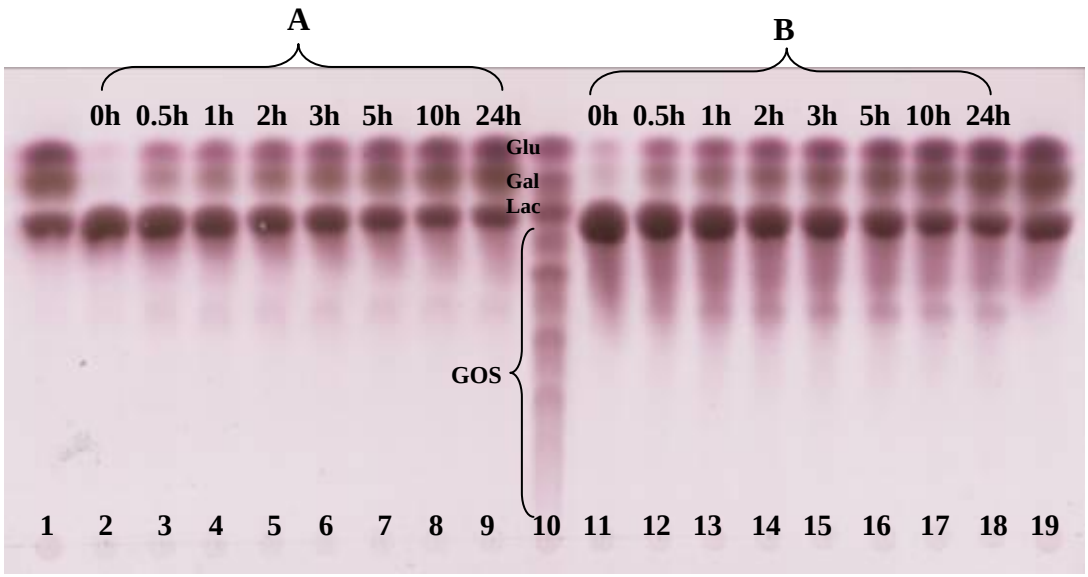


Figure 4.46 TLC of lactose hydrolysis by purified β-galactosidase from *B. licheniformis* DSM 13 at 60^{o}C and 300 rpm constant agitation using 50 g/L (A) and 200 g/L (B) lactose as substrate. Lane1 and 19, standard LGG (lactose, glucose and galactose), 10 g/L each; lane 2-9 (A), GOS production at various interval times; lane 10, standard Elix'or, 30 g/L; lane 11-18 (B), GOS production at various interval times. Abrev.; Glu, glucose; Gal, galactose; Lac, Lactose.

almost zero and the derivatized products migrate towards an anode (Kazmaier *et al.*, 1998). The mechanism of the reductive amination is based on the reaction of the primary amine function of the derivatization reagent with the reducing function of the carbohydrates forming a Schiff base in the first step which is accomplished subsequently by sodium cyanoborohydride to produce a stable secondary amine (Pfaff *et al.*, 1999). Lactose hydrolysis products were analyzed by capillary electrophoresis as shown in Figure 4.47 to Figure 4.53. They shows a very low products of lactose hydrolysis using 200 g/L lactose as initial substrate at 37^{o}C for 0,

1, 3.5 10, 24 and 36 h, respectively. Glucose has the highest migration velocity and migrates as an anion into the opposite direction to the electroosmotic flow (Kazmaier *et al.*, 1998). Althought, GOS production was performed at the initial time; it was still less products at 36 h. Remarkable features of capillary electrophoresis with pre-column derivatization of the carbohydrates are its simplicity and ruggedness. However, quantification of oligosaccharides and determination of the oligomer distribution may cause problems because of the derivatization reaction, which was incompletes, and the risk of hydrolysis of higher molecular weight carbohydrates in the derivatization process (Kazmaier *et al.*, 1998).

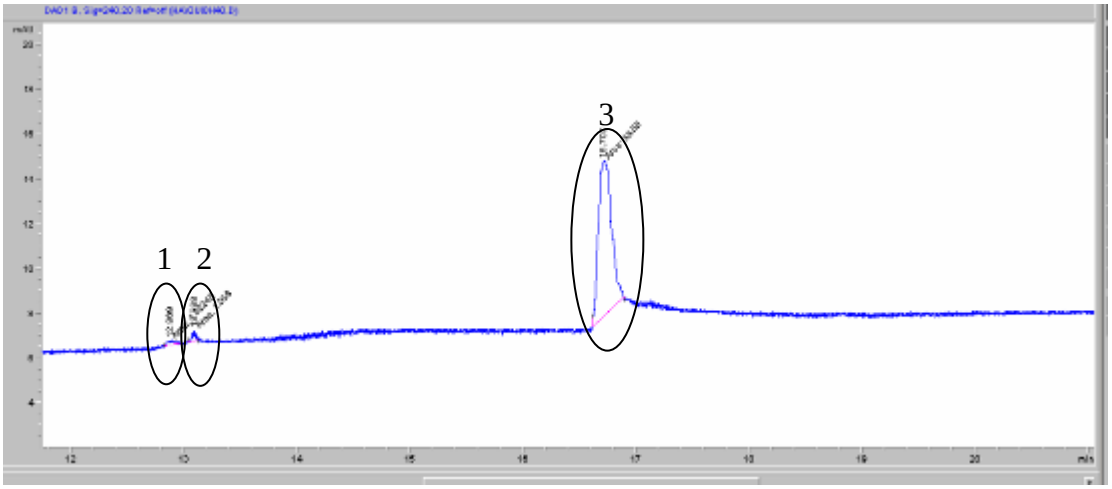


Figure 4.47 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37^{o}C for 0 h, using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3).

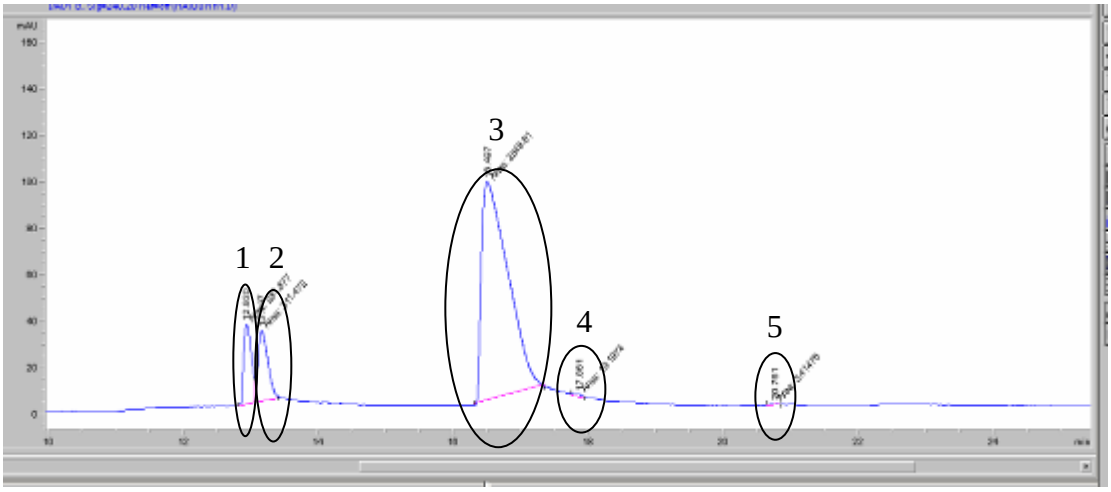


Figure 4.48 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37°C for 1 h, using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3), GOS (4 and 5).

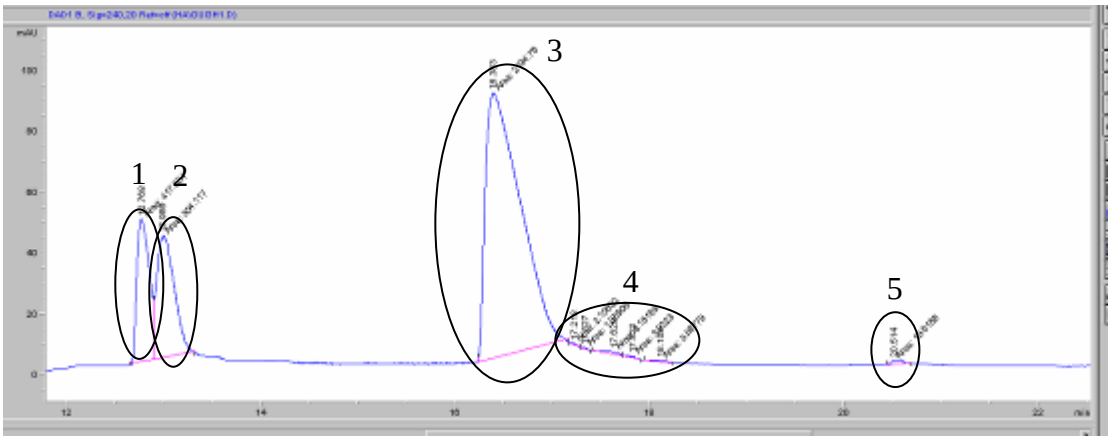


Figure 4.49 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37°C for 3 h, using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3), GOS (4 and 5).

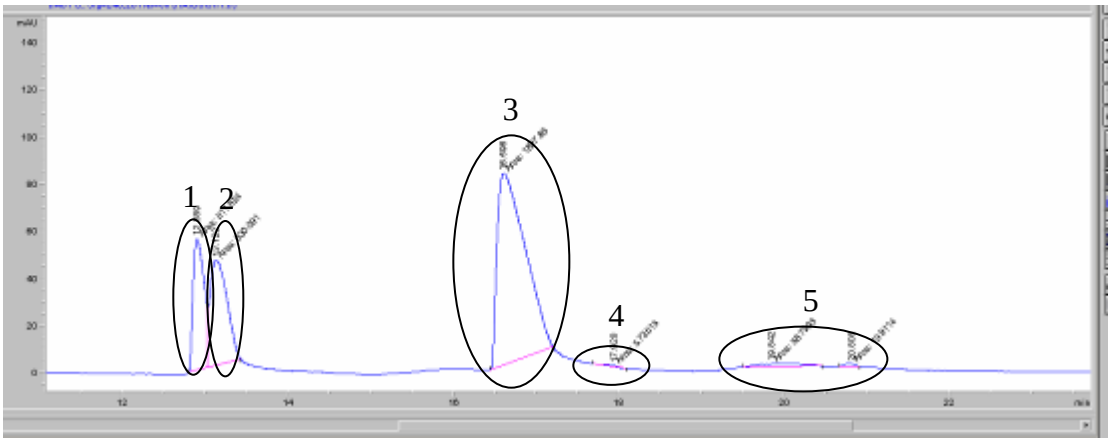


Figure 4.50 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37°C for 5 h, using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3), GOS (4 and 5).

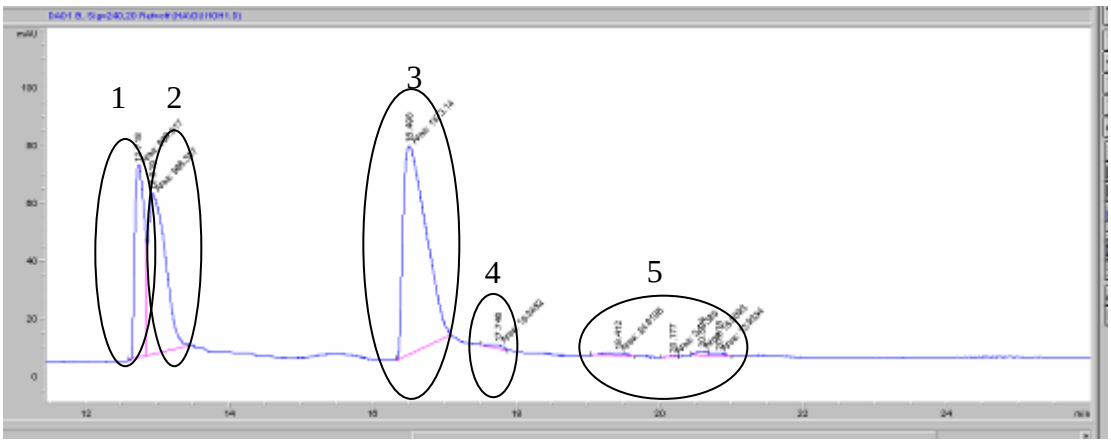


Figure 4.51 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37°C for 10 h using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3), GOS (4 and 5).

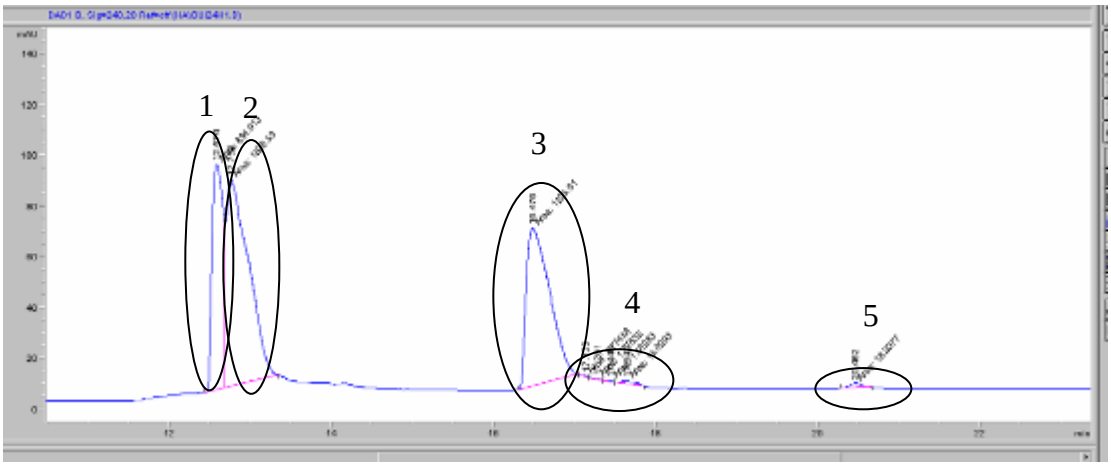


Figure 4.52 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37°C for 24 h, using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3), GOS (4 and 5).

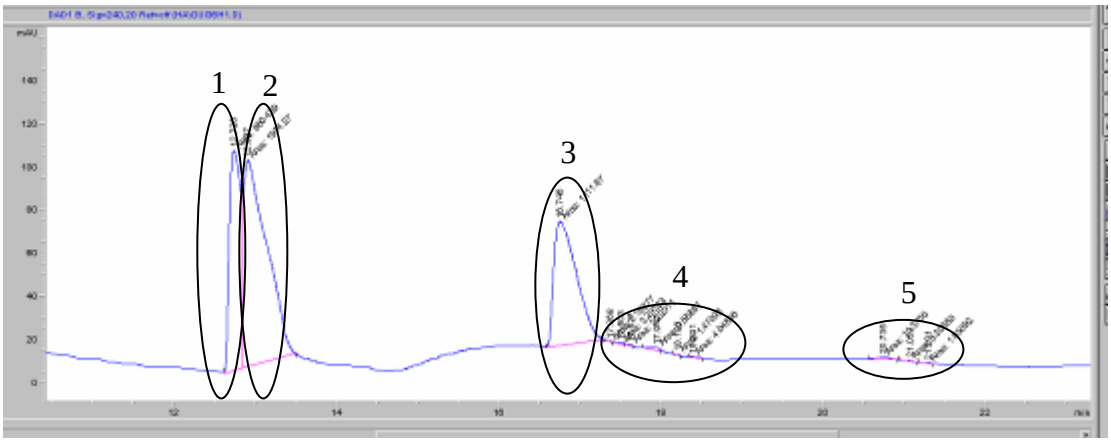


Figure 4.53 CE analysis chromatogram of products from batch reaction of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 37°C for 36 h using 200 g/L lactose as initial substrate. Glucose (1), galactose (2), lactose (3), GOS (4 and 5).

The HPAEC-PAD is a powerful tool for analysis of oligo- and polysaccharides to evaluate changing in chain length distribution of GOS and conveniently apply in the characterization of GOS at different degree of polymerization to evaluate their prebiotic properties. The high sensitivity and specificity of integrated pulsed amperometric detection for the analyzed carbohydrates minimized possible interferences and simplified sample preparation (Corradini *et al.*, 2004). A major advantage of HPAEC-PAD is, that it does not require any derivatization reaction of the carbohydrates (Pfaff *et al.*, 1999). Figure 4.54 to 4.56 shows that the gradient results of HPAEC-PAD chromatograms of the lactose hydrolysis at 55^{o}C using 200 g/L lactose as initial substrate for 0, 10 and 24 h, respectively, are slightly increased until the end of the reaction. However, the GOS product are still very less when compared with the other publication. Figure 4.57 to 4.59 shows the gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis at 60^{o}C using 200 g/L lactose as initial substrate for 0, 10 and 24 h reapectively. The chromatograms reveal that β-galactosidase enzyme from *B. licheniformis* DSM 13 was more appropriate for lactose hydrolysis than the transglycosylation, therefore, the GOS product from this experiment is quite lower than that of the other microorganisms. Hence, it is suitable for the low lactose for dairy manufacture.

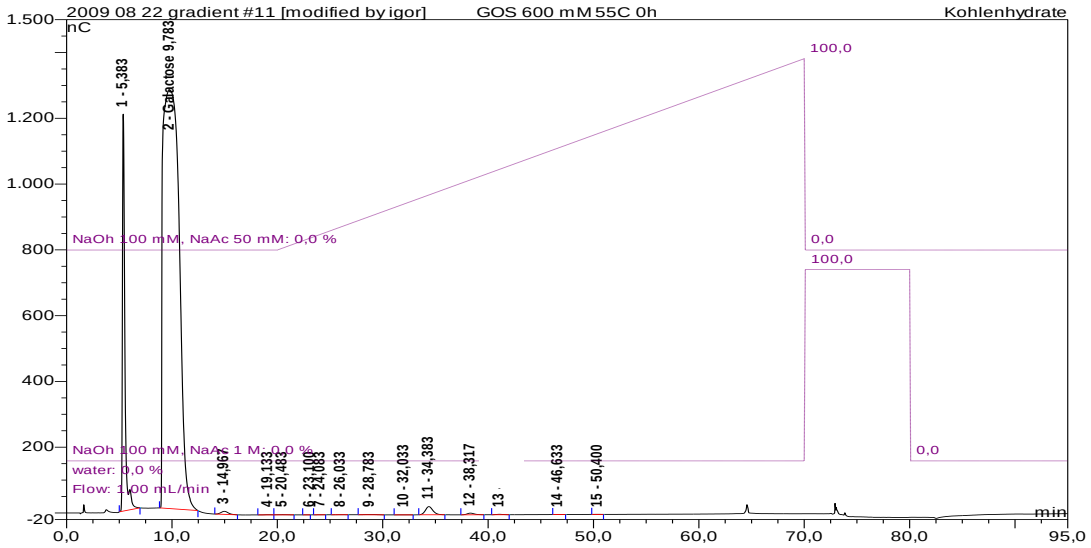


Figure 4.54 The gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 55°C for 0 h using 200 g/L lactose as initial substrate.

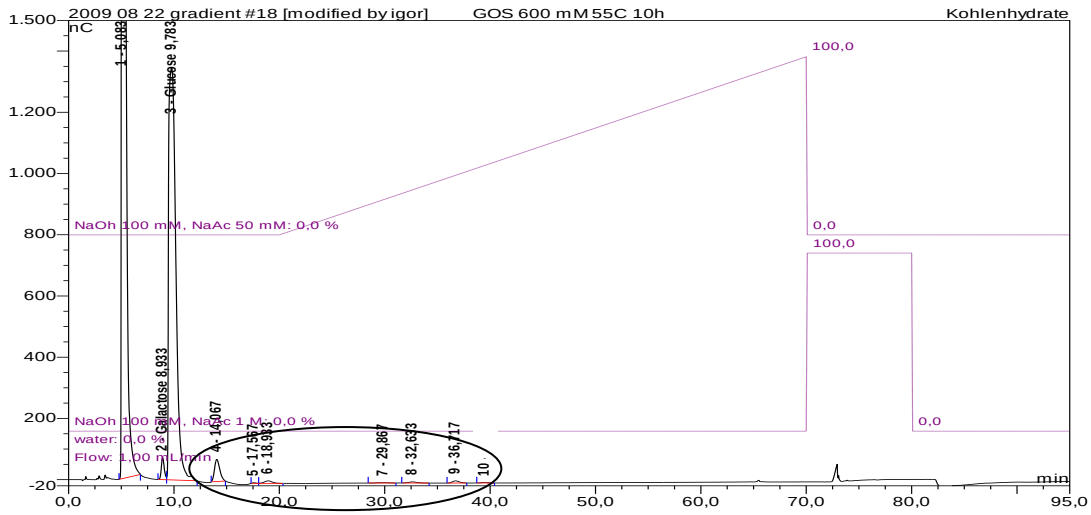


Figure 4.55 The gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 55°C for 10 h using 200 g/L lactose as initial substrate. The circle shows the GOS production.

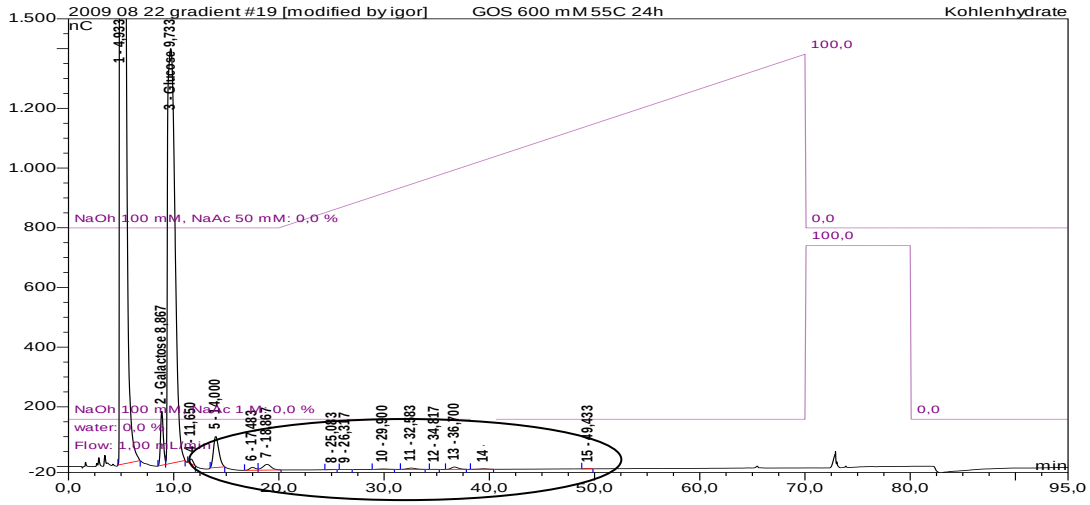


Figure 4.56 The gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis by β-galactosidase from *B. licheniformis* DSM 13 at 55°C for 24 h using 200 g/L lactose as initial substrate. The circle shows the GOS production.

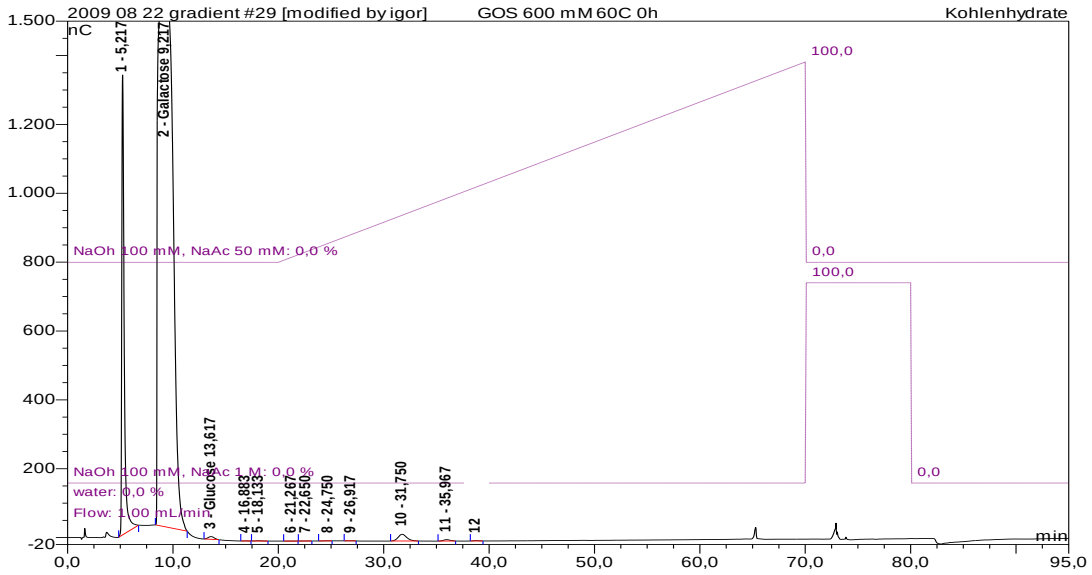


Figure 4.57 The gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis by β-galactosidase of *B. licheniformis* DSM 13 at 60°C for 0 h using 200 g/L lactose as initial substrate.

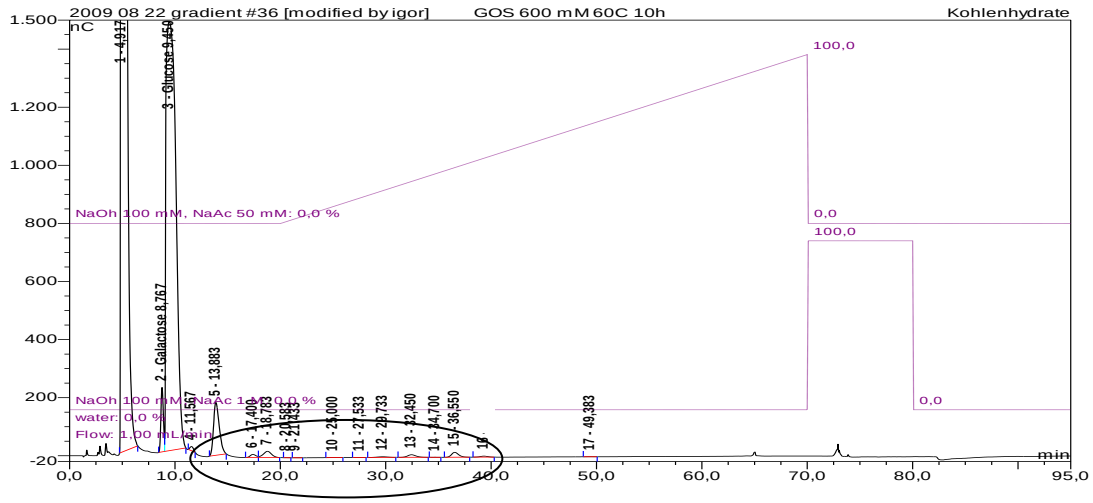


Figure 4.58 The gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis by β-galactosidase of *B. licheniformis* DSM 13 at 60°C for 10 h using 200 g/L lactose as initial substrate. The circle shows the GOS production.

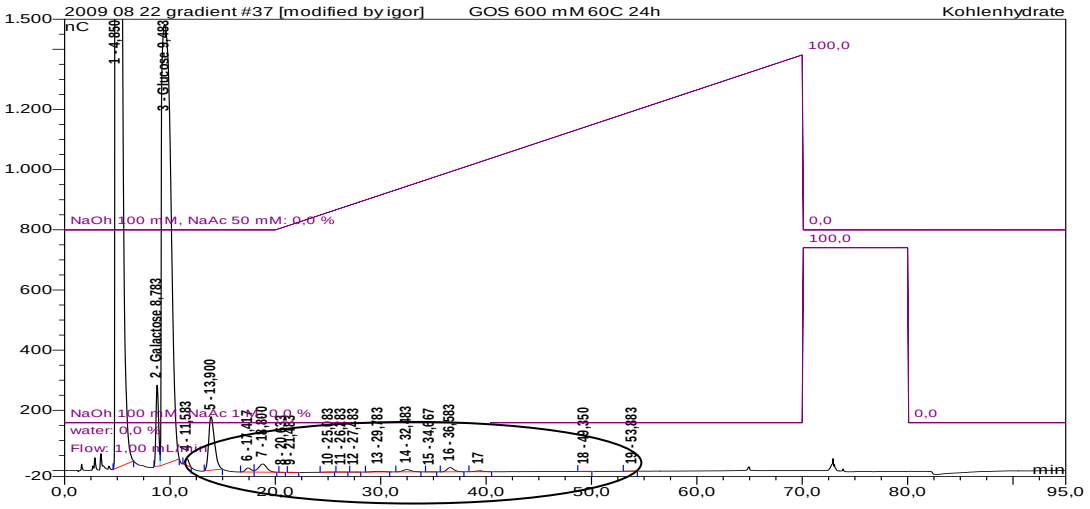


Figure 4.59 The gradient result of HPAEC-PAD chromatograms of the lactose hydrolysis by β-galactosidase of *B. licheniformis* DSM 13 at 60°C for 24 h using 200 g/L lactose as initial substrate. The circle shows the GOS production.

It is clear that the lactose was more hydrolyzed at high temperature (60^{o}C) than that at the low temperature (37^{o}C) (Figure 4.60). At the end of reaction time, 30 and 40% lactose residual for 60 and 37^{o}C, respectively. Therefore, the end products, glucose and galactose, were less than 40% for both temperatures. Anion exchange chromatohraphy with pulsed amperometric detection does not mees any pre-column derivatization steps. Disadvantages of HPAEC-PAD are its need for skilled operators and sophisticated equipment with expensive columns and with the need for continuous helium degassing of the mobile phases to prevent dissolution of even traced of atmospheric carbondioxide (Kazmaier *et al.*, 1998).

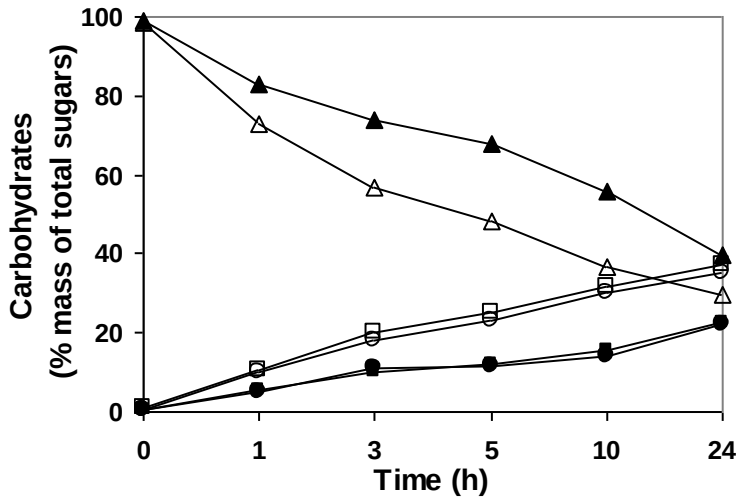


Figure 4.60 Time course reaction of lactose conversion in discontinuous batch processes. The reactions were carried out using 50 g/L lactose initial concentration in 50 mM sodium phosphate buffer and 8 U_{Lac}/mL recombinant enzyme at 37^{o}C (lactose (▲), glucose (■) and galactose (●)) and at 60^{o}C (lactose (∆), glucose (□) and galactose (○)).

GOS formation reached approximately 12% with initial lactose concentration of 200 g/L at the process temperature of 60°C whereas approximately 7% GOS formation reached the highest from 25% lactose hydrolysis with 50 g/L lactose concentration as initial substrate (Figure 4.61). It was reported by many authors (Prenosil *et al.*, 1987) that GOS yield is significantly influenced by initial lactose concentration or the percentage of lactose converted to oligosaccharides during the hydrolysis of lactose rose with an increase in the initial lactose concentration (Yang and Tang, 1988). The increase in initial lactose concentration is one of the main factors for GOS formation beside enzyme source, temperature, and pH (Zarate and Lopez-Leiva, 1990) for GOS formation. To compare the GOS yield using 200 g/L lactose as initial substrate, 12% GOS formation was produced at 60^{o}C whereas only 5% GOS formation was produced at 37^{o}C (Figure 4.62). The GOS yield observed in this work was not as high as expected but was lower than the level reported for *B. subtilis* KL88 (20%) (Rahim and Lee, 1991). This may be due to product inhibition of the enzyme; hence, high rates of lactose conversion were not achieved. Interestingly, the hydrolysis of lactose by this β-galactosidase could be benefit for lactose intolerant people, who lack lactase in the intestine and for improves the process in the product of diary product such as increase the solubility and sweetness in the product. It is generally accepted that a 50-80% lactose reduced milk will satisfy the physiological requirements or the majority of intolerant groups, although extreme intolerance may require an exclusively lactose-free milk (Indyk *et al.*, 1996).

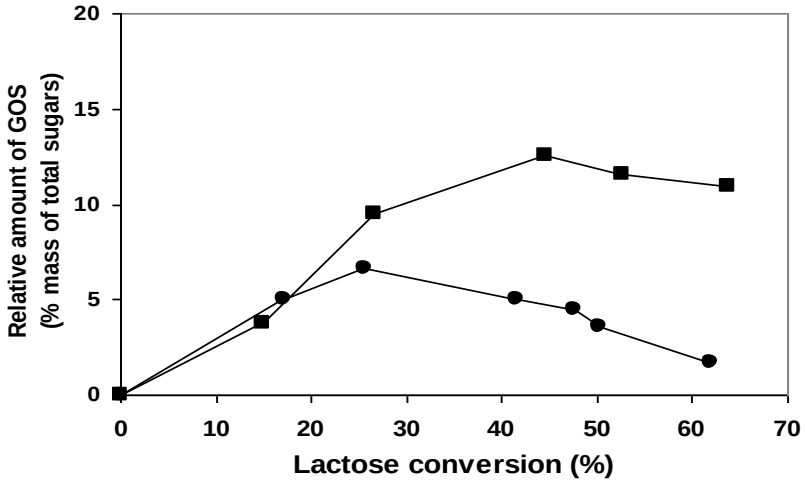


Figure 4.61 Formation of GOS during lactose conversion at different initial lactose concentrations by recombinant β-galactosidase from *B. licheniformis* DSM 13 over-expressed in *E. coli*. The reactions were performed at 60°C using 50 g/L (●) and 200 g/L (■) lactose in 50 mM sodium phosphate buffer (pH 6.5).

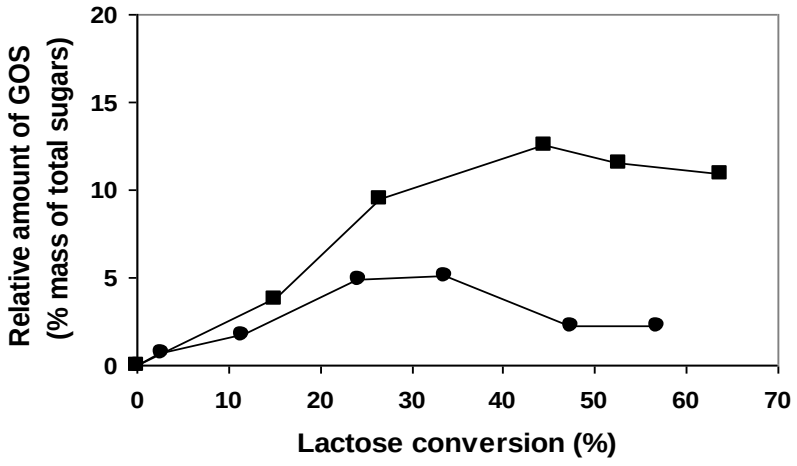


Figure 4.62 Formation of GOS during lactose conversion at different process temperatures by recombinant β-galactosidase from *B. licheniformis* DSM 13 over-expressed in *E. coli*. The reactions were performed using

200 g/L lactose in 50 mM sodium phosphate buffer (pH 6.5) at 37°C (●) and 60°C (■).

Figure 4.63 shows the effect of temperature on the lactose hydrolysis and GOS production by β-galactosidase from *B. licheniformis* DSM 13 using 50 g/L lactose as initial substrate at 10 h. The lactose hydrolysis at 55 and 60°C gave the GOS production higher than that at 37°C similar to that when used 200 g/L lactose as initial substrate (Figure 4.64). Nevertheless, there were still very low amount of GOS. High temperatures enable increased initial lactose concentration (Boon *et al.*, 2000). Moreover, high incubation temperatures favored oligosaccharide formation over lactose hydrolysis and the optimum yield of GOS was achieved under denaturing condition at 60°C.

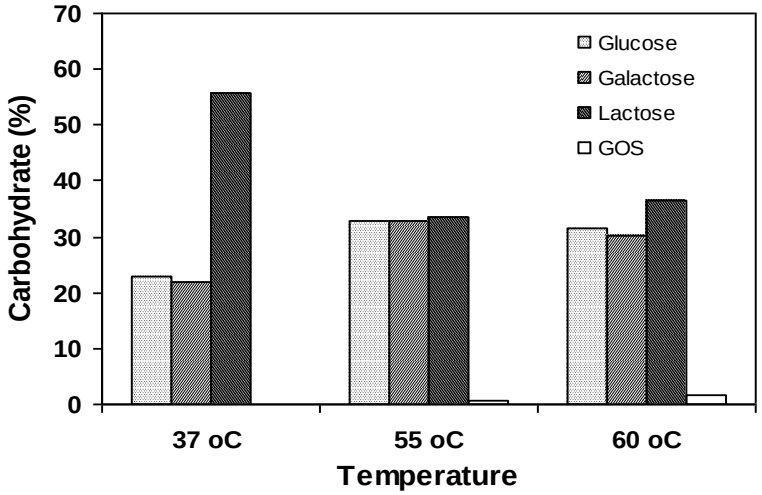


Figure 4.63 Effect of temperature on the lactose hydrolysis and GOS production by β-galactosidase from *B. licheniformis* DSM 13 using 50 g/L lactose as initial substrate at 10 h. The reaction was performed at various temperatures (37, 55 and 60°C) with 300 rpm constant shaking.

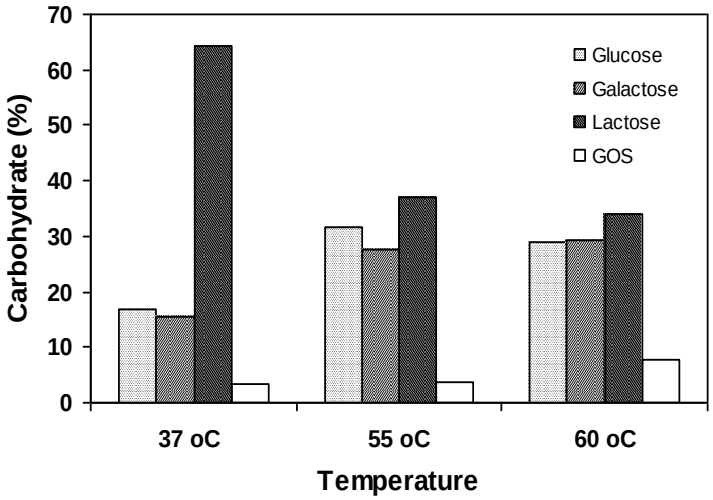


Figure 4.64 Effect of temperature on the lactose hydrolysis and GOS production by β-galactosidase from *B. licheniformis* DSM 13 using 200 g/L lactose as initial substrate at 10 h. The reaction was performed at various temperatures (37, 55 and 60^{o}C) with 300 rpm of the constant shaking.

The relationship among galactose, glucose and lactose were investigated. The β-galactosidase functions as a transgalactosidase so that the GOS production involved the transfer of galactose as the acceptor molecules. As a result, free galactose presented less than free glucose in this batch fermentation. The formation of GOS from the lactose hydrolysis reaction of batch fermentation by β-galactosidase from *B. licheniformis* DSM 13 at 37^{o}C slightly increased from initial reaction while lactose was slowly hydrolyzed. There was no GOS yield at 50 g/L lactose as initial substrate compare with 200 g/L lactose (Figure 4.65). It was found that GOS production with 50 g/L lactose slightly higher than that of 200 g/L lactose at the initial time to 5 h after that reduced and less than that at 200 g/L (Figure 4.66). In Figure 4.67 also demonstrates time course of lactose hydrolysis at 60^{o}C, GOS production was then increased at the initial lactose concentrartion of 200 g/L and slightly increased more

than that at 50 g/L and decreased to nearly zero at 24 h. Noticed that the lactose hydrolysis had increased when the reaction temperature and amount of lactose increased and also increased with time course according to that of the β-galactosidase from *B. stearothermophilus* (Chen *et al.*, 2008). In contrast, increasing temperature did not enhance the synthesis of GOS at 37^{o}C (Chen *et al.*, 2003).

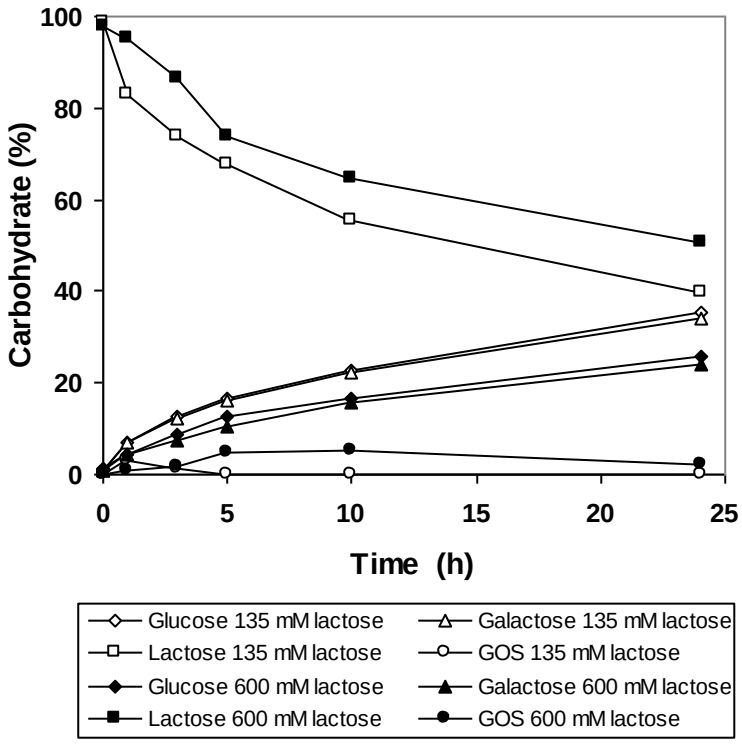


Figure 4.65 Time course of the lactose hydrolysis and the GOS formation from the batch reaction by β-galactosidase from *B. licheniformis* DSM 13 at 37^{o}C using 50 and 200 g/L lactose as initial substrate. The reaction was performed at pH 6.5 with 300 rpm of the constant shaking.

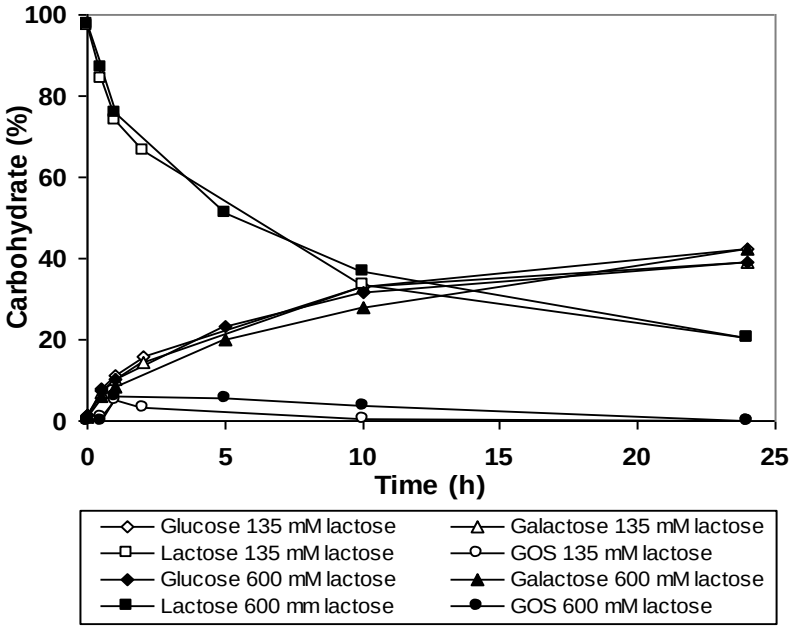


Figure 4.66 Time course of the lactose hydrolysis and the GOS formation from the batch reaction by β-galactosidase from *B. licheniformis* DSM 13 at 55^{o}C using 50 and 200 g/L lactose as initial substrate. The reaction was performed at pH 6.5 with 300 rpm of the constant shaking.

Following the lactose conversion, it was found that 26% of lactose conversion revealed approximately 6% of GOS production using 50 g/L lactose as substrate at 60^{o}C (Figure 4.68) and continuely decreased whereas approximately 10% GOS production was the highest GOS product using 200 g/L lactose as substrate at 60^{o}C (Figure 4.69) and then rapidly decreased. The percentage of lactose converted to GOS during the hydrolysis of lactose raised with an increase the initial lactose concentration (Yang and Tang, 1988; Iwasaki *et al.*, 1996). GOS productions from various microorganisms have been reported. A maximum oligosaccharide conversion of over 30% by β-galactosidase from *A. oryzae* at 40^{o}C and pH 4.5 was obtained with a lactose concentration greater than 1.11 mol/liter for the lactose concentration

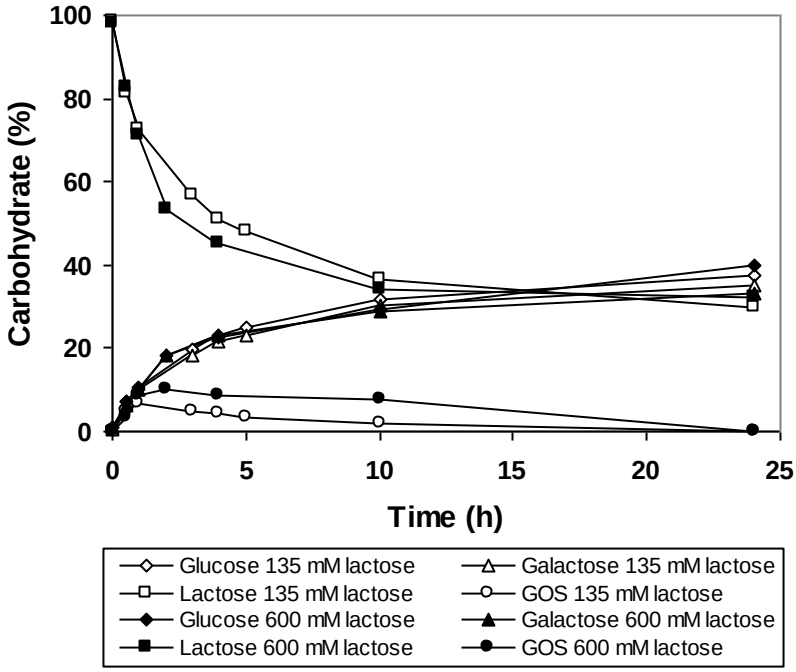


Figure 4.67 Time course of the lactose hydrolysis and the GOS formation from the batch reaction by β-galactosidase from *B. licheniformis* DSM 13 at 60^{o}C using 50 and 200 g/L lactose as initial substrate. The reaction was performed at pH 6.5 with 300 rpm of the constant shaking.

ranging from 0.139 to 1.67 mol/liter (Iwasaki *et al.*, 1996). Chen *et al.* (2003) reports that β-galactosidase from *E. coli* can produce 44% (w/w) GOS under experiment condition of pH 6.5 at 37^{o}C (Chen *et al.*, 2003). β-Galactosidase from *Enterobacter cloacae* B5 synthesized galacto-oligasaccharide with a high yield of 55% from 275 g/L lactose at 50^{o}C for 12 h (Lu *et al.*, 2009). A maximum yield of 32.5% (w/w) GOSs were achieved from 40% lactose solution at 45^{o}C, pH 6.8 when the lactose conversion was 59.4% (Hsu *et al.*, 2007). The yield of GOS from a thermostable *Sulfolobus solfataricus* β-galactosidase was obtained more than 50% for 56 h from 600 g/L lactose solution at 80^{o}C, pH 6.0 (Park *et al.*, 2008). It has been recognized that the origin of enzyme plays a significant role in the formation of GOS.

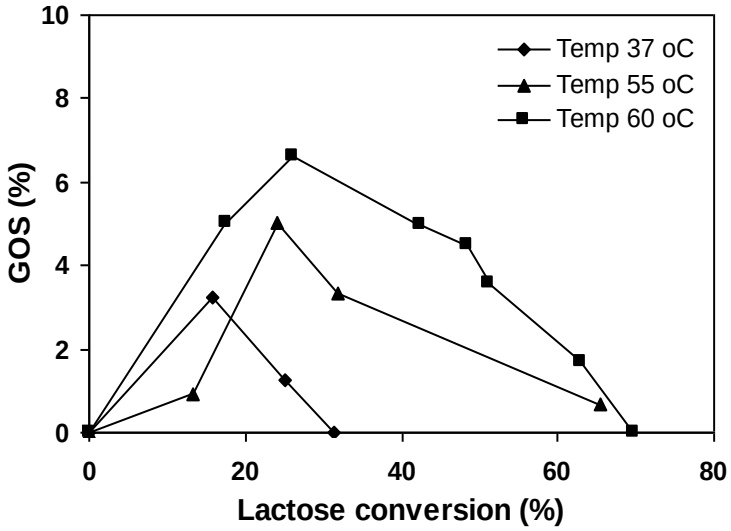


Figure 4.68 GOS formation during lactose conversion by β-galactosidase from *B. licheniformis* DSM 13 using 50 g/L lactose as initial substrate. The reaction was performed at pH 6.5 with various temperatures (37, 55 and 60^{o}C) of batch reaction and 300 rpm constant shaking.

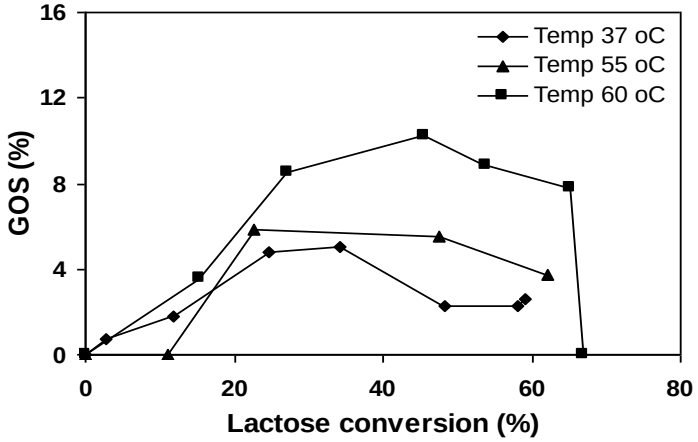


Figure 4.69 GOS formation during lactose conversion by β-galactosidase from *B. licheniformis* DSM 13 using 200 g/L lactose as initial substrate. The reaction was performed at pH 6.5 with various temperatures (37, 55 and 60^{o}C) of batch reaction and 300 rpm constant shaking.

Considering the enzyme properties, temperature and its hydrolytic activity of lactose, the *B. licheniformis* DSM 13 β-galactosidase has a potential for enzyme application in a low-lactose milk production for milk pasteurization process. In addition, *B. licheniformis* DSM 13 is generally regarded as safe status and never been reported to be pathogenic for either animals or plants. Consequently, *B. licheniformis* DSM 13 β-galactosidase could be suitable for application in the low-lactose milk production. The availability of lactose hydrolysed milk is recognized to be of special importance to the high proportion of human populations with intolerance to lactose. Since most populations can tolerate low levels of intact lactose, it generally accepted that a 50-80% lactose reduced milk will satisfy the physiological requirements of the majority of intolerant groups (Indyk *et al.*, 1996).

CHAPTER V

CONCLUSION

In this studied the β-galactosidase enzyme from *B. licheniformis* DSM 13 has been cloned and sequenced and the properties of recombinant β-galactosidase enzyme from *B. licheniformis* DSM 13 were investigated. This is the first studied about the lactose hydrolysis of β-galactosidase from *B. licheniformis* different from the previous studied of this microorganism (Trân *et al.*, 1998). So that, this studied was cloning and characterization of the recombinant β-galactosidase from *B. licheniformis* DSM 13 expressed in *E. coli*, especially reveal the high level of lactose hydrolysis and produced the low level of GOS production. Furthermore, the purification procedure of this enzyme is only single step. For the sequencing analysis indicates that *lacA* gene from recombinant *B. licheniformis* DSM 13 β-galactosidase should belong to the glycosyl hydrolase family 42. Some divalent cations Cu^{2+}, $Zn^{2+}$and Fe^{2+} can inhibit 66-100% of the enzyme activity. The optimum pH and temperature were 6.5 and 50^{o}C that led this suitable for the hydrolysis lactose in milk. This enzyme can be used in the production of lactose-reduced milks and dairy products low in GOS as the GOS formation were at lower levels compared with other sources. This enzyme should be the advantage for lactose malabsorbers and the dairy industry in the near future for reduces lactose in dairy product. Consequently, it will be continued to utilize for food application such as to produce low lactose-milk.

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APPENDICES

APPENDIX A

PLASMID MAPS

1. pET-21a(+) vector

The pET-21a(+) vector carry an N-terminal T7-Tag sequence plus an optional C-terminal His-Tag sequence. These vectors differ from pET-24a-d(+) only by their selectable marker (ampicillin vs. kanamycin resistance). Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (from http://www.biovisualtech.com/bvplasmid/pET-21_a_(+).htm).

pET-21a(+) sequence landmarks:

T7 promoter	311-327
T7 transcription start	310
T7-Tag coding sequence	207-239
Multiple cloning sites	
(BamH I - XhoI)	158-203
HisiTag coding sequence	140-157
T7 terminator	26-72
lacIcoding sequence	714-1793
pBR322 origin322	7
bla coding sequence	3988-4845
f1 origin	4977-5432

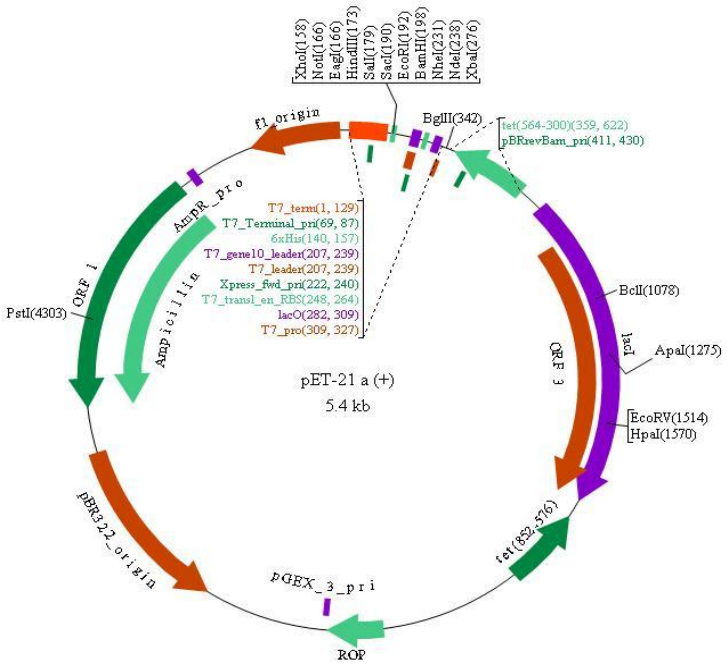


Figure A1. Schematic overview of pET-21a(+) (http://wolfson.huji.ac.il/expression/ commercial-vectors/pet-21-map.pdf).

2. pFLAG-CTS vector

Expression and secretion of C-terminal FLAG fusion proteins under control of the *tac* promoter. The *Escherichia coli* FLAG ® expression vectors allow expression, detection and purification of recombinant FLAG fusion proteins in *E. coli*. FLAG® vectors offer a choice of periplasmic (+*ompA*) or cytoplasmic expression with either amino- or carboxy-terminal tagging. All of the *E. coli* expression vectors confer ampicllin resistance for easy selection of positive transformants. Vectors using the strong tac promoter (a hybrid of the trp and lac promoters) offer protein expression levels in excess of 10 mg/L of culture when using IPTG as a de-repressor. They can be used to express protein in any established expression host (http://www.sigmaaldrich.com/ life-science/molecular-biology/molecular-biology-products.html? TablePage=9614873).

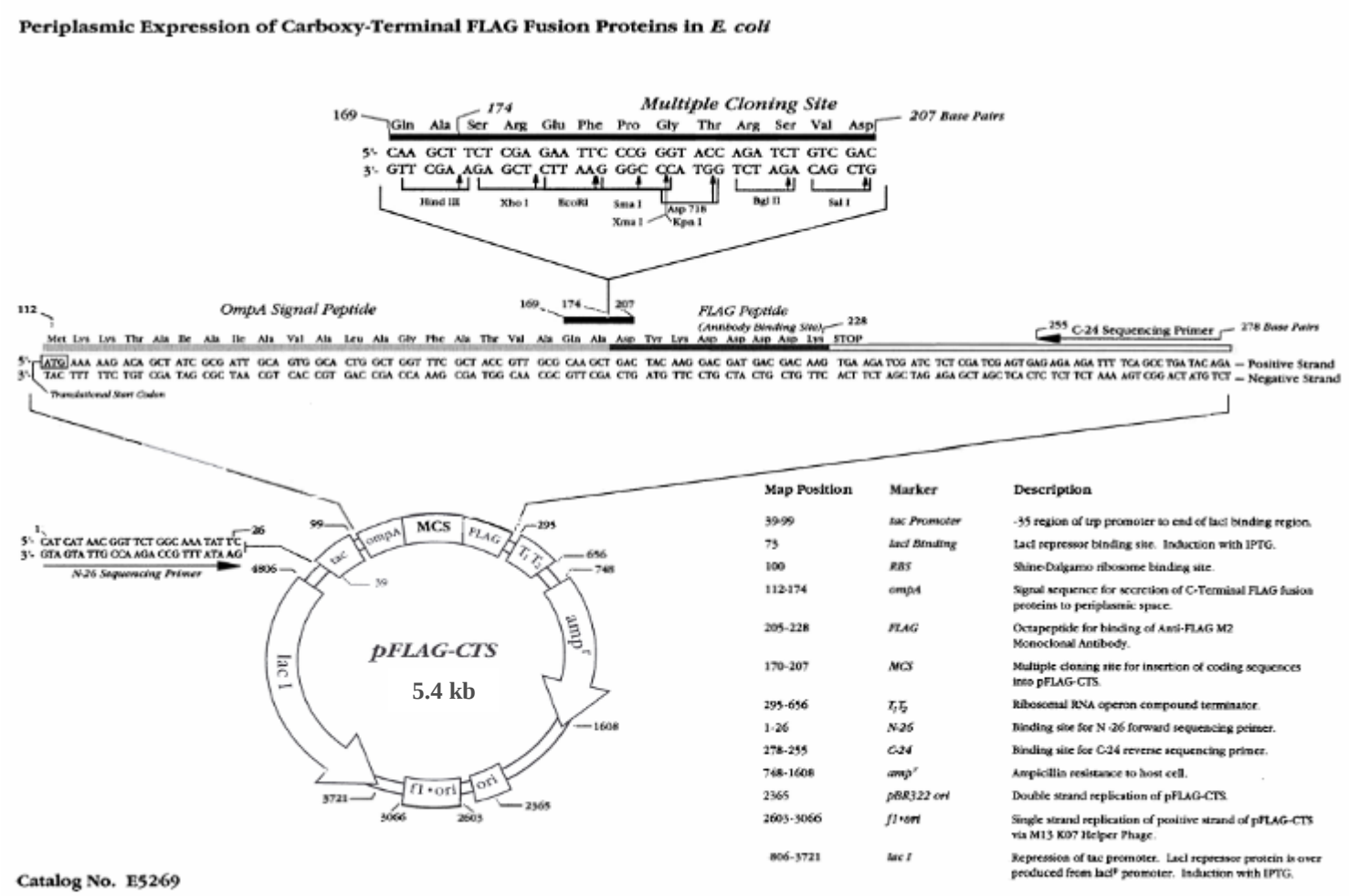


Figure A2. Schematic overview of pFLAG-CTS from Sigma Aldrich Corp.

APPENDIX B

ELECTROTRANSFORMATION OF *E. COLI*

1. Solution and reagents

1. LB broth

2. LB agar containing 100 mg/mL ampicillin

2. 10% (v/v) glycerol

3. SOC medium; 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

2. Preparation of electrocompetent cells

2.1 Inoculate 1% inoculum size of overnight *E. coli* (TOP10 and DH5α) into 250 mL of LB media in 1 L flask.

2.2 Incubate at 37°C at 150 rpm until OD_{600} of approximately 0.5–0.7 (the best results are obtained with the cells that are harvested at early to mid log phase; the appropriate cell density)

2.3 Chill cells on ice for 20 min. (also chill everything on ice bath before use such as 10% glycerol and all containers)

2.4 Harvest cells by centrifugation at 4000 rpm for15 min at 4°C

2.5 Pour off and discard supernatant and keep the cell pellet. It is better to sacrifice the yield by pouring off a few cells than to leave any supernatant behind (the supernatant contains some salts that make low yield efficiency).

2.6 Gently resuspend the cell pellet in 125 mL of cold sterile 10% glycerol (use eppendorf instead of vortex (vortex not allow).

2.7 Centrifuge at 4000 rpm for 15 min at 4°C.

2.8 Carefully pour off and discard the supernatant.

2.9 Resuspend the pellet in 125 mL of cold sterile 10% glycerol. Centrifuge at 4000 rpm for 15 min at 4°C; carefully pour off and discard the supernatant.

2.10 Resuspend the pellet in 20 mL of cold sterile 10% glycerol and then transfer into a 50 mL sterile cold tube.

2.11 Centrifuge at 4000 rpm for 15 min. at 4°C and then carefully pour off and discard the supernatant.

2.12 Resuspend the cell pellet in 1 mL of cold sterile 10% glycerol. The cell concentration should be at least $1x10^8$ cells/mL. Divide this suspension in 50 μL in sterile and cold eppendorf tubes and freeze them immediately in liquid nitrogen. These aliquots are then stored at -70°C. The cells are stable for at least 6 months under these conditions until used.

3. Electroporation

3.1 Place a 1.5 microfuge tube and 0.1 cm electroporation cuvette on ice.

3.2 Thaw the electrocompetent cell on ice.

3.3 Pipett 2-5 μL of DNA into the electrocompetent cell, mix well and incubate on ice 1 min.

3.4 Set the micropulser to “EC 1” when using 0.1 cm cuvettes.

3.5 Transfer the mixture of the cell and DNA to a cold electroporation cuvette, tap the mixture to the bottom, and then put the cuvette into the chamber slide. Put the slide into the base of the base of the chamber. Pulse two times (the time constant should be 4.6-5.5 milliseconds.

3.6 Remove the cuvette from the chamber and immediately add 600 μL of SOC medium into the cuvette. Transfer it quickly but gently resuspened the cells with a sterile pipette. Delaying this transfer by even 1 minute causes a 3-fold drop in transformation. This phenomenon continues to decrease 20-fold drop by 10 min.

3.7 Transfer the cell suspension to a microfuge tube and incubate at 37°C for 45 min at 200 rpm.

3.8 Plate on LB agar containing 100 mg/mL ampicillin.

APPENDIX C

SDS-PAGE METHOD (MANUALLY)

1. Solution and reagents

1. Composition of sepatating gel and stacking gel as following:

Composition	**Separating gel**	**Stacking gel**
H_2O	1.7 mL	0.75 mL
30% Acrylamide	2.0 mL	0.325 mL
1.5 M Tris-HCl, pH 8.8	1.25 mL	-
1.5 M Tris-HCl, pH 6.8	-	0.313 mL
10% SDS	50 μL	12.5 μL
10% APS	50 μL	12.5 μL
TEMED	7.5 μL	5 μL

2. 3x Gel loading buffer

Prepare 5% β-mercaptoehtanol, 6% SDS, 0.1% bromophenol blue, 30% glycerol in 150 mM Tris pH 6.8

3. 5x Running buffer

Prepare 15 g Tris, 72 g glycine, 5 g SDS and then, adjust volume to 1 liter.

4. Coomassie blue stain

Dissolve 0.25 g Coomassie brilliant blue R250 in 90 mL methanol : DI (40 : 50) and add 10 mL glacial acetic acid.

5. Destaining solution

Prepare 800 mL methanol, 200 mL glacial acetic acid and 1000 mL DI

2. Method

2.1 To prepare acrylamide gel, prepare the gel according to the general standard method using the reagents for separating gel and stacking gel from the table.

2.2 To prepare the sample, centrifuge100 μL cell suspension for 2 min, 14000 rpm at 37^{o}C adding 100 μL of 3x gel loading buffer, vigorously vortex, and then heat at 100 ^{o}C for 5-10 min after that centrifuge for 5 min, 14000 rpm.

2.3 Load 20 μL samples into the gel and run the gel at 15 mA in 1x running buffer for 30 min and then run at 30 mA until the sample reach to the end of the gel.

2.4 Place the gel in a plactic box and then stain the gel with Coomassie Blue until the band was appeared.

2.5 Destain the gel with destaining solution for overnight. Pour off the destaining solution and rinse with water.

2.6 Dry the gel onto the gel dryer at 50^{o}C for 1.30 h.

APPENDIX D

PROTEIN PURIFICATION: NI-NTA COLUMN

1. Chemical preparation

Equilibrium buffer

Dissolve20 mM Tris pH 8.0 and 150 mM NaCl in DI.

Wash solution 1

Dissolve 20 mM Tris pH 8.0, 150 mM NaCl and 5 mM Imidazole in equilibrium buffer.

Wash solution 2

Dissolve 20 mM Tris pH 8.0, 150 mM NaCl and 20 mM Imidazole in equilibrium buffer.

Elution buffer

Dissolve 20 mM Tris pH 8.0, 150 mM NaCl, 250 mM Imidazole in equilibrium buffer.

2. Protein purification (manually)

Bead Ni-NTA was soaked in a 70% ethanol. 400 μL of Bead Ni-NTA was spun down at 4000 rpm at 4^{o}C for 2 min. Supernatant was pour out and approximately 200 μL of slurry was collected. After that, the slurry was washed with 1 mL distilled water (DI) and then centrifuged at 4000 rpm at 4^{o}C for 2 min (repeat this step for 4 times, washing with DI and centrifuging). The slurry was added with 1 mL of equilibrium buffer and gently inverted and then centrifuged at 4000 rpm at 4^{o}C for 2 min (repeat this step for 4 times, adding equilibrium buffer and

centrifuging). 1 mL sample was added onto the bead and equilibrated for 5-10 min and then centrifuged at 4000 rpm at 4^{o}C for 2 min. The flow through fraction was collected. The column was then added with wash solution 1 for 5 column volume (= 1 mL) and gently inverted until well-mixed and then centrifuged at 6000 rpm at 4^{o}C for 5 min. The wash 1.1 fraction was collected in the eppendorf. The column was then added with wash solution 1 again for 5 column volume (cv) (200 μL x 5 cv = 1 mL) gently invert until well-mixed and then centrifuged at 6000 rpm at 4^{o}C for 5 min. The wash 1.2 fraction was collected in the eppendorf. The column was then added with wash solution 2 for 5 column volume (1 mL) and gently inverted until well-mixed and then centrifuged at 6000 rpm at 4^{o}C for 5 min. The wash 2 fraction was collected in the eppendorf. The column was then added with elution buffer 1 for 100 μL, gently inverted until well-mixed and left it for 5 min and then centrifuged at 6000 rpm at 4^{o}C for 5 min. The Elution fraction 1 was collected in the eppendorf (repeat this step for 2 times, adding the elution buffer and centrifuging). The elution fractions, E1, E2, E3, were then collected, respectively.

APPENDIX E

PROTEIN DETERMINATION: BRADFORD METHOD

The protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. It is sensitive to about 5 to 200 micrograms protein, depending on the dye quality.

Material

Bradford reagent : one bottle contains 450 mL solution of dye (Bio-Rad); phosphoric acid and methanol (or dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol, add 100 mL 85% (w/v) phosphoric acid and adjust volume to 1 liter distilled water). Dilute 1 volume of dye reagent concentrate (Bio-Rad) with 4 volumes of high quality distilled water. Filter through a Whatman #1 paper and store the reagent in a brown glass bottle at room temperature.

Method

Pipette 1000 µL Bradford reagent into the disposal cuvette. Add 20 µL appropriate dilutions sample and well-mixed the solution. Leave the cuvette for 15 min at room temperature. Measure the sample with the Beckmann DU-800 at 595 nm (warm up the visible lamp at least 15 min)**.**

Standard curve

Prepare stock BSA solution: Dissolve 1 mg BSA in DI and adjust to 1 mL. Dilution BSA solution in a series from 1 mg/mL to 0.1 mg/mL as the following:

BSA conc.(mg/mL)	BSA (μL)	DI (μL)
0	0	100
0.1	10	90
0.2	20	80
0.4	40	60
0.6	60	40
0.8	80	20
1.0	100	0

APPENDIX F

METALLIC ACTIVATION

1. Solution Preparation

1. Dissolve 10 mM Bis-tris in distilled water and adjust pH to 6.5 with HCl.

2. Dissolve1 M NaCl in distilled water and then make the dilution to 10 mM, 100 mM, and 250 mM in Bis-tris.

3. Dissolve 1 M KCl in distilled water and then make the dilution to 10 mM, 100 mM, and 166.67 mM in Bis-tris.

4. Prepare 166.66 mM all chemicals, $MnCl_2$, $MgCl_2$, $FeCl_2$, $CaCl_2$, $Cu(SO_4)$, and $ZnCl_2$ with Bis-tris and then diluted all chemical to 16.66 mM in Bis-tris.

5. Prepare 24.558 mM *o*NPG in Bis-tris.

* This method diluted enzyme with 10 mM bis-tris before measure the enzyme activity.

2. Treatments

1. Na^+ : 1 mM, 10 mM, 100 mM
2. K^+ : 1 mM, 10 mM, 100 mM
3. K^+ 1 mM + Na^+ 10 mM
4. K^+ 10 mM + Na^+ 10 mM
5. Mn^{2+} 1 mM + Na^+ 10 mM
6. $Mn2^+$ 10 mM + Na^+ 10 mM
7. Mg^{2+} 1 mM + Na^+ 10 mM

8. Mg^{2+} 10 mM + Na^{+} 10 mM
9. Fe^{2+} 1 mM + Na^{+} 10 mM
10. Fe^{2+} 10 mM + Na^{+}10 mM
11. Ca^{2+} 1 mM + Na^{+}10 mM
12. Ca^{2+} 10 mM + Na^{+}10 mM
13. Cu^{2+} 1 mM + Na^{+}10 mM
14. Cu^{2+} 10 mM + Na^{+}10 mM
15. Zn^{2+} 1 mM + Na^{+}10 mM
16. Zn^{2+} 10 mM + Na^{+}10 mM

3. Enzyme activity measument

1. For the control

Pipett 430 µL *o*NPG into 1 mL eppendorf tube.

Add 50 µL Bis-tris.

Add 20 µL enzyme.

Incubate at 30^{o}C, 600 rpm for 10 min and then stop reaction with 600 mL of 0.4 M Na_2CO_3

2. For Na^{+} or K^{+} : 1 mM, 10 mM and 100 mM

Pipett 430 µL *o*NPG into 1 mL eppendorf tube.

Add 50 µL Na^{+} or K^{+}; 10mM, 100 mM and 1000 mM each tube. It would be 1 mM, 10 mM, 100 mM final concentration.

Add 20 µL enzyme.

Incubate at 30^{o}C, 600 rpm for 10 min ,and then stop reaction with 600 mL of 0.4 M Na_2CO_3

3. For mixed of cation

4.1 For example; K^+ 1 mM + Na^+ 10 mM

4.1.1 Pipett 430 µL *o*NPG into 1 mL eppendorf tube.

4.1.2 Add 30 µL of 16.67 mM K^+ (final conc. is 1 mM K^+).

4.1.3 Add 20 µL of 250 mM Na^+.

4.1.4 Add 20 µL enzyme.

4.1.5 Incubate at 30^{o}C, 600 rpm for 10 min, and then stop reaction with 600 mL of 0.4 M Na_2CO_3.

4.2 For example; K^+ 10 mM + Na^+ 10 mM

4.2.1 Pipett 430 µL *o*NPG into the 1 mL eppendorf tube.

4.2.2 Add 30 µL of 166.67 mM K^+ (final conc. is 10 mM K^+).

4.2.3 Add 20 µL of 250 mM Na^+.

4.2.4 Add 20 µL enzyme.

4.2.5 Incubate at 30^{o}C, 600 rpm for 10 min, and then stop reaction with 600 mL of 0.4 M Na_2CO_3.

APPENDIX G

REGENERATION OF NI-CHARGED RESIN

Regenerate Ni-charged resin step in the affinity chromatography

Ni Sepharose 6 fast flow column (GE Healthcare Bio Science AB, Uppsala, Sweden) is used for protein purification. Resin in the column is regenerated after use to purify protein. There are 10 steps of the regenerate Ni-charged resin.

Step 1. Distilled water (10 column volume; cv)

Step 2. 0.2 M EDTA

Step 3. 0.5 M NaCl

Step 4. 1 M NaOH

Step 5. Buffer A (50 mM KH_2PO_4 / 500 mM NaCl / 20 mM Imidazol), pH 6.5

Step 6. 50 mM Na-acetate / 0.3 M NaCl / pH 4 (5 cv)

Step 7. 0.2 M $NiCl_2$

Step 8. 50 mM Na-acetate / 0.3 M NaCl / pH 4 (5 cv)

Step 9. Distilled water

Step 10. Buffer A (50 mM KH_2PO_4 / 500 mM NaCl / 20 mM Imidazol) pH 6.5

* All reagents should be degassed before use.

APPENDIX H

SONICATION AND HOMOGENIZATION

1. Sonication Procedure

1. Prepare the sample in 50 mM NaH_2PO_4 buffer pH 6.5 (1 : 3 to 1 : 5 dilutions of the cells), then put it on ice box to cool down at least 5 min.

2. Pulse for 30 sec. at 80% intensity, the sample always on the ice box during sonication.

3. Stop pulse for 30 sec. and the sample still on the ice box and pulse again for 30 sec, then repeat from 2 to 3 for 4 times.

4. Wash the sonicator with alcohol and distilled water and then wipe it dry.

Notice: wear ear protector and glasses when working with the sonicator.

2. Homogenization Procedure

1. Prepare the sample in 50 mM NaH_2PO_4 buffer and the dilution should be 1 : 3 to 1 : 5 of the cells (it must be no rest of cotton in the sample before use).

2. Start the cooling of the homogenizer at 4°C for 30 min before the run.

3. Fill tap water into the vessel and purge without pressure. Remove most of water and add the sample. Circulate your sample while adjusting the pressure (100 bar max) and then change the outlet tubing to a separate vessel.

4. When finishes, wash the homogeniser with 1 liter hot tap water and add detergent (0.5 liter of 0.5 M NaOH) for 30 min (circulating) and then add twice 1 liter hot tap water. Empty the homogenizer completely and turn off.

Notice: wear ear protector and glasses when working with the homogenizer.

APPENDIX I

CALCULATION METHOD OF GOS PRODUCTION

1. GOS production using 600 mM lactose as substrate

At 600 mM lactose in 50 mM NaH_2PO_4, pH 6.5 (~ 200 g/L, we need the final volume of the reaction equal 2 mL).

The activity of the pool A (purified enzyme) equal to 88 U/mL of lactose.

The activity 1 mL = 88 Units

1 µl = 0.088 Units

Definition 1 Unit = 1 µmol of glucose released per min

So, 1000 mL lactose solution has a concentration of 0.6 mol

2 mL lactose solution " $\frac{0.6x2}{1000}$ = 0.0012 mol lactose

= 1200 µmol lactose

= 1200 µmol glucose released

For 24 h = 1440 min (time for GOS production)

So, the unit of enzyme used per min = $\frac{1200\mu mol}{1440\,min}$ = 0.83 Units

The activity 88 unit 1 mL

0.83 unit $\frac{0.83}{88}$ = 0.00943 mL (= 9.4 µl of enzyme)

We have to add 20 times of enzyme = 0.83x20 = 16.6 units

That means the volume of enzyme we need = 9.4x20 = 188 µL of enzyme

∴ The volume of 600 mM lactose solution = 2000-188 = 1,812 µL

2. GOS production using 135 mM lactose as substrate

At 135 mM lactose in 50 mM NaH_2PO_4, pH 6.5 (~ 50 g/L, we need the final volume of the reaction equal 2 mL).

The activity of the pool B (purified enzyme) equal to 60 U/mL of lactose.

The activity 1 mL = 60 Units

1 μL = 0.060 Units

Definition 1 Unit = 1 μmol of glucose released per min

$\therefore$ 60 Units = 60 μmol of glucose released per min

So, 1000 mL lactose solution has a concentration of 0.135 mol

2 mL lactose solution " $\frac{0.135x2}{1000}$ = 0.00027 mol lactose

= 270 μmol lactose

= 270 μmol glucose released

For 24 h = 1440 min (time for GOS production)

So, the unit of enzyme used per min = $\frac{270\mu mol}{1440\,min}$ = 0.1875 Units

The activity 60 unit 1 mL

0.1875 unit $\frac{0.1875}{60}$ = 0.00312 mL (= 3.1 μl of enzyme)

We have to add 20 times of enzyme = 0.1875x20 = 3.75 units

That means the volume of enzyme we need = 3.1x20 = 62 μL of enzyme

$\therefore$ The volume of 135 mM lactose solution = 2000-62 = 1,938 μL

APPENDIX J

B-GALACTOSIDASE ASSAY

(Thu-Ha Nguyen : personal communication, BOKU, Vienna, Austria)

1. β-Galactosidase assay with natural substrate lactose

Lactose or milk sugar, a natural substrate of β-galactosidases, is a disaccharide consisting of D-glucose and D-galactose units linked through a β1→4-glycosidic linkage. Lactose is commonly used as a natural substrate for the detection of the hydrolytic activity of β-galactosidases. The release of D-glucose, a hydrolytic product, can be determined colorimetrically (Kunst *et al.*, 1988). There are many methods of lactose and galactose determination by gas chromatography, by HPLC or by TLC. The majority of determination of lactose is performed by enzymatic techniques. There are a large number of methods for determining lactose which are based on hydrolysis by means of β-galactosidase. One unit of lactase activity is defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the specified assay conditions.

Some points should be kept in mind before using this assay method:

- Each enzyme differs in pH and temperature optima as well as the stability in certain ranges of pH and temperature.

- Enzyme assays must be performed at *temperatures at which the enzyme is active* and, for the sake of comparison, must always be performed at the same temperature.

- Enzyme assays must be performed using *an appropriate buffer* with an adequate pH.

- The concentration of the substrate solution used for the assay is another point to consider. Since measuring the activity of the enzyme is dependent on having an ample supply of substrate, you want the substrate to always be present in excess so that it is not limiting your ability to measure the enzyme.

- Appropriate dilution of the enzyme should be used.

- The reaction continues as long as there is substrate available; you will need to stop the reaction after a *designated time*. This is done by heating the assay solution at 99°C for 5 minutes to denature the enzyme and render it inactive.

Materials

1. 50 mM sodium phosphate buffer, pH 6.5 ($NaH_2PO_4.2H_2O$; Mr = 156.01g/mol) Dissolve 1.9501 g sodium phosphate in DI and then adjust volume to 250 mL. Titrate with NaOH to obtain pH 6.5.

2. 600 mM lactose solution (in 50 mM sodium phosphate buffer, pH 6.5) Lactose monohydrate (Mr = 360.32 g/mol). Dissolve 10.8096 g/50 mL buffer.

Methods

Enzyme assay

- Add 20 µL of enzyme solution to 480 µL of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5.

- Incubate the reaction mixture at 30°C using an Eppendorf thermomixer compact with agitation speed of 600 rpm.

- After 10 minutes, stop the reaction by heating the reaction mixture at 99°C for 5 minutes.

- After cooling to room temperature, the release of D-glucose is determined colorimetrically using the GOD/POD assay (Kunst *et al.*, 1988).

Blank sample: use 20 µL of water instead of enzyme solution

Samples: at least in duplicate, experimental error should not exceed 5%

Calculation of the activity:

Note: c_{glucose} [mM] = c_{glucose} [µmol/mL]

$$\frac{U}{ml} = c_{glucose}[mM] \times \left(\frac{1}{t_{reaction}(\text{min})} \right) \times \left(\frac{V_{enzyme} + V_{lactose}}{V_{enzyme}} \right) \times (enzyme\ dilution\ \ factor)$$

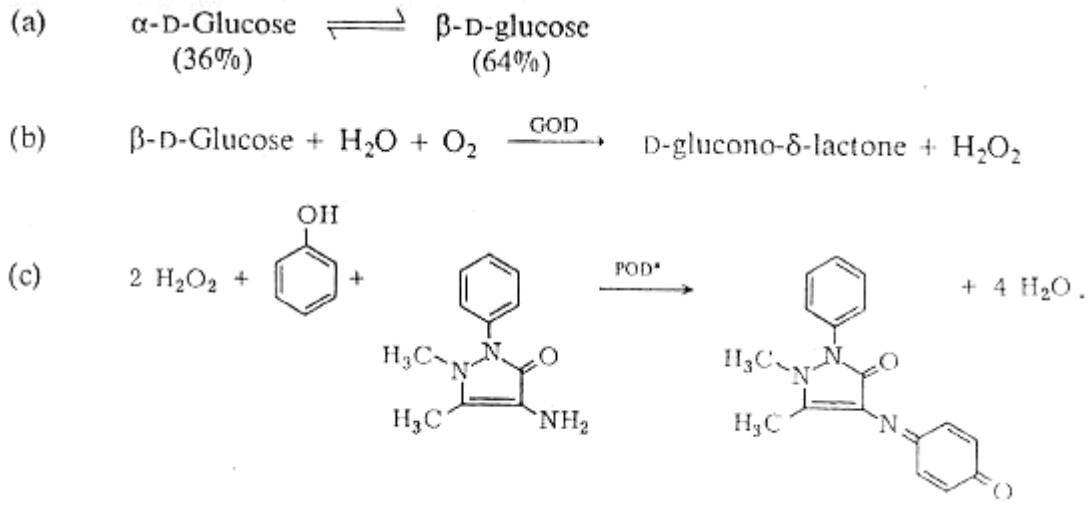
$$\frac{U}{ml} = c_{glucose}[mM] \times \left(\frac{1}{10} \right) \times \left(\frac{20 + 480}{20} \right) \times (enzyme\ dilution\ \ factor)$$

$$\frac{U}{ml} = c_{glucose}[mM] \times 2.5 \times (enzyme\ dilution\ \ factor)$$

2. Enzymatic method for determination of D-glucose with glucose oxidase (GOD) and peroxidase (POD)

This method is used for the determination of D-glucose in a solution. In aqueous solution D-glucose exists in two configurational modifications, α-D-glucose (36%) and β-D-glucose (64%). Although glucose oxidase specifically oxidizes β-D-glucose, it can be used to determine the total glucose content of a sample since the remaining α-D-glucose is converted to the β-form by spontaneous mutarotation (Pigman and Anet, 1972).

Principle of the method:



(Kunst *et al.*, 1988)

β-D-Glucose is oxidized by GOD to D-glucono-δ-lactone. The hydrogen peroxide produced in the glucose oxidase reaction (b) is determined by the means of phenol and 4-aminophenazone in the presence of peroxidase, yielding a coloured product. The amount of dye formed is a measure of the glucose concentration in the sample. The absorption of the dye can be measured between 470 and 550 nm and the measured absorbance of the sample is compared with that of a glucose standard (Kunst *et al.*, 1988).

Optimized conditions for measurement: the equilibria of reaction (b) and (c) lie completely on the right. As result of consumption of β-D-glucose, all α-D-glucose is transformed into the β-formed into the β-form by mutarotation and thus become susceptible to the action of GOD, GOD is most active at pH 5.6 while POD is most active between pH 4 and 8. On the other hand, mutarotation is dependent on pH and phostphate concentration. The measurements in this method are made at pH 6.5 and

in phosphate buffer, 50 mM/L. Under these conditions quantitative oxidation of glucose is obtained within 35-60 min at room temperature.

Materials

1. Solution 1: GOD/POD solution

Dissolve 2.77 g KH_2PO_4 and 32.1 mg 4-amino anti-pyrine in 160 mL water. Adjust to pH 7.0 with 1M NaOH. Add 21 mg GOD (glucose oxidase from *Aspergillus niger*, lyophilized, Fluka, 205 U/mg enzyme preparation, -20°C). Add 1.5 mg POD (peroxidase from horseradish, lyophilized, Sigma, 277 mg enzyme preparation contain 50 000 Units → 180 U/mg enzyme preparation, -20°C). Make up the solution to 200 mL and store in a dark bottle. This solution can be kept at 4°C for 2-3 weeks.

2. Solution 2: phenol (560 mmol/L)

Dissolve 211 mg phenol in 4 mL water. This phenol solution is stable indefinitely.

3. Assay solution

Mix 50 mL of solution 1 and 1 mL of solution 2 and store in a dark bottle. This assay solution is stable for 1 week at 4°C. This assay solution contains GOD (~ 21.5 U/mL), POD (~ 1.35 U/mL), 4-aminoantipyrine (157 µg/mL) and phenol (11 mmol/L) in 0.1 M potassium phosphate buffer (pH 7.0).

4) Glucose standard solutions (for the standard curve)

Glucose monohydrate; Mr = 180.16 g/mol, Stock solution: 1 g/L (dissolve 0.1 g anhydrous glucose in 100 mL water). Glucose standard solution should be prepared each day.

Methods

1. Glucose standard curve

Solutions:	**1**	**2**	**3**	**4**	**5**	**6**
$V_{STOCK\ SOLUTION}$ (parts)	1	2	3	4	5	6
V_{WATER} (parts)	9	8	7	6	5	4
Concentration (g/L)	0.1	0.2	0.3	0.4	0.5	0.6

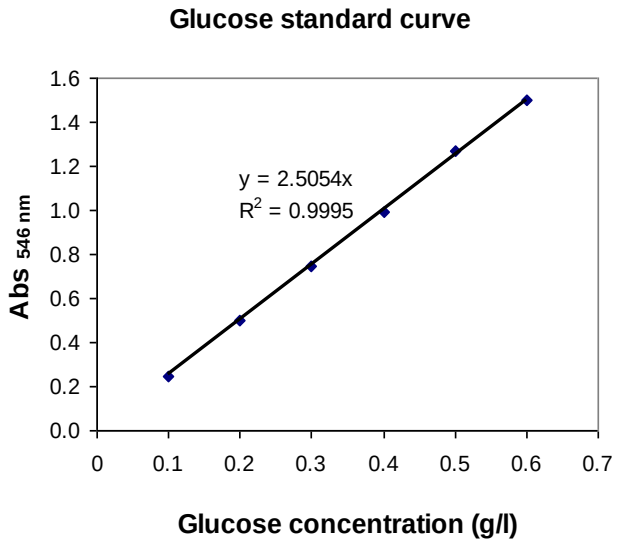
60 µL glucose solutions + 600µL assay solution

↓

Incubate in the dark at room temperature for 40 minutes

↓

Abs $_{546nm}$



2. GOD/POD assay

60 µL sample + 600µL assay solution

↓

Incubate in the dark at room temperature for 40 minutes

↓

Abs $_{546nm}$

Blank sample: use 60 µL of water instead of enzyme solution

Samples: at least in duplicate, experimental error should not exceed 5%

Results

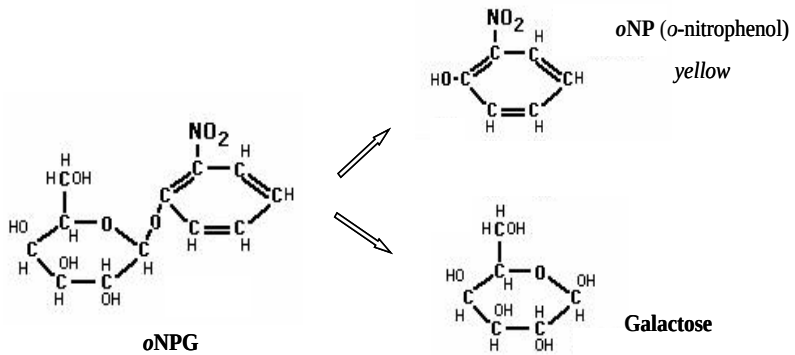
Calculation of glucose concentration:

$$c\,[g/L] = \left(\frac{Abs_{546nm} - Blank}{slope_{\text{glucosestandardcurve}}} \right) \times (sample\ dilution\ \ factor)$$

$$c\,[mM] = \frac{c\,[g/L]}{Mr_{glucose}} \times 1000$$

3. β-Galactosidase assay with chromogenic glycoside

A number of chromogenic, fluorogenic, and luminogenic substrates specific for β-galactosidase has been designed, developed and used. For a list of these substrates refer to Nakayama and Amachi (1999). It should be mentioned that the affinity of enzymes towards different substrates differs significantly therefore β-galactosidase activity units measured with one substrate do not reflect its activity units on other substrates. A chromogenic substrate, ortho-nitrophenyl-β-D-galacto-pyranosideside (*o*NPG), is the common synthetic substrate used for the determination of β-galactosidase activity. The hydrolysis of *o*NPG is catalyzed by β-galactosidase at



***o*NPG**

o-nitrophenyl-β-D-galactopyranoside

the nonreducing end of the β-galactosidic bond yielding galactose and *o*-nitrophenol, which give yellow colour and absorbs maximally at 420 nm. One unit of *o*NPG

activity is defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the specified assay conditions.

Some points should be kept in mind before using this assay method:

- Each enzyme differs in pH and temperature optima as well as the stability in certain ranges of pH and temperature.

- Enzyme assays must be performed at *temperatures at which the enzyme is active* and, for the sake of comparison, must always be performed at the same temperature.

- Enzyme assays must be performed using *an appropriate buffer* with an adequate pH.

- The *concentration of the o*NPG solution used for the assay is another point to consider. Since measuring the activity of the enzyme is dependent on having an ample supply of substrate, you want the substrate to always be present in excess so that it is not limiting your ability to measure the enzyme. Therefore, if the assay tube turns bright yellow immediately, the substrate is being used too quickly and the assay will not be accurate.

- When more enzyme is present, more product will be formed and more yellow colour will be seen. *Appropriate dilution of the enzyme* should be used.

- The reaction continues as long there is substrate available; you will need to stop the reaction after a *designated time* in order to measure the absorbance consistently. This is done by adding $NaCO_3$, a strong base (pH 10-11) that denatures the enzyme and renders it inactive.

Materials

1. 50 mM sodium phosphate ($NaH_2PO_4.2H_2O$; Mr = 156.01 g/mol) buffer pH 6.5. Dissolve 1.950125 g sodium phosphate in DI 250 mL and adjust to pH 6.5 with NaOH.

2. 22 mM *o*NPG ($C_{12}H_{15}NO_{8;}$ Mr = 301.3 g/mol) in 50 mM sodium phosphate buffer, pH 6.5. Dissolve 0.16571 g in DI and adjust volume to 25 mL. (*o*NPG should be kept at -20°C and is expensive so just prepare the amount enough for your experiment. *o*NPG is not easy to dissolve so magnetic stirrer can be used but it should be done very quickly to avoid warming up the solution. *o*NPG is sensitive to light so wrap the solution container with foil and should be always kept on ice. It should be prepared freshly everyday, however, if after the day of experiment there is still some solution left, do not throw it away. It could be kept in -20°C for the next time.)

3. 0.4 M Na_2CO_3 (Mr = 105.99 g/mol)

   Dissolve 10.599 g Na_2CO_3 in 250 mL DI or 4.2396 g in 100 mL DI.

4. *o*NP standard solution (in 50 mM sodium phosphate buffer, pH 6.5). *o*-nitrophenol ($C_6H_5NO_3$, Mr = 139.1 g/mol) Stock solution: 2 mM (Prepare 20 mM stock solution → required mass: 0.1391 g in 50 mL phosphate buffer and then make 1 : 10 dilution to obtain 2 mM stock solution).

Methods

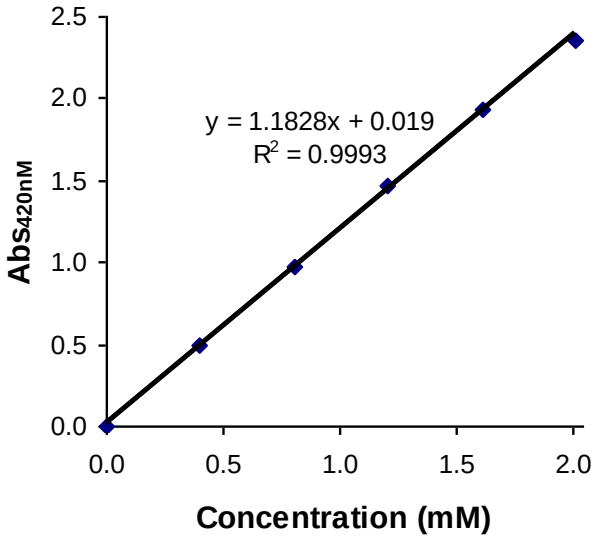
1. *o*NP standard curve

Solutions:	**1**	**2**	**3**	**4**	**5**	**6**
$V_{STOCK\ SOLUTION}$ (parts)	0	1	2	3	4	5
V_{BUFFER} (parts)	5	4	3	2	1	0
Concentration (mM)	0	0.4	0.8	1.2	1.6	2.0

0.5 mL *o*NP solutions + 0.75 mL Na_2CO_3

↓

Abs $_{420}$



An example of the standard curve:

2. Enzyme assay

480 µL *o*NPG
(22 mM in 50 mM sodium phosphate buffer, pH 6.5)

↓

Add 20 µL of enzyme solution (appropriate dilution)

↓

Incubate at 30°C for 10 min, 600 rpm using thermomixer
(To start the reaction)

↓

Add 750 µL Na_2CO_3 (0.4 M)
(To stop the reaction)

↓

Abs_{420}

Blank sample: use 20 µL of water instead of enzyme solution

Samples: at least in duplicate, experimental error should not exceed 5%

Results

Calculation of the activity:

$$\frac{U}{ml} = \left(\frac{Abs_{420nm} - Blank}{slope_{\text{oNP standard curve}}} \right) \times \left(\frac{1}{t_{reaction}(\text{min})} \right) \times \left(\frac{V_{enzyme} + V_{oNPG}}{V_{enzyme}} \right) \times (enzyme\ dilution\ \ factor)$$

$$\frac{U}{ml} = \left(\frac{Abs_{420nm} - Blank}{slope_{\text{oNP standard curve}}} \right) \times \left(\frac{1}{10} \right) \times \left(\frac{20 + 480}{20} \right) \times (enzyme\ dilution\ \ factor)$$

$$\frac{U}{ml} = \left(\frac{Abs_{420nm} - Blank}{slope_{\text{oNP standard curve}}} \right) \times 2.5 \times (enzyme\ dilution\ \ factor)$$

APPENDIX K

MEDIA AND CHEMICAL PREPARATION

1. Media preparation

1.1 LB Medium (Luria-Bertani Medium)

Dissolve 10 g Tryptone, 5 g Yeast Extract and 5 g NaCl in distilled water and make to 1 L final volume (adjust pH to 7 with 1 N NaOH) and then autoclave at 121^{o}C for 15 min.

1.2 TB Medium (Terrific Broth)

Dissolve 12 g Peptone from casein, 24 g yeast extracts, 4 mL glycerol, 15 g agar, in 900 mL distilled water and then autoclave at 121^{o}C for 15 min. (prior to use add 100 mL of 1 M KH_2PO_4 pH 7.5).

1.3 YT medium (Yeast extract-Tryptone Medium)

Dissolve 8 g Bacto tryptone, 5 g Yeast Extract, 5 g NaCl in distilled water and make to 1 L final volume (adjust pH to 7 with 1 N NaOH) and then autoclave at 121^{o}C for 15 min.

1.4 NB Medium (Nutrient Broth)

Dissolve 5 g Peptone and 3 g Meat extract in distilled water and make to 1 L final volume (adjust pH to 7 with 1 N NaOH) and then autoclave at 121^{o}C for 15 min.

1.5 SOC medium

Dissolved 2% Tryptone, 0.5% Yeast Extract, and 10 mM NaCl, 2.5 mM KCl,

10 mM $MgCl_2$, and 10 mM $MgSO_4$ in distilled water and then autoclave at 121^{o}C for 15 min. Add 20 mM glucose (by passing glucose through a 0.22 μm filter for sterilization) into the solution.

2. Chemical preparation

2.1 TAE solution

Stock Solution/Liter	Working Solution
50x 242 g of Tris base	1x
57.1 mL of glacial acetic acid	40 mM Tris-acetate
100 mL of 0.5 M EDTA (pH 8.0)	1 mM EDTA

2.2 Gel-loading buffers

Dissolve 0.25% (w/v) bromophenol blue in 40% (w/v) sucrose in distilled water.

2.3 Dimethylsulfoxide (DMSO)

Divide the solution into 1 mL aliquots in sterile tubes. Close the tube tightly and store at -20^{o}C. Use aliquot only once and then discard.

2.4 Ethidium Bromide (10 mg/mL)

Add 1 g of ethidium bromide to 100 mL of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or store it in the dark cupboard.

2.5 Glygerol (40% v/v)

Dissolve 40 mL of glycerol into 60 mL of distilled water and autoclave at 121^{o}C for 15 min.

2.6 Isopropyl β-D-thiogalactopyranoside (IPTG; 1 M)

Dissolve 2.383 g of IPTG into distilled water. Adjust the volume of solution to 10 mL with distilled water. Sterilize passing it through a 0.22 μm disposable filter. Dispense the solution into 1 mL aliquots and store them at -20^{o}C.

2.7 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 2% w/v)

Dissolve 20 mg/mL of X-gal in dimethyformamide or dimethylsulfoxide at concentration of 20 mg/mL solution. Dispense the solution into 1 mL aliquots, wrap it with the aluminum foil to prevent damage by light and store it in -20^{o}C. It is not necessary to sterilize the x-gal solution by filtration.

2.8 Ampicillin (stock solution 100 mg/mL)

Dissolve 100 mg/mL of ampicillin in distilled water. Sterilize the ampicillin stock solution by filtration through a 0.22 μm filter, and store it in -20^{o}C.

2.9 4-methylumbelliferyl-β-D-galactopyranoside (4MUGal; 3.5 mg/mL)

Dissolve 3.5 mg 4MUGal in 1 mL 50 mM NaH_2PO_4 buffer pH 6.5. Incubate at 25^{o}C for 1000 rpm in a thermomixer before used.

2.10 2-Aminopyridine (AP)

Dissolve 1 g of 2-aminopyridine ($C_5H_6N_2$) in 600 μL methanol and 470 μL acetic acid (it need to add methanol first).

2.11 Sodium cyanoborohydride

Dissolve 59 mg Sodium cyanoborohydride ($NaCNBH_3$) in 1 mL of 30% acetic acid.

2.12 Britton-Robinson buffer (20 mM each of Boric acid, acetic acid and phosphoric acid)

Dissolve 0.309 g boric acid (H_3BO_3) in distilled water. Add 0.303 mL acetic acid ($C_2H_4O_2$) and 0.576 mL ortho-phosphoric acid (85%; H_3PO_4). Adjust final volume to 250 mL with distilled water.

2.13 SDS buffer (Thu-Ha Nguyen : personal communication, BOKU, Vienna, Austria)

Prepare 47 mM Tris-HCl buffer and adjust pH to 6.8 with 1 M HCl. Dissolve 34 mg/mL SDS, 0.1 mg/mL bromophenol blue 5% v/v mercaptoethanol and 15% v/v glycerol in 10 mL of 47 mM Tris-HCl pH 6.8 (this solution can be used with the protein sample and incubate at 60^{o}C).

2.14 Z-buffer

Dissolve 16.1 g $Na_2HPO_4.7H_2O$ (0.06 M), 5.5 g $NaH_2PO_4.H2O$ (0.04 M), 0.75 g KCl (0.01 M), 0.246 g $MgSO_4.7H_2O$ (0.001 M) and 2.7 ml β-mercaptoethanol (0.05 M) into distilled water and adjust to 1 L final volume. Do not autoclave. Adjust pH to 7.0.

BIOGRAPHY

Mrs. Onladda Juajun was born on December 31, 1968 in Bangkok, Thailand. She received her Bachelor's Degree in Biotechnology from Khonkaen University in 1991. She continued her Master's Degree in Biotechnology at King Mongkut Institute of Technology Thonburi, Bangkok during 1992-1994. After graduation, she has been employed in a position of lecturer at Faculty of Food Science and Technology, Rajamangala University of Technology Isan, Surin Campus. She has been working there for eleven years and then she has continued her Doctoral Degree at Suranaree University of Technology, Nakhon Ratchasima. During her study, she has experience on her thesis work in Division of Food Biotechnology at BOKU, the University of Natural Resources and Applied Life Sciences, Vienna, Austria, during June 16, 2008 to September 30, 2009. She has expressed two of her posters in the topic of "Cloning, Purification and Characterization of β-galactosidase from *Bacillus licheniformis* DSM13" on Milk conference at BOKU during September 17-18, 2009 and also in the topic of "Cutting Edge with Immobilization of β-galactosidase on Chitin" on the international meeting of Bio-ethanol: status and future at Hanoi University of Technology, Hanoi, Vietnam during March 25-26, 2009. Besides, she has one article, Milk Intolerance Problem and Approach Problems Resolved, in Kasetsuranaree 08, Suranaree University of Technology, Nakhon Ratchasima, pp. 91-99.