การผลิตนมและไอศกรีมแลคโตสต่ำโดยใช้เทคโนโลยีเอนไซม์

นางสาวน้ำฝน ไทยวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE PRODUCTION OF LOW LACTOSE MILK AND ICE CREAM USING ENZYME TECHNOLOGY

Numphon Thaiwong

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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THE PRODUCTION OF LOW LACTOSE MILK AND ICE CREAM USING ENZYME TECHNOLOGY

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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วัตถุประสงค์ของการศึกษานี้เพื่อผลิตนมและไอศกรีมแลคโตสต่ำ โดยใช้เอนไซม์เบต้า -กาแลคโทสิเดสลูกผสมจากเชื้อ *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 โดยใช้เชื้อ *L. plantarum* TLG02 เป็นเซลล์เจ้าบ้านสำหรับผลิตเอนไซม์เกรดอาหาร ในขั้นแรกของการผลิตเอนไซม์เกรดอาหารนี้เป็นการเปลี่ยนเวคเตอร์ pSIP409-*lacZ* และ pSIP409-*lacZ*-His ซึ่งมียีนต้านยาปฏิชีวนะอีริโทรมัยซินให้เป็นยีนอะลานีนราซิเมส ทำให้ได้เวคเตอร์ ใหม่คือ pSIP609-*lacZ* และ pSIP609-*lacZ*-His เวคเตอร์ดังกล่าวนี้เป็นเวคเตอร์ที่ไม่ต้องใช้ ยีนต้านยาปฏิชีวนะเป็นยีนเครื่องหมายในการคัดเลือก ต่อมาเวคเตอร์เหล่านี้ได้ถูกถ่ายโอนไปยังเชื้อ *L. plantarum* TLG02 ซึ่งเป็นเซลล์เจ้าบ้านที่ถูกดัดแปลงพันธุกรรมให้เหมาะกับ การถ่ายโอนเวคเตอร์ที่มียีนอะลานีนราซิเมสเป็นเครื่องหมายในการคัดเลือก เอนไซม์เบต้า-กาแลคโทสิเดสลูกผสม บริสุทธิ์ที่ได้ในข้างต้นนี้ มีค่ากิจกรรมจำเพาะของเอนไซม์ 165±5 ยูนิตต่อมิลลิกรัม และอุณหภูมิที่เหมาะสม ในการทำงานของเอนไซม์นี้คือ ในช่วงอุณหภูมิ 55–60 องศาเซลเซียส เมื่อใช้แลคโตสเป็นสารตั้งต้น ค่าคงที่ไมคีลิส-เมนเทนที่ดีที่สุดของเอนไซม์เบต้า-กาแลคโทสิเดสลูกผสม บริสุทธิ์นี้มีค่าประมาณ 5.6 มิลลิโมลาร์ ที่อุณหภูมิ 30 องศาเซลเซียส ซึ่งค่า ความเร็วสูงสุ ดของเอนไซม์ ที่อุณหภูมินี้มีค่าประมาณ 153 ไมโครโมลต่อนาทีต่อมิลลิกรัม ทั้งนี้ยังได้มีการศึกษา เสถียรภาพ ของเอนไซม์ดังกล่าวข้างต้นในบัฟเฟอร์ต่างชนิด (โซเดียมฟอสเฟตบัฟเฟอร์, โซเดียมฟอสเฟตบัฟเฟอร์ที่มีแมกนีเซียมไอออน 1 มิลลิโมลาร์, โซเดียมฟอสเฟตบัฟเฟอร์ที่มีแมกนีเซียมไอออน 10 มิลลิโมลาร์ และบัฟเฟอร์นม ซึ่งพบว่าโซเดียมฟอสเฟตบัฟเฟอร์ที่มีแมกนีเซียมไอออน 1 มิลลิโมลาร์สามารถยืดอายุการเก็บรักษาของเอนไซม์ได้ การศึกษาผลของการใช้เอนไซม์สกัดหยาบที่ความเข้มข้น 3 ระดับ (1, 5 และ 10 ยูนิตต่อมิลลิลิตร) ร่วมกับแลคโตสที่ความเข้มข้น 2 ระดับ (125 และ 165 มิลลิโมลาร์) ที่อุณหภูมิแตกต่างกัน (4 และ 65 องศาเซลเซียส) ที่มีต่อปฏิกิริยาแลคโตสไฮโดรไลซิสในบัฟเฟอร์นม พบว่าการใช้เอนไซม์สกัดหยาบ ที่ความเข้มข้น 5-10 ยูนิตต่อมิลลิลิตร สามารถไฮโดรไลซ์แลคโตสได้อย่างน้อยร้อยละ 50 ทั้งที่ 4 และ 65 องศาเซลเซียส นอกจากนี้การศึกษาปฏิกิริยาแลคโตสไฮโดรไลซิสในนมไขมันต่ำ ที่ได้จากเปรียบเทียบการใช้ เอนไซม์เบต้า -กาแลคโทสิเดสลูกผสม ต่างชนิด (เอนไซม์สกัดหยาบ และเอนไซม์บริสุทธิ์) และเอนไซม์เชิงการค้า (Lactozym 2600L) ด้วยเทคนิคโครมาโทกราฟีเหลวความดันสูง พบว่า เอนไซม์ทุกชนิด สามารถย่อยแลคโตสในนมไขมันต่ำได้มากกว่าร้อยละ 85 ภายใน 18 ชั่วโมง ที่ 4 องศาเซลเซียส ซึ่งในปฏิกิริยาดังกล่าวนี้พบว่า กาแลคโต-โอลิโกแซคคาไรด์สามารถเกิดขึ้นได้มากที่สุด (3.63 มิลลิกรัมต่อมิลลิลิตร) ที่เวลา 3 ชั่วโมง โดยผล

ของปฏิกิริยาแลคโตสไฮโดรไลซิสด้วยเอนไซม์ต่างชนิดกันในข้างต้นได้ถูกศึกษาในนมที่มีปริมาณไขมันที่ต่างกัน (ไขมันต่ำและไขมันปกติ : ร้อยละ 3.5) ซึ่งพบว่าปริมาณไขมันในนมไม่มีผลกระทบต่อปฏิกิริยาแลคโตสไฮโดรไลซิส จากการใช้ เอนไซม์ทุกชนิด ผลดังกล่าวนี้ ยืนยันได้ โดยการตรวจสอบด้ วยเทคนิ คโครมาโตกราฟี แผ่นบาง ส่วนผลการทดสอบทางประสาทสัมผัสพบว่าคะแนนเฉลี่ยความชอบรวมของนมไขมันต่ำหรือนมไขมันปกติที่มีการใช้เอนไซม์แต่ละชนิดและนมตัวอย่างควบคุมมีความแตกต่างกัน อย่างมีนัยสำคัญ ($p<0.05$) ในขณะที่คะแนนเฉลี่ยความชอบรวมของไอศกรีมจากนมไขมันต่ำที่มีปริมาณแลคโตสต่ำจากการใช้เอนไซม์สกัดหยาบหรือเอนไซม์บริสุทธิ์และไอศกรีมตัวอย่างควบคุมมีความแตกต่างกันอย่างมีนัยสำคัญ ($p<0.05$) แต่ไม่พบความแตกต่างในไอศกรีมจากนมไขมันปกติที่มีปริมาณแลคโตสต่ำจากการใช้เอนไซม์ทั้งสองชนิดและไอศกรีมตัวอย่างควบคุม ($p>0.05$) ดังนั้นเอนไซม์เบต้า-กาแลคโทสิเดสลูกผสมทั้งชนิดเอนไซม์สกัดหยาบและเอนไซม์บริสุทธิ์สามารถ นำมาประยุกต์ใช้ ได้ในการผลิตนมและไอศกรีมที่มีปริมาณแลคโตสต่ำ เพื่อตอบสนองความต้องการของผู้บริโภคที่มีปัญหาในเรื่องการย่อยแลคโตสในนม

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NUMPHON THAIWONG : THE PRODUCTION OF LOW LACTOSE MILK AND ICE CREAM USING ENZYME TECHNOLOGY. THESIS ADVISOR : ASST. PROF. SIWATT THAIUDOM, Ph.D., 220 PP.

RECOMBINAT β-GALACTOSIDASE/*LACTOBACILLUSPLANTARUM*/ pSIP VECTOR/OVEREXPRESSION/LACTOSE HYDROLYSIS/MILK BUFFER

The objective of this study was to produce low lactose milk and ice cream using a recombinant β-galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081, overexpressed in *L. plantarum*, a food-grade expression host. The food-grade expression vector (pSIP-based) was constructed by replacing the erythromycin resistance gene (erm^R) of the pSIP409-*lacZ* and pSIP409-*lacZ*-His with the alanine racemase (*alr*) gene, allowing an antibiotic-free selection condition. Subsequently, the food-grade expression vectors, designated as pSIP609-*lacZ* and pSIP609-*lacZ*-His, were transformed into the *L. plantarum* TLG02, which is a D-alanine auxotroph. Consequently, a recombinant β-galactosidase was produced from these bacteria. The purified recombinant β-galactosidase showed the specific activity was 165±5 U/mg and the optimal temperature was in the range of 55–60°C when lactose was used as a substrate. The best value of Michaelis-Menten constant (K_m) was approximately 5.6 mM at 30°C, of which the maximal velocity ($V_{max,Glc}$) at this temperature was approximately 153 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Then, the catalytic stability was determined in different buffers (Sodium phosphate buffer, Sodium phosphate buffer + 1 mM Mg^{2+}, Sodium phosphate buffer + 10 mM Mg^{2+}, and milk buffer). The result showed that 1 mM of Mg^{2+} in the sodium phosphate buffer could enhance the enzyme stability. The

effects of different crude enzyme concentrations (1, 5, and 10 U/mL), lactose concentrations (125 and 165 mM), and temperatures (4 and 65°C) in the milk buffer on lactose hydrolysis were also studied. The crude enzyme concentration from 5-10 U/mL could hydrolyze lactose by at least 50% in both studied temperatures. Moreover, low-fat milk as a lactose source was used for the comparison of different types of recombinant β-galactosidase (crude and purified forms) and commercial enzyme (Lactozym 2600L) on lactose hydrolysis. HPLC analysis indicated that all enzyme preparations could hydrolyze more than 85% of lactose within 18 hours at 4°C. The highest galacto-oligosaccharides formation (3.63 mg/mL) was found at 3 hours in this reaction condition. The effect of fat content (low-fat milk and regular milk: 3.5% milk fat) on the hydrolysis of these enzymes was also investigated. The results showed that the different fat content in milks (low-fat milk and regular milk) did not affect the lactose hydrolysis for all enzyme preparations, which were detected by TLC. For the sensory evaluation, the overall acceptance of low-fat lactose-hydrolyzed milks and regular-fat lactose-hydrolyzed milks were significantly different from that of the control ($p<0.05$). The overall acceptance of all ice cream from low-fat lactose-hydrolyzed milk using crude or purified enzymes and the control was significantly different ($p<0.05$) but no significant difference was found among those samples prepared by regular-fat milk ($p>0.05$). In conclusion, both crude and purified recombinant β-galactosidase could be applied for the preparation of low-lactose milk and ice cream for lactose-intolerant consumers.

School of Food Technology

Academic Year 2016

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LIST OF ABBREVIATIONS

A	=	remaining enzyme activity
A_t	=	activity measured at the beginning
ANOVA	=	Analysis of Variance
alr	=	Alanine racemase gene
C	=	glucose concentration
°C	=	Degree Celsius
Ca^{2+}	=	Calcium ion
$Ca_3(PO_4)_2$	=	Calcium Phosphate
$CaHPO_4$	=	Calcium Hydrogen Phosphate
$Ca(HPO_4)_2$	=	Monocalcium phosphate monohydrate
cfu	=	a colony-forming unit
DE	=	Dextrose equivalent
DH	=	Degree of hydrolysis
erm	=	Erythromycin antibiotic resistance gene
FOS	=	Fructo-oligosaccharides
g	=	gram
g	=	relative centrifugal force
GalOS	=	Galacto-oligosaccharides
GH	=	Glycoside hydrolase
Glu	=	Glutamic acid

LIST OF ABBREVIATIONS (Continued)

GOD	=	Glucose Oxidase enzyme
HPLC	=	High-performance liquid chromatography
HPLC	=	High-performance liquid chromatography
IP673	=	Peptide pheromone
LAB	=	Lactic acid bacteria
kbp	=	kilo base-pair
k_{cat}	=	Turnover number
kDa	=	kilodalton
k_{in}	=	Inactivation constant
K_m	=	Michaelis constant
L	=	Liter
M	=	Molar
mg	=	Milligrams
Mg^{2+}	=	Magnesium ion
$MgCl_2$	=	Magnesium chloride
min	=	minute
mL	=	milliliter (10^{-3}l)
mM	=	millimolar (10^{-3}mol l^{-1})
Mr	=	molecular mass
MRS	=	Man-Rogosa-Sharpe medium
MSNF	=	Milk solids-not-fat

LIST OF ABBREVIATIONS (Continued)

ηg	=	nanograms
Ni	=	Nickel
NICE	=	Nisin-Controlled Expression
nm	=	nanometer
ns	=	non-significant
Nu	=	Galactosyl acceptor
OD	=	Optical density
*o*NP	=	*ortho*-Nitrophenol
*o*NPG	=	*ortho*-Nitrophenyl β-D-galactopyranoside
POD	=	Peroxidase enzyme
R_f	=	retardation factor
pSIP	=	pheromone-inducible
rpm	=	Revolutions per minute
S	=	second
SDS-PAGE	=	Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis
spp.	=	several species
subsp.	=	subspecies
t	=	various time points
$\tau_{1/2}$	=	half-life value
TAS	=	Thai agricultural standard

LIST OF ABBREVIATIONS (Continued)

TLC	=	Thin layer chromatrography
TPC	=	Total plate count
U	=	Unit
UHT	=	Ultra-high-temperature processing
µL	=	microliter
µmol	=	micromoles
V	=	Volt
$V_{max,Glc}$	=	Maximal velocity

CHAPTER I

INTRODUCTION

1.1 Introduction

About 90% of the people in Africa and Asia are lactose intolerant (Bulhões et al., 2007; The National Digestive Diseases Information, 2012; Curry, 2013). The cause of lactose intolerance is the inability to digest significant amounts of lactose because of a genetically inadequate amount of the β-galactosidase enzyme at the intestinal brush border (Swagerty et al., 2002; Wang et al., 1998) where the β-galactosidase enzyme breaks down the lactose into the glucose and galactose molecules for absorption into the body (Suarez et al., 1995). When the amount of β-galactosidase is not adequate to break down the lactose, the body cannot absorb the lactose directly. Therefore the lactose remains in the intestines and eventually finds its way into the large intestine. In the large intestine, millions of bacteria use the lactose for their own nourishment, multiplying rapidly, and producing a large number of by-products. Usually a variety of gases, organic acids, and other irritating chemicals result from the activity of these micro-organisms, of which the effects are excessive flatus, abdominal bloating, pain, loose stools, or diarrhea, and general distress in the large intestine (Swagerty et al., 2002; The National Digestive Diseases Information, 2011).

Most mammals normally become lactose intolerant after weaning (Saavedra and Perman, 1989; Wang et al., 1998), and this can also happen in adults (Ferguson et

al., 1984). Due to the world population ageing structure, there will soon be a much more elderly population than at present; therefore, the number of lactose intolerant people will correspondingly increase (The Department of Economic and Social Affairs of the United Nations Secretariat, 2009). This will also affect Thailand, where the numbers of people aged 60 and above will rise to 19% (14 million) in 2025 and will constitute 25% (19 million) in 2050 (Knodel et al., 2011). Although common in adults, lactose intolerance is rarely dangerous, but lactose intolerant people should avoid drinking milk or consuming other dairy products containing lactose (Densupsoontorn et al., 2004; Jelen and Tossavainen, 2003). Consequently, dairy companies have developed products which are suitable for lactose intolerant people.

Likewise, the market trend of lactose-reduced products has been driving demand for lactose-free products (Mahoney, 2003). The market survey (the Statistics Portal) of lactose-free dairy products in the United States of America, the largest market for lactose-free food, has shown an increased demand for lactose-free food products of 701.5 million US dollar in 2012 to 807.7 million US dollar in 2015. The Thai dairy market had total revenues of 1.6 billion US dollar in 2012, representing a compound annual growth rate (CAGR) of 3.3% between 2008 and 2012 (Senadisai et al., 2015).

Most production of lactose free and low lactose products use the β-galactosidase enzyme to break down the lactose molecules by using either the free (soluble) or, rarely, the immobilized enzyme variant of the process, from which the hydrolysis of lactose or related compounds of β-galactosidase are used to improve digestibility and the sweetness of dairy products. In addition, some β-galactosidases catalyze transgalactosylation reactions in which lactose, as well as the released

glucose and galactose, serve as galactosyl acceptors, yielding a series of di, tri-, and higher oligosaccharides called galacto-oligosaccharide (GalOS) (Park and Oh, 2010; Reyes-Torres et al., 2010; Rodriquez-Colinas et al., 2012). GalOS are non-carcinogenic, reduce the level of cholesterol in serum, prevent colon cancer, and exhibit prebiotic properties. However, the properties of GalOS depend on their chemical composition, structure, and degree of polymerization (Cardelle-Cobas et al., 2011; Rodriquez-Colinas et al., 2012). Moreover, the transferase reaction of the hydrolyzed lactose can be used to attach galactose to other chemicals which has potential applications in the production of food ingredients, pharmaceuticals, and other biologically active compounds (Mahoney, 1998).

Lactose-hydrolyzed milk production by β-galactosidase has been used for the preparation of flavored milk, cheese, and yogurt. It also prevents lactose crystallization in condensed milk products and frozen foods such as ice milk, and ice cream (Panesar et al., 2010). The result of lactose-hydrolyzed milk is sweeter than ordinary milk because of the glucose and galactose, which are obtained after the conversion of lactose (Akcan, 2011; Harju et al., 2012; Panesar et al., 2006). It has been suggested that limiting hydrolysis to 80-90% avoids excessive sweetness. Flynn and co-workers (1994) had used potassium chloride for lactose hydrolysis, which the optimal condition was at 15-45 mmol/L. In 1996, Vasala et al patented a method to reduce the sweetness of lactose-hydrolyzed UHT milk by adding the potassium salt of an organic acid, such as citrate, malate, or gluconate. Moreover, Harju and co-workers (2012) reduced the sweetness of lactose-hydrolyzed milk by potassium chloride treatment. Nevertheless, many consumers can feel unnatural taste of lactose free or low lactose products that are much sweeter than fresh milk. Additionally,

β-galactosidase can be used for converting lactose in whey into a very useful product like sweet syrup, which can be used in various processes of dairy, confectionary, baking, and soft drink industries (Panesar et al., 2010; Pivarnik et al., 1995; Tweedie et al., 1978). Therefore, lactose hydrolysis not only allows milk consumption by lactose intolerant populations, but can also solve the environmental problems linked with whey disposal (Champluvier et al, 1988; Gekas and Lopez-Leiva, 1985; Martinez and Speckman, 1988; Panesar et al., 2010).

Beta-galactosidases from microorganisms are the major source of β-galactosidase for lactose hydrolysis in bioprocess technology and biotechnological applications due to their easy approaches (Halbmayr et al., 2008; Husain, 2010; Juajun et al., 2011; Nakayama and Amachi, 1999; Nguyen et al., 2006; Park and Oh, 2010; Rahim and Lee, 1991; Rajakala and Karthigai, 2006). Main β-galactosidase from microorganisms are derived from *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Aspergillus niger*, respectively, with the remainder being those from *Escherichia coli* (*lacZ*) (Henrissat, 1991). Also, *Aspergillus oryzae* (Chen et al., 2002), *Aspergillus japonicas* (Saad, 2004), *Pyrococcus furiosus* (Li et al., 2013), *K. lactis* (Li et al, 2007; Novalin et al., 2005), and *Bacillus circulans* (Rodriguez-Colinas et al., 2012) are the sources of β-galactosidase. Beta-galactosidases from *E. coli* cannot be used in the food industry, mainly because of the unacceptability of the bacterium from consumers or humans (Nakayama and Amachi, 1999). Beta-galactosidase from different microorganisms has different enzyme properties, such as molecule weight, protein chain length, and the position of the active site, but they have the same amino acid residue, glutamic acid, as their catalytic site (Harju et al., 2012; Mahoney,1997).

Up to date, many species of *Lactobacillus* spp. have been studied for the enzyme production and application because they are recognized as the GRAS organism (Generally Recognized As Safe) and play important roles in foods. There have been a number of investigations of enzymes from this source and their possible application for the production of galacto-oligosaccharides (Toba et al., 1981). Garman and co-workers (1996), in a study of a number of species of *Lactobacillus* and *Streptococcus thermophilus*, found that *Lactobacillus delbrueckii* subsp. *bulgaricus* strain possesses a β-galactosidase with transgalactosylation acidity similar to the enzyme from *S. thermophilus*. Kobayashi and co-workers (1990) patented a method for producing a processed milk containing galacto-oligosaccharide. In their patent, milk was treated with a β-galactosidase derived from *S. thermophilus* or *L. delbrueckii* subsp. *bulgaricus* which could change at least 15% of the lactose in the milk into galacto-oligosaccharide. Moreover, β-galactosidase from *L. reuteri* L103 and L461 also exhibited high transgalactosylation activity (Nguyen et al., 2006).

Although β-galactosidase naturally from native *Lactobacillus* spp. is considered as a product from GRAS organism, safeness of using recombinant β-galactosidase expressed in *E. coli* system is doubtful and likely to apply for the non-food industry. Few examples of β-galactosidase from *Lactobacillus* spp. with *E. coli* expression system, which has been applied to lactose-hydrolyzed milk include: the cold-adapted recombinant β-galactosidases from *L. acidophilus* (Pan et al., 2010) and the acid tolerant β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (Rhimi et al., 2009).

In order to replace the *E. coli* system, the production of β-galactosidase from *Lactobacillus* spp. can be obtained using the Nisin-Controlled Expression (NICE) or pheromone-inducible (pSIP) systems, which are well-known as overexpression systems for Lactic acid bacteria (LAB) (Sorvig et al., 2003; Sorvig et al., 2005; Halbmayr et al., 2008; Mathiesen et al., 2008; Straume et al., 2006).Furthermore, the vectors in the pSIP system permit all parts of the plasmid to be easily modified and used among different LAB, especially *Lactobacillus* spp., while plasmids in the NICE system are suitable mainly for *Lactococcus* spp. (Sorvig et al., 2003; Maischberger et al., 2010). However, it was shown that the pSIP system resulted in higher levels of overexpressed enzyme than the NICE system (Nguyen et al., 2011[b]).

Although, the use of the pSIP system has been increasingly used in the food industry recently, there is a major limitation of pSIP system. Because of the original system, the erythromycin antibiotic resistance gene (*erm*) is used as a selective marker for the cell selection stage of enzyme production, which might affect the microflora in the human body. In addition, the *erm* gene may be transferred to other organisms, resulting in resistance to this antibiotic. To avoid this undesirable effect, the alanine racemase gene (*alr*) is used as a selection marker, instead of the *erm* gene, which is safer in terms of human food consumption. The alanine racemase enzyme is important for cell wall biosynthesis, especially in LAB. It converts L-alanine to D-alanine, which is an essential component for the growth and cell wall biosynthesis of prokaryotic cells (Hols et al., 1997). Additionally, pSIP vectors carrying the *alr* gene as a selection marker have been successfully applied in complementation approaches both in Lactococci and Lactobacilli (Nguyen et al., 2011[a]). Thus, expression of the *lacZ* gene, coding for β-galactosidase, using the *alr* gene as a selection marker can

provide a safer new method to produce recombinant β-galactosidase in a completely food-grade manner. At this point, little research has been conducted utilizing an expression vector carrying the *alr* gene with a suitable *alr*-deletion strain to overexpress a β-galactosidase of the *LacLM* type (Nguyen et al., 2011[a]). This *lacLM* protein is heterodimeric and is encoded by two partially overlapping genes, which might be more complicated to express and study as a model system. Thus, the expression of homodimeric *lacZ* β-galactosidase encoded by the *lacZ* gene with the *alr* gene as a selective marker in a pSIP vector, known as a recombinant system, might be more beneficial, in terms of cost, time, and safety, than the heterodimeric system.

Beta-galactosidase from *Lactobacillus* spp. has not been widely applied for the production of lactose-free or low lactose milk due to three complicated methods (Rand, 1981; Mahoney, 1997; Jelen and Tossavainen, 2003; Mahoney, 2003). First, β-galactosidase is added to the milk before heat treatment (pre-treatment). Then, the milk is heated, packed, and sold at 6-8°C to avoid microbial growth. As this method is not performed in the optimum temperature of the enzyme, the hydrolysis time of this method is rather long (24-30 hours) depending upon the enzyme dosage. However, in good quality raw milk, hydrolysis at high temperature (38-40°C) can be used (Fuquay et al, 2011). Moreover, the milk should always be pasteurized or at least thermalized prior to hydrolysis for safety purposes. The second method involves the application of β-galactosidase after heat treatment of milk (post-treatment). In this process, milk is packaged after a sterile β-galactosidase is added in-line immediately following UHT treatment of milk, allowing the lactose to be hydrolyzed in the final package at ambient temperature. Since active enzyme is present in the milk during

storage, this method requires a high quality of β-galactosidase preparation. The last method is applying β-galactosidase into milk products by the consumer by adding a few drops of β-galactosidase prior to consumption of the milk. Consumers can buy small packages of neutral β-galactosidase, and the lactose will be hydrolyzed within 12–24 hours depending upon the dosage (Rand, 1981; Pivarnik et al, 1995; Mahoney, 1997; Jelen and Tossavainen, 2003; Mahoney, 2003). Nevertheless, the β-galactosidase from *Lactobacillus* spp. can be use similarly for lactose-free milk production. The commercial β-galactosidases are commonly used for milk lactose hydrolysis to obtain the pasteurized-milk product, and there were few studies of recombinant β-galactosidase expressed in *Lactobacillus* spp. for lactose-hydrolyzed milk in complete milk pasteurization processing. Jokar and Karbassi (2011) demonstrated the hydrolysis of sterile milk using the cell free extracts of β-galactosidases from *L. bulgaricus* CHR Hansen Lb-12 in comparison with the commercial enzyme (DSM Food specialist Maxilact 12000, France) for the sensory evaluation. When β-galactosidase is applied to milk for the production of lactose free or low lactose products, the price of lactose-hydrolyzed milk is higher than for ordinary milk (Jelen and Tossavainen, 2003). Variable prices of β-galactosidase are depending on enzyme, the level of production, and purification (Akcan, 2011; Panesar et al., 2006).

In Thailand, most lactose free or low lactose products are imported from abroad by large companies, so the price of these products is even higher. Beta-galactosidase is mainly used to produce the lactose-free or low lactose products in the hospitals for lactose intolerant people. Moreover, the application of β-galactosidase is used for improving texture-quality and stability of dairy products, such as, ice cream

in small or medium company. There is only little research on the production lactose-hydrolyzed milk and ice cream by recombinant β-galactosidases from *L. bulgaricus* with pSIP-based system. This project includes enzyme production through the molecular cloning and utilization of the recombinant enzyme to produce lactose-hydrolyzed milk and ice cream as a prototype of enzyme production and application in Thailand for the future.

The objectives of this study were to produce the recombinant β-galactosidase (*LacZ*) from *L. bulgaricus* expressed in *L. plantarum*, using a pSIP-based food-grade expression system and to study the food-grade β-galactosidase expression, enzyme characterization, and kinetic parameters, in order to study the application of food grade β-galactosidase in low-lactose milk and ice cream for their physical, microbial, and sensory properties.

1.2 Research objectives

The objectives of this study were:

1. To produce the recombinant β-galactosidase (*LacZ*) from *L. bulgaricus* expressed in *L. plantarum*, using a pSIP-based food-grade expression system.

2. To determine the food-grade β-galactosidase expression, enzyme characterization, and kinetic parameters.

3. To apply food grade β-galactosidase in milk and ice cream and determine for their physical, microbial, and sensory properties.

1.3 Research hypotheses

Beta-galactosidases from the heterologous overexpression of single-gene encoded β-galactosidase (*LacZ*) from *L. bulgaricus* expressed in *L. plantarum* can be used as an enzyme for low lactose milk, which may be comparable to the commercial enzyme. In addition, milk and ice cream produced by this enzyme possess favorable qualities suitable for consumers.

1.4 Scope of the study

The study was divided into three parts which were: 1) the production of recombinant β-galactosidase expressed in *L. plantarum*, using a pSIP-based food-grade expression system; 2) the food-grade β-galactosidase expression and enzyme characterization; and 3) the application of β-galactosidase (*LacZ*) from *L. bulgaricus* in milk and ice cream.

In phase 1, the plasmids (pSIP609-*lacZ* and pSIP609-*lacZ*-His) were constructed from pSIP vectors; pSIP409-*lacZ* and pSIP409-*lacZ*-His and *L. plantarum* WCFS1 bacterial host (provided by Food Biotechnology Laboratory, Department of Food Sciences and Technology, BOKU-University of Natural Resources and Life Sciences Vienna, Austria). The single-gene encoded β-galactosidase (*LacZ*) from *L. bulgaricus* in pSIP vectors was cloned into *L. plantarum* TLG02, which was a food-grade host in order to produce a food grade β-galactosidase

In phase 2, expression of food-grade, recombinant β-galactosidase produced from *L. plantarum* TLG02 harboring pSIP609-*lacZ* or pSIP609-*lacZ*-His was determined by the denaturing sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and the measuring enzyme activity. The recombinant enzyme was purified and characterized for the optimal temperature, the steady-state kinetic, and the catalytic stability. The optimal temperature was measured at temperatures ranging from 20-90^{o}C in two different substrates (*ortho*-Nitrophenyl-β-galactoside (*o*NPG) and lactose) both milk buffer and sodium phosphate buffer. The steady-state kinetic was analyzed in milk buffer from 4-65°C. The catalytic stability of recombinant enzyme was compared with different substrates (*o*NPG and lactose) and different buffers (sodium phosphate buffer, sodium phosphate buffer with 1 mM Mg^{2+}, sodium phosphate buffer with 10 mM Mg^{2+}, and a milk buffer) at temperatures ranging from 4-55^{o}C.

Finally, the crude enzyme of recombinant β-galactosidase was applied for lactose hydrolysis in the milk buffer. The recombinant β-galactosidase activity was determined in the presence of lactose, glucose, galactose, and galacto-oligosaccharides (GalOS) content from low-fat milk hydrolysis by the Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). Low lactose milk and ice cream were produced by recombinant β-galactosidase. The qualities of the low lactose milk and ice cream were examined for their physical, microbial, and sensory properties. These qualities were compared with low lactose milk and ice cream which was hydrolyzed by commercial enzyme. The low-lactose milk samples were qualitative analysis by thin layer chromato-graphy (TLC).

1.5 Expected results

We expected that the *LacZ* gene from *L. bulgaricus* using pSIP vectors could overexpress in *L. plantarum* TLG02 as a host. The result of lactose hydrolysis by

crude enzyme of β-galactosidase, recombinant β-galactosidase, and commercial enzyme (Lactozym 2600L) in milk buffer should show the suitable condition for further application in regular milk and low-fat milk that can be benefit to the dairy industry. Moreover, these enzymes can be used as the superior substitutes for current commercial enzymes that will be more readily accepted by consumers. These advancements will lead to more suitable conditions for the production of low lactose milk and ice cream.

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CHAPTER II

LITERATURE REVIEWS

2.1 Lactose

Lactose (β-D galactopyranosidases) is the principle carbohydrate in the milks of all mammals; non-mammalian sources are very rare. The range of concentrations of lactose is about 2-10% (w/v), and the average lactose content of bovine milk is about 4.8%, ranging between 4.4% and 5.2% (Ganzle et al., 2008; Reyes-Torres et al., 2010). Milk contains only trace amounts of other sugars, including glucose (50 mg/L), fructose, glucosamine, galactosamine, neuraminic acid and neutral and acidic oligosaccharides (Fox and McSweeney, 1998). Lactose is less sweet than sucrose. The sweetness of 1% sucrose is equal to 16% lactose. The low level of sweetness makes lactose useful as a bulking agent (McSweeney and Fox, 2010). In addition, it is important in the manufacture of fermented dairy products because it serves as a carbon source for lactic acid bacteria (LAB) which produce lactic acid (McSweeney and Fox, 2010). However, lactose is a hygroscopic sugar. As such, it has a strong tendency to adsorb flavors and odors. It also causes various defects in refrigerated foods such as crystallization in dairy foods, development of sandy or gritty texture, and formation of deposits (Carrara and Rubiolo, 1994; Panesar et al., 2010).

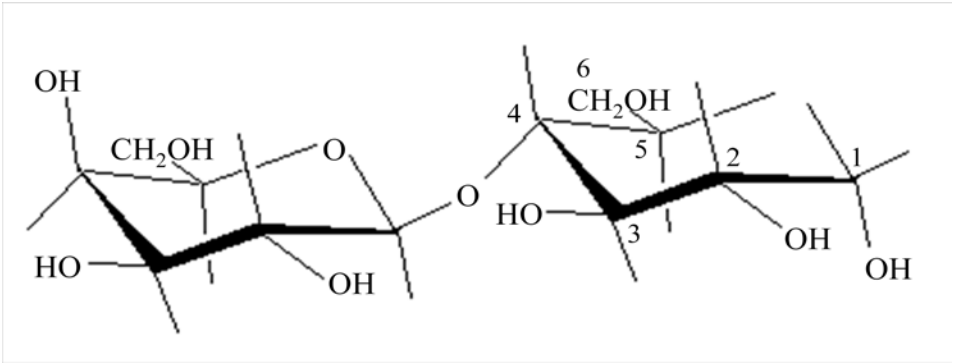
2.1.1 Lactose Physical Properties

Lactose dissolves in the serum (whey) phase of fluid milk can be found in two forms, α-anomer and β-anomer. These forms can convert back and forth between

each other (Figure 2.1). The solubility of these two anomers is temperature-dependent, therefore the equilibrium concentration of the two forms will be different at different temperatures. At room temperature (20°C), the equilibrium ratio is approximately 37% α- and 63% β-lactose. At temperatures above 93.5°C, the β-anomer is less soluble, so there is a higher ratio of α- to β-lactose. The type of anomer does not affect the nutritional properties of lactose, but it does affect the physical properties of lactose crystals (Dincer, Parkinson, Rohl, and Ogden (1999).

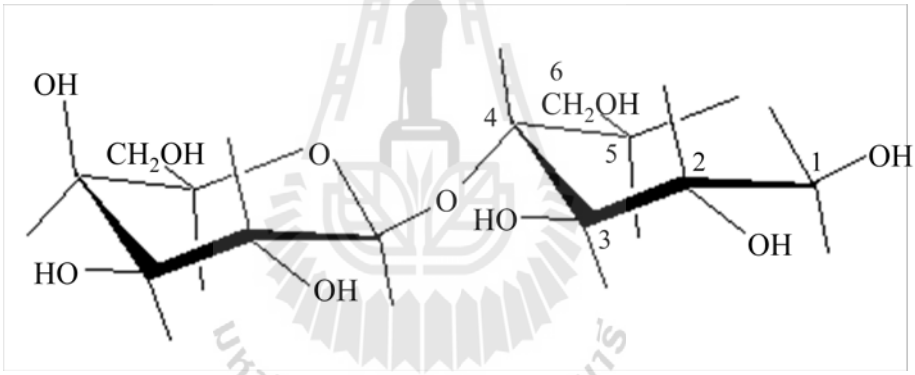
Lactose crystals formed at temperatures below 20°C are mainly α-lactose crystals (Fox and McSweeney, 1998; Holsinger, 1988; Holsinger, 1997; O'Brien, 1995; O'Brien, 1997). The α-monohydrate lactose crystals are very hard, and they can form when ice cream goes through numerous warming and freezing cycles (Ganzle et al., 2008; Fox and McSweeney, 1998; Holsinger, 1988; Holsinger, 1997; O'Brien, 1995; O'Brien, 1997). They create an undesirable, gritty, sandy texture in the ice cream (Abboud, 1999). This problem can be improved by the addition of gums, which are often used in ice cream to inhibit lactose crystallization. The crystal form of β-lactose is sweeter and more soluble than the α-monohydrate lactose, and may be preferred in some bakery applications. (Fox and McSweeney, 1998; Holsinger, 1988; Holsinger, 1997; O'Brien, 1995; O'Brien, 1997).

(A)



D-galactose D-glucose

(B)



D-galactose D-glucose

Figure 2.1 The molecular structure of (A) α-lactose and (B) β-lactose. The figure was modified from Dincer and co-workers (1999).

2.1.2 Significance of lactose in dairy products

Due to the fact that lactose is less soluble, crystallizing and hygroscopic than most sugars, it causes a problem in food processing, especially concentrated, dehydrated and frozen dairy products. Although concentrated and dehydrated dairy products comprise a small portion of the overall dairy market, these physiochemical

properties also cause problems in the production of frozen milk. These problems can negatively affect dairy economics, especially if the milk is pre-concentrated. However, the problem of lactose crystallization formation in frozen dairy product relates to the casein micelles, which are destabilized during frozen storage and aggregate on thawing. Destabilization is caused by a decrease in pH and an increase in Ca^{2+}, both due to the formation of $Ca_3(PO_4)_2$ from $CaHPO_4$ and $Ca(HPO_4)_2$ on reducing the amount of solvent water on the formation of ice and which is reduced further by the crystallization of α-lactose monohydrate (Fox and McSweeney, 1998).

Lactose is a type of reducing sugar that can participate in the Maillard reaction, principally with the ε-amino group of lysine, resulting in impaired functionality and nutritional value, and the formation of brown-colored pigments of volatile flavored compounds. The Millard reaction is particularly severe in heated products but also occurs in milk powders, especially during storage in adverse temperatures and humidities. It can also cause problems with cheese that is subjected to severe heating, e.g. Mozzarella, or in grated cheese during storage (Fox and McSweeney, 2009).

The use of galactose-negative stains of lactobacilli and *Streptococcus thermophilus*, which cannot metabolize galactose, may produce sufficient galactose to cause browning-related problems in certain types of cheese and whey during drying. The accumulated galactose is significantly different from those of lactose, and has strong tendency to cause problems in whey powders (Turner and Martley, 1983; Robitaille, et al., 2007; Fuquay et al., 2011; Anbukkarasi et al., 2013). The monosaccharides, glucose and galactose, are more reactive than lactose, which make dairy products containing hydrolyzed lactose particularly susceptible to Maillard

browning. The hydrolysis of lactose by β-galactosidase markedly increases the heat stability of milk and concentrated milk more than 20%, especially around the pH of minimum solubility (Tan and Fox, 1996). The mechanism of stabilization has not been fully elucidated but proposed that carbonyls are formed via the Maillard reaction. Then, the lactose-hydrolyzed milk products are sensitive to the maillard reaction, effect the consumer perception (Fox and McSweeney, 2009; Fuquay et al., 2011).

2.1.3 Lactose Intolerance

All humans are born with the ability to produce the β-galactosidase enzyme which is secreted by the cells of the small intestine. β-galactosidase breaks down lactose into glucose and galactose molecules, which are then absorbed into the bloodstream. As humans age, their ability to produce adequate amounts of β-galactosidase enzyme decreases. This condition is called lactase non-persistence (Miller et al.,1999; Savaiano et al.,2006; Suarez et al., 1995; The National Digestive Diseases Information Clearinghouse, 2009; Vesa et al., 2000). Inadequate amounts of β-galactosidase enzyme causes inadequate lactose-hydrolyzation, a condition in which lactose cannot be broken down into glucose and galactose molecules. Therefore, ingested lactose remains undigested in the small and large intestines. Millions of bacteria in the large intestine use lactose for their own nourishment. They multiply rapidly and produce a large number of by-products, usually a variety of gases, organic acids, and other irritating chemicals, typically causing excessive flatus, abdominal bloating, pain, loose stool, diarrhea, and general distress in the lower intestines (Panesar et al., 2010; Shukla and Wierzbicki, 1975; Swagerty et al., 2002; The National Digestive Diseases Information, 2011). Lactose malabsorption is the

condition where people suffer these effects after eating dairy products (Miller et al., 1999; Savaiano et al., 2006; Vesa et al., 2000).

Lactose intolerance due to β-galactosidase deficiency has two causes; 1) a genetically inadequate production of β-galactosidase at the intestinal brush border and 2) damage to small intestine's brush border. Genetic β-galactosidase deficiency develops over time and begins after about age 2, when the body begins to produce less β-galactosidase. Most children who have β-galactosidase deficiency do not experience symptoms of lactose intolerance until late adolescence or adulthood (Swagerty et al., 2002; The National Digestive Diseases Information Clearinghouse, 2009; Wang et al., 1998). Injury acquired β-galactosidase deficiency occurs commonly with severe intestinal illness, such as severe diarrhea, celiac disease, Crohn's disease, and chemotherapy. This type of β-galactosidase deficiency can occur at any age but is more common in infancy (The National Digestive Diseases Information Clearinghouse, 2009).

In general, humans lose 90-95% of birth β-galactosidase levels by early childhood, and there is a continuous decline in β-galactosidase during the course of a life- time (Ferguson et al., 1984). The number of lactose intolerant people is increasing due to the world population ageing structure (The Department of Economic and Social Affairs of the United Nations Secretariat, 2009). The National Digestive Diseases Information (2012) showed; 1) total percentage of lactose intolerant people is around 33%, 2) total percentage of adults that have a decrease in β-galactosidase activity is 75%, and 3) total percent of Asian-Americans that are lactose intolerance is 90% which is more likely to become lactose intolerant than others (Bulhões et al., 2007; Curry, 2013: Figure 2.2). The percentage of Thai adults at age 60 years and older who

will be lactose intolerant in 2025 is around 19% (14 million), and they will constitute 25% (19 million) in 2050 (Knodel et al., 2011).

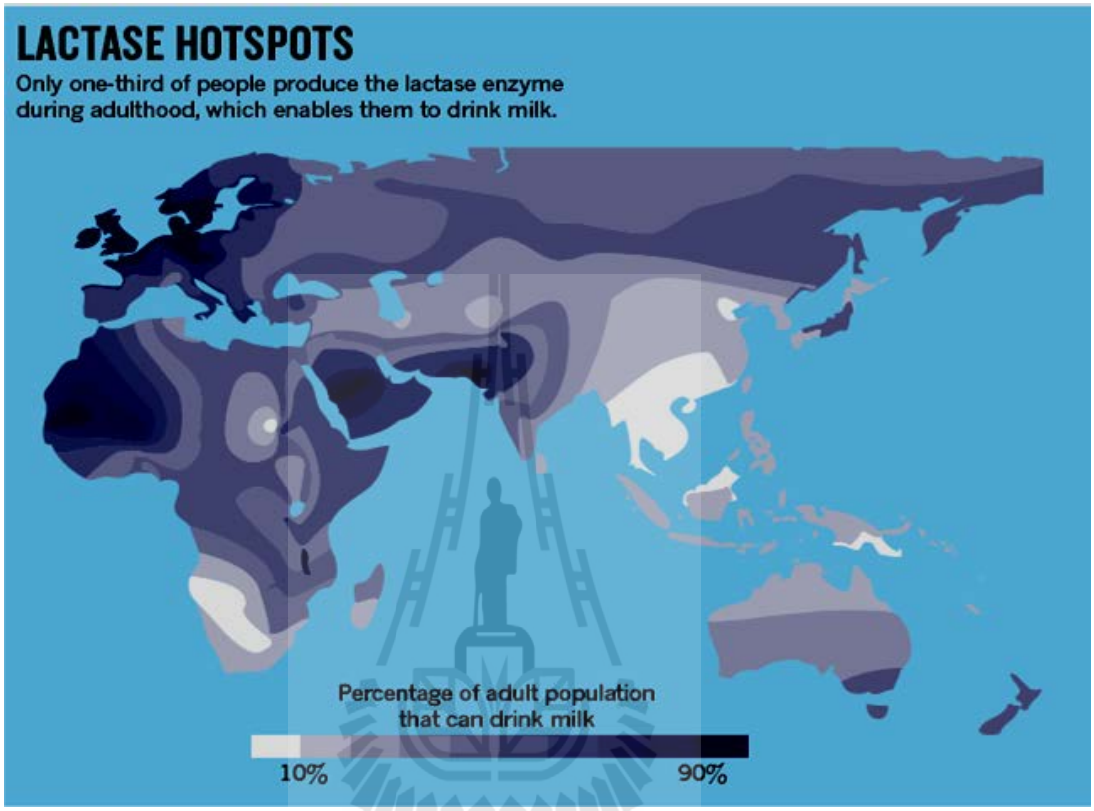


Figure 2.2 Worldwide prevalence of lactose intolerance.

Source: Curry, 2013**.**

Lactose intolerant people often avoid dairy products. Long term avoidance of dairy products resulting from perceived lactose malabsorption can result in decreased consumption of calcium, potassium, magnesium and other minerals and vitamins necessary to support proper health. However, regularly consuming small amounts of dairy can minimize symptoms of lactose intolerance by slowly adapting the digestive system to the presence of lactose. In addition, the consumption of fermented dairy foods containing lactic acid producing bacteria that have lactase

present provide additional active enzymes to assist with human digestion. Fermented dairy products usually have less lactose because some has been consumed by the bacteria to produce the desirable flavors and textures of products like yogurt and cheese. Lactose-reduced dairy products are another option as well as pills containing the β-galactosidase enzyme, which can be taken prior to consuming dairy products to ease digestion (Miller et al.,1999; Savaiano et al., 2006; Vesa et al., 2000).

2.2 Beta-Galactosidase

Beta-galactosidase (β-gal, EC 3.2.1.23), most commonly known as lactase, catalyzes the hydrolysis and transgalactosylation of β-D galactopyranosidases by the attaching of β-galactosidase at the o-glucosyl group of lactose (Reyes-Torres et al., 2010). The general mechanism of enzymatic lactose hydrolysis has a transgalactosylic nature, involving a multitude of sequential reactions with a series of di, tri-, and higher oligosaccharides called galacto-oligosaccharides (GOS), as intermediate products (Reyes-Torres et al., 2010; Rodriguez-Colinas et al., 2012; Wallenfels and Malhotra, 1960). Beta-galactosidase is found widespread in nature that has been isolated and characterized from many different sources including microorganisms, plants, and animals. Beta-galactosidases from microorganisms are of major interest due to their easy application in bioprocess technology and biotechnological applications (Halbmayr et al., 2008; Husain, 2010; Juajun et al., 2011; Nakayama and Amachi, 1999; Nguyen et al., 2006; Park and Oh, 2010; Rahim and Lee, 1991; Rajakala and Karthigai, 2006).

Beta-galactosidase is used in lactose hydrolysis in order to catalyze the reaction by broking down lactose into glucose and galactose, which in turn serves as

an easily metabolizable and renewable substrate for a number of different fermentations (Juajun et al., 2011; Shukla and Wierzbicki, 1975; Panesar et al., 2010). β-galactosidase is widely known for applications in the dairy industries to improve digestibility, solubility and sweetness of lactose (Nakayama and Amachi., 1999; Panesar et al., 2010; Shukla and Wierzbicki, 1975). Furthermore, β-galactosidase can possess transgalactosylation activity, which has recently gained interest for the production of galacto-oligosaccharides (GOS) - prebiotic that can stimulate the growth of beneficial bacteria such as Bifidobacteria and Lactobacilli (Juajun et al., 2011; Macfarlane et al., 2008; Rastall and Maitin, 2002). During the past years, the β-galactosidase-catalyzed trans-galactosylation has proved to be useful for structural and functional modification of food materials, medicines, and other biologically active compounds. At present, more than a hundred putative β-galactosidase sequences can be deduced from various databases, and these can be classified in to four different glycoside hydrolase (GH) families GH-1, GH-2, GH-35, and GH-42, based on functional similarities (Cantarel et al., 2009). In addition, microbial β-galactosidases are used as marker enzymes for coliform bacteria, which are indicators of the fecal contamination of water (Leitner, 2009; Nakayama and Amachi, 1999).

Major sources of β-galactosidase are derived in order from *K. lactis*, *K. marxianus*, and *A. niger*, respectively, with the remainder being those from *E. coli*. Henrissat (1991) has classified these glycosyl hydrolases into four families based on sequence similarities. According to this system, β-galactosidases are grouped into four families: 1, 2, 35 and 42. The members of family 2 include the β-galactosidase from *E. coli* (*lacZ*), the most extensively studied β-galactosidase. Unfortunately, enzymes from *E.coli* cannot be used in the food industry, mainly because of the unacceptability

of the bacterium (Leitner, 2009; Nakayama and Amachi, 1999). Beta-galactosidase from different microorganisms has different enzyme properties, such as molecule weight, protein chain length, and the position of the active site, but they have the same amino acid residue, glutamic acid, as their catalytic site, as shown in Table 2.1.

Table 2.1 Properties of microbial β-galactosidase**.**

Source	Molecular weight ($\times 10^3$)	pH optimum[a]	Temperature operation range (°C)	Activators	Ionic inhibitors[b]
Aspergillus niger	124	3.0-4.0	55-60	None needed	None
Aspergillus oryzae	90	5.0-6.2	50-55	None needed	None
Kluyveromyces lactis	228	6.5-7.3	35	K, Mg, Mn	Ca, Na
Kluyveromyces marxianus	201	6.6	37	K, Mg, Mn	Ca, Na
Escherichia coli	464	7.2	40	Na, K, Mg	-
Bacillus circulans	240	6.0	60	None needed	-
Bacillus subtilis	88	6.5-7.0	50	None needed	-
Bacillus stearothermophilus	116	5.8-6.4	65	Mg	-
Lactobacillus acidophilus	540	6.2-6.6	55	Mg	-
Streptococcus thermophilus	464	7.1	55	Na, K, Mg	Ca

Note: [a] Dependent on strain/source. [b] Ionic species likely to be found in dairy products; a dash indicates data not available.

Source: Harju et al (2012) and Mahoney (1997).

2.2.1 *Lactobacillus* spp. for β-galactosidase production

Lactobacilli are one of the most beneficial to human health among the species of bacteria present in the human intestine. Several beneficial functions such as vitamin production, production of digestive enzymes, and stimulation of the immune system have been suggested for the members of this genus as well as for other probiotic strains (Holzapfel and Schillinger, 2002; McNaught and MacFie, 2000; Nguyen et al., 2006; Sanders, 1998).

Many species of Lactobacillus (*L. acidophilus, L. rhamnosus, L. casei, L. plantarum,* and *L. reuteri*) have been evaluated for clinical effects in humans, including modulation of intestinal flora, lowering fecal enzyme activities, prevention and treatment of antibiotic-associated diarrhea, and effects on superficial bladder cancer and cervical cancer (Holzapfel and Schillinger, 2002; Saarela et al., 2000)Because of these proven and assumed positive effects, it is desirable to increase their number in the colon of human hosts (Nguyen, 2006).Then, β-galactosidases from *Lactobacillus* spp. have been able to produce the galacto-oligosaccharides possibility (GalOS) (Toba et al., 1981), apart from the main benefit that possess the high enzyme activity. The various sources of *Lactobacillus* spp. such as *L. delbrueckii* subsp. *bulgaricus* and *L. reuteri* are proven that they possessed the β-galactosidases activity and transgalactosylation such as *L. delbrueckii* subsp. *bulgaricus* (Garman et al., 1996; Kobayashi et al., 1990; Nguyen et al., 2006).

In general, the production of recombinant β-galactosidase from *Lactobacillus* spp. can be expressed in two well-known overexpression systems for lactic acid bacteria, which are the Nisin-Controlled Expression (NICE) or pheromone-

inducible (pSIP) systems (Sorvig et al., 2003; Sorvig et al., 2005; Halbmayr et al., 2008; Mathiesen et al., 2008; Straume et al., 2006).

The NICE system is appropriate mainly for plasmids from *Lactococcus* spp., while the pSIP system is easily adapted and applied to the different LAB, especially *Lactobacillus* spp. (Sorvig et al., 2003; Maischberger et al., 2010). Nguyen and co-workers (2011[b]) reported that the pSIP system gave the higher levels of overexpression of β-galactosidase from *L. plantarum* than that of the NICE system. Thus, the production of β-galactosidase from Lactobacillus spp., recognized as the GRAS organism (Generally Recognized As Safe), is even more interesting in the application to food industry.

Nevertheless, the pSIP system has a major limitation in food application from the original system. Since it possesses the erythromycin antibiotic resistance gene (*erm*), this gene is the selection marker one for the cell selection stage of enzyme production. The *erm* gene might have an effect on the microflora in human body and it may be transferred to other organisms, resulting in resistance to this antibiotic. In order to avoid this undesirable effect, the alanine racemase gene (*alr*) is used to be a selection marker, instead of the *erm* gene in the system for safety in terms of human food consumption. The *alr* gene could be produce the alanine racemace enzyme which this enzyme could convert L-alanine to D-alanine that is an essential component for the growth and cell wall biosynthesis of prokaryotic cells (Hols et al., 1997). Recently, Nguyen and co-workers (2011[a]) have been successfully used the pSIP vectors for carrying the *alr* gene as a selection marker in complementation approaches both in lactococci and lactobacilli (Nguyen et al., 2011[a]). Therefore, the expression of the *lacZ* gene, coding for β-galactosidase with the *alr* gene as a

selection marker can afford a safer new method to produce a recombinant β-galactosidase in a completely food-grade manner. Nonetheless, there has been only a work of Nguyen and co-workers (2011[a]) that revealed the successful expression of β-galactosidase from *LacLM* type with the *alr*-based. The *LacLM* as a expression gene of β-galactosidase is more complex in terms of protein structure than *LacZ* gene. The expression of β-galactosidase from *LacZ* gene has not been studied in the *alr*-based vector.

2.2.1.1 Substrates

Many chromogenic, fluorogenic, and luminogenic substrates that are specific for β-galactosidase have been developed (Nakayama and Amachi, 1999). In nature, the substrate for β-galactosidases is lactose which is similar to *ortho*-nitrophenyl-β-D-galactopyranoside (*o*NPG) structure. The respective reaction products of lactose and *o*NPG are shown in Figure 2.3 (Leitner, 2009).

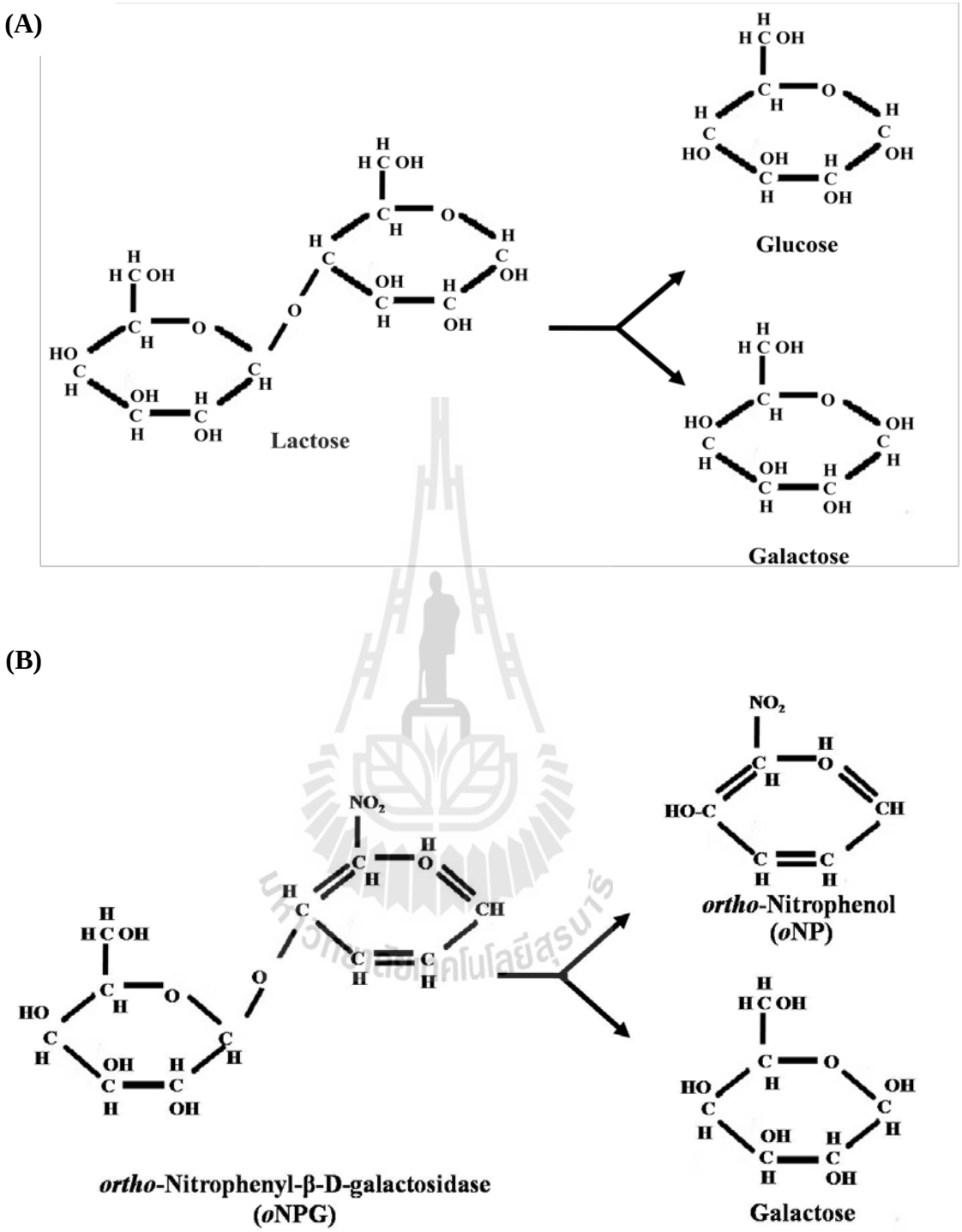


Figure 2.3 Substrate degradation by β-galactosidase (A) Natural substrate lactose (B) *ortho*-Nitrophenyl-β-D-galactosidase (*o*NPG). The figure was modified from Leitner (2009).

2.2.1.2 Hydrolysis and transgalactosylation reaction of lactose

The mechanism of lactose hydrolysis can be explained on the structure. β-galactosidase has 2 glutamic acid residues (Glu482 and Glu551). These residues function simultaneously as proton donors and as a nucleophile/base in the enzymatic reaction. The first step is the formation of enzyme–galactosyl complex and the simultaneous glucose liberation. In the second step, the enzyme–galactosyl complex is transferred to an acceptor containing a hydroxyl group as shown Figure 2.4. While in a diluted lactose solution or water, lactose can be a more competitive acceptor than other sugars such as glucose. Therefore, galactose is formed and released from the active site. In addition, in a high lactose content solution, the lactose molecule has more chances to act as the acceptor, binding with the enzyme–galactose complex to form oligosaccharides, which explains the formation of the Galacto-oligosaccharides (Huh et al., 1990; Lopez-Leiva and Guzman, 1995; Mahoney, 1998; Reyes-Torres et al., 2010; Rustom et al., 1998; Sheu et al., 1998).

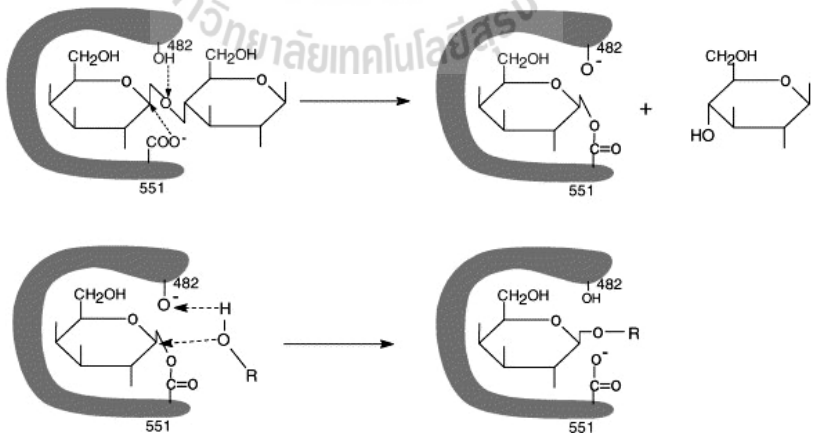


Figure 2.4 Schematic mechanism of the lactose hydrolysis by β-galactosidase.

Source: Zhou and Chen (2001).

2.2.1.3 Galacto-oligosaccharides (GalOS)

Galacto-oligosaccharides can be produced from lactose in milk through the enzymic transgalactosylation reactions of β-galactosidase. GalOS appear as undesirable by-products in processes for low lactose or lactose-free products. However, GOS has been shown to promote the growth of *Bifidobacteria*, health microbes, in the large intestine of humans (Chen et al., 2002; Ito et al., 1990; Matsumoto et al., 1989; Reyes-Torres et al., 2010; Tanaka et al., 1983; Yanahira et al., 1995).

The formation of GalOS is shown in Figure 2.5. During intramolecular trans-galactosylation lactose is broken down, and the glucose molecule binds immediately again with a different glycosidic linkage to the galactose- moiety, which is not replaced from the active site by a water molecule. This process is how allolactose is formed. When high concentrations of lactose are present, lactose can act as a galactosyl acceptor (Nu; Figure 2.4) with a transfer product of galactosyllactose. The galactosyllactose can act again as galactosyl acceptor to produce tetrasaccharides (intermolecular transgalactosylation) such as β-6′galactosyllactose.

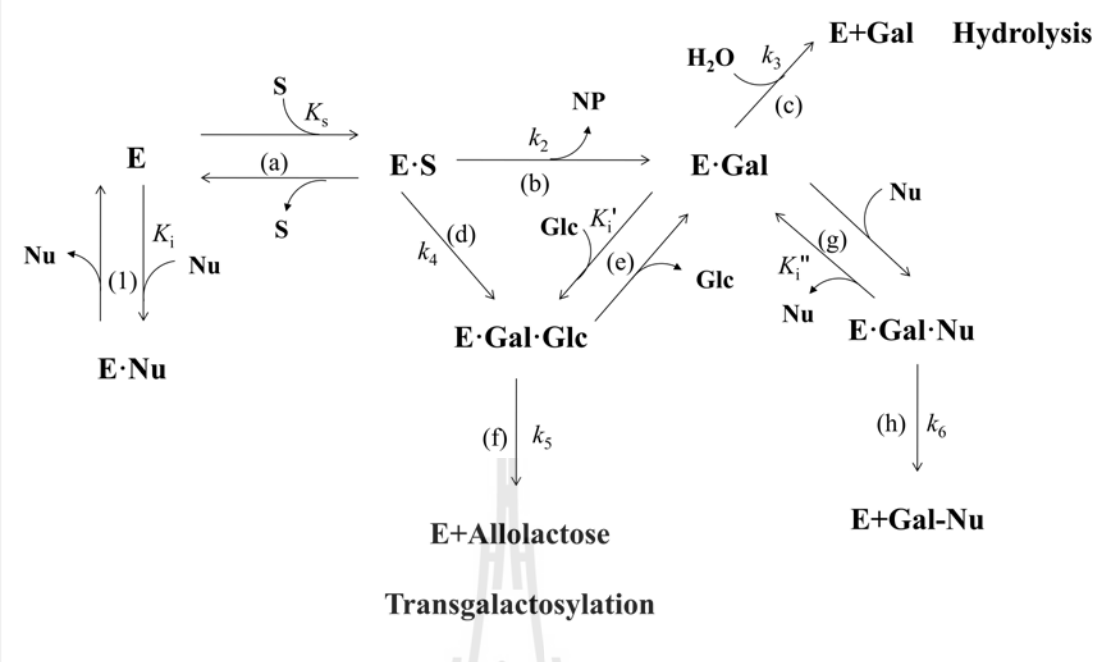


Figure 2.5 Hydrolysis and transgalactosylation of lactose catalyzed by *E. coli LacZ* β-galactosidase: E: enzyme (β-galactosidase); S: substrate (lactose); Lac: lactose; Gal: galactose; Glc: glucose; Nu: nucleophil. The figure was modified from Nakayama and Amachi (1999).

The global market size of GOS was estimated to be 20,000 tons with a compound annual growth rate of 10-20% (Affertsholt-Allen 2007; Reyes-Torres et al., 2010). While the global market size of non-digestible oligosaccharides is estimated at 50,000-80,000 tons annually. Oligosaccharides derived through enzymatic synthesis from lactose, particularly GOS, lactulose, and lactosucrose, account for a major share, approximately 40%, of annual production (Crittenden and Planyne, 1996; Gänzle, 2012; Seibal and Buchholz, 2010).

2.2.1.4 Treatment of milk with β-galactosidase

Beta-galactosidase treatment for lactose-hydrolyzed milk production has been used for the preparation of flavored milk, cheese, and yogurt. It

also prevents lactose crystallization in condensed milk products and frozen foods such as ice milk, and ice cream (Panesar et al., 2010). Moreover, β-galactosidase can be used for converting lactose in whey into a very useful product like sweet syrup, which can be used in various processes of dairy, confectionary, baking, and soft drink industries (Panesar et al., 2010; Pivarnik et al., 1995; Tweedie et al., 1978). Therefore, lactose hydrolysis not only allows milk consumption by lactose intolerant populations, but can also solve the environmental problems linked with whey disposal (Champluvier et al, 1988; Gekas and Lopez-Leiva, 1985; Martinez and Speckman, 1988; Panesar et al., 2010). Lactose content can also be reduced in liquid milk products by lactic acid fermentation, because lactose is the main source of carbon for lactic acid bacteria (LAB) (McSweeney and Fox, 2010). Approximately 30% of lactose is converted by these bacteria during the fermentation (Harju et al., 2012).

The process of lactose hydrolysis is simple and, therefore, does not require special equipment in dairy plants. Three techniques are used commercially to perform lactose hydrolysis: 1) single-use batch system, 2) recovery systems (enzyme reuse) and 3) immobilized enzymes (Mahoney, 1997). Most production of lactose free- or low lactose products uses β-galactosidase to hydrolyze lactose molecules by using either the free (soluble) or, rarely, the immobilized enzyme. The free enzyme treatment has several factors that must be considered: substrate concentration, pH of operation, maximum temperature, contact time permissible, enzyme activity, and cost. In addition, maintaining consistent temperatures must be considered because this factor affects microbial growth. Overnight holding of production at refrigeration temperature may be more suitable than using the temperature at 35-45°C for preventing microbial growth (Zadow, 1986). The

production of lactose-hydrolyzed milk by β-galactosidase treatment affects the physical properties of milk by decreasing the freezing point (Nijpels et al., 1980; Kreft et al., 2001; Nagaraj et al., 2009). Lactose-hydrolyzed milk is sweeter than ordinary milk because of the glucose and galactose, which are obtained after the conversion of lactose (Akcan, 2011; Harju et al., 2012; Panesar et al., 2006). It has been suggested that limiting hydrolysis to 80-90% avoids excessive sweetness. Vasala et al (1996) patented a method to reduce the sweetness of lactose-hydrolyzed UHT milk by adding the potassium salt of an organic acid, such as citrate, malate, gluconate or lactate at up to 80 mmol/L, optimally 15-45 mmol/L. Flynn et al (1994) reduced the sweetness of lactose-hydrolyzed milk using potassium chloride (Harju et al, 2012).

Heat treatment is also a problem of lactose-hydrolyzed milk production. The increased numbers of reduced sugars (i.e., the glucose and galactose) from increased hydrolysis reaction create significant Maillard browning (Harju et al., 2012). Nevertheless, UHT (Dahlqvist et al., 1977) and sterilization treatments (Mendoza et al., 2005) are used for lactose-hydrolyzed milk to avoid the Maillard reactions problem. As a result, β-galactosidase treatment increases the cost of fluid milk by ~$0.06-$0.08/L (McSweeney and Fox, 2010). However, the immobilization of enzyme can be applied to pasteurization or ultra-high temperature sterilization of milk for a more heat stable benefit of enzyme (Panesar et al., 2010). For the yeast β-galactosidase treatment of lactose-free milk production, three principle ways have been recommended for enzyme application (Rand, 1981; Mahoney, 1997; Jelen and Tossavainen, 2003; Mahoney, 2003). Firstly, pre-treatment, the enzyme is performed in milk before heat treatment then the product is heated, packed, and sold at 6-8°C to avoid microbial growth. However, the temperature of this method is not the optimal

temperature for enzyme activation. Thus, the duration of enzyme application should be longer around 24-30 hours depending on the enzyme dosage. Secondly, post-treatment, the enzyme is performed in milk after heat treatment. For this process, a sterile β-galactosidase is supplied in-line immediately following UHT treatment of milk. Then, the product is subsequently packaged. The lactose is hydrolyzed in the final package at ambient temperature. However, the high quality of β-galactosidase preparation should be kept in mind in this method because the active enzyme must be presented in the milk during storage. Lastly, a few drops of enzyme is added in milk products prior by consumer in order to produce the milk lactose-hydrolyzed products. This process time is within 12–24 hours depending upon the dosage of enzyme (Rand, 1981; Pivarnik et al, 1995; Mahoney, 1997; Jelen and Tossavainen, 2003; Mahoney, 2003). Although, these ways of lactose-free milk production can be used with the β-galactosidase from yeast but they can also utilize the enzyme from *Lactobacillus* spp. in order to produce the lactose-hydrolyzed milk.

2.2.1.5 Application of β-galactosidase

2.2.1.5.1 Milk

Beta-galactosidase treatment for the production of lactose-free or low lactose products occurs only in hospitals for lactose intolerant people. Otherwise, β-galactosidase treatment for decreasing sandiness or stability improvement of ice cream does not exist in manufacturing, which may be explained by the high costs of investment in β-galactosidase treatment.

The application of β-galactosidases from different *Lactobacillus* spp. to hydrolyze lactose with milk as substrate has been shown a few studies. The milk lactose has been hydrolyzed about 80 and 89% after 10 hours

incubation at 42°C with the original and mutated-enzyme form, respectively, these enzymes were form an acid tolerant β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, expressed in *E. coli* (Rhimi et al., 2009). While a recombinant enzyme from *L. acidophilus*, produced through *E. coli* expression system, has hydrolyzed the milk lactose about 73% within 30 hours at 10°C (Pan et al., 2010; Oliveira et al., 2011). Nonetheless, these researches have reported the recombinant β-galactosidase, which produced in the *E. coli* expression systems apply to the probable non-food products.

In term of lactose hydrolysis in milk by a recombinant β-galactosidase expressed in *Lactobacillus* spp. has not been shown a research now, except of which Jokar and Karbassi (2011). Beta-galactosidases from *L. bulgaricus* CHR Hansen Lb-12 has been mentioned from a native or recombinant cell, the milk lactose has been hydrolyzed with the cell free extracts of β-galactosidases for sterilized milk production that the sensory evaluation of lactose-hydrolyzed milk by the cell free extract of enzyme did not exhibit any significant difference when compared with ordinary UHT milk (Jokar and Karbassi; 2011).

2.2.1.5.2 Ice cream

Lactose makes up over one third of the solid matter in milk, and approximately 20% of the carbohydrates in ice cream (Marshall and Arbuckle, 1996). The lactose in ice cream mixes is from milk and milk solids not fat (MSNF). The percentage of lactose in ice cream is dependent on the amount of MSNF and fat in the mixture. Increasing D-glucose and D-galactose in lactose hydrolysis by β-galactosidase leads to an increase of the total solids in solution without contributing to the caloric content of the ice cream mix (Bakken et al., 1992; Matak et al., 1999).

Lactose-hydrolyzed milk is sweeter than non-hydrolyzed milk. About 70% of the hydrolyzed lactose in milk increases the sweetness of milk, equivalent to adding 2% of sucrose in milk (Zadow, 1986). In addition, the monosaccharides produced from hydrolyzed lactose can decrease the freezing point of ice cream mixes, increase relative sweetness, and promote the ease of dipability of ice cream (Iversen, 1983). The low freezing point is normally responsible for the accelerated melting of ice cream (Marshall and Arbuckle, 1996). The freezing point of ice cream mixes is directly proportional to the number of particles in solution and decreases proportionately to the level of lactose hydrolysis processing (Lindamood et al, 1989; Mitchell, 1989). Invariably, the hydrolysis of lactose in ice cream results in a smoother product (McSweeney and Fox, 2010). Therefore, β-galactosidase treated milk might be a new choice to improve the stability and texture of ice cream.

However, the application of β-galactosidase from *Lactobacillus* spp. with pSIP system to hydrolyze milk lactose and to improve ice cream properties has been still few studies that are interesting to study more. Because this enzyme, which produced in food-grade host might be appropriate in food industry in soon.

2.3 Ice cream

Ice cream is a frozen dessert made by freezing a pasteurized mix of ingredients, which the main ingredients of ice cream are cream, non-milk-solid, sweeteners and flavoring. In addition, the main components are milk fat, lactose, milk protein, sugar, and corn syrup solids (Marshall et al., 2003). Then, the regulatory standards for chemical composition of ice cream are usually determined by the fat

content, total solids, and protein content. The processing of ice cream is basically two steps; mix making and mix freezing (Schmidt, 2004). Mix is the liquid product consisting of milk ingredients that the optional mix ingredients, such as corn syrup solids, whey, whey protein powders, caseinates, coloring, egg solids, stabilizers, and emulsifiers may be used depending on the desired end product (Schmidt, 2004).

2.3.1 Ingredients

Common ingredients of ice cream include cream, butter or vegetable fats as the main sources of fat, condensed skim or whole milk, skim milk powder and/or whey powder or whey protein products as concentrated milk-solids-not-fat (MSNF), sucrose and/or corn starch hydrolysates as sweeteners, polysaccharides (such as locust bean gum, guar gum, carbozymethyl cellulose and/or carrageenan) as stabilizers, mono- and di-glycerides and polysorbate 80 as emulsifiers, and milk or water as main sources of water in the formula to balance the total solids of the components (Marshall et al., 2003). One mix is usually used for the production of a variety of flavors (McSweeney and Fox, 2010). Components of typical ice cream mix formulations are shown in Table 2.2. Each ingredient is an indicator of the perceived quality of ice cream.

2.3.1.1 Fat

The fat content is indicative of the perceived quality and/or value of ice cream. It increases the richness of ice cream, helps to give body, produces a characteristic smooth texture by lubricating the palate, and aids in producing desirable melting properties (Berger, 1997; Goff, 1997; Marshall and Arbuckle, 1996; McSweeney and Fox, 2010).

2.3.1.2 Milk solids-not-fat (MSNF)

Serum solids or milk solids-not-fat (MSNF) contain protein, carbohydrate, water-soluble vitamins, and minerals. Limitations on MSNF content may lead to problems of excessive freezing point depression and/or lactose crystallization (McSweeney and Fox, 2010).

2.3.1.3 Sweeteners

Sweeteners enhance flavors and improve the texture and palatability of ice cream. Their stabilization of a solution lowers the freezing point and imparts a measure of control over the temperature-hardness relationship (Berger, 1997). The most common sweetening agent is sucrose, which is used alone or in combination with other sugars. Sucrose and lactose are found most commonly in ice cream in the supersaturated glassy state, with few crystals present (Berger, 1997; Caldwell et al., 1992). In many ice cream formulations, sweeteners derived from corn syrup are substituted for either all or a portion of the sucrose. The use of corn starch hydrolyzed products (corn syrups of glucose solids) in ice cream is generally perceived to provide greater smoothness by contributing to a firmer and more chewy body, providing better melt-down characteristics, reducing heat shock potential (which improves the shelf-life of the finished product), and providing an economical source of solids (McSweeney and Fox, 2010).

2.3.1.4 Stabilizers

Ice cream stabilizers are a group of hydrocolloid ingredients (usually polysaccharides) used in ice cream formulations to produce smoothness in body and texture, to reduce or retard the formation of ice and lactose crystals during storage, and to provide melt resistance and uniform texture. They also increase the

viscosity of the mix, stabilize the mix by preventing serum separation (e.g., carageenan), aid in suspension of flavoring particles, produce a stable foam with stiffness and easy cut-off at the barrel freezer for packaging, slow down moisture migration from the product to the package, and help prevent shrinkage of the product volume during storage (Marshall and Arbuckle, 1996). Commonly used stabilizers include: locust bean (carob) gum, guar gum, carboxymethyl cellulose, sodium alginate, xanthan, gelatin and carrageenan (McSweeney and Fox, 2010).

2.3.1.5 Emulsifiers

Emulsifiers have been used in the manufacturing of ice cream mix for many years. They are usually integrated with stabilizers in proprietary blends, but their function and action are very different from that of stabilizers. They are used to improve the whipping quality of the mix, produce a drier ice cream to facilitate molding (fancy extrusion and novelty product manufacture), produce a smoother body and texture in the finished product, and promote superior drawing qualities at the freezer to produce a product with good stand-up properties and melt resistance (Goff, 1997; Marshall and Arbuckle, 1996; McSweeney and Fox, 2010). All ingredients must be analyzed for quality and composition to ensure that the preparation of the final product complies with legal requirements, company, specifications, and consumer expectations (Schmidt, 2004).

Table 2.2 Components of typical ice cream mix formulations

Component	Range (%)
Milk fat	10-16
Milk solids-not-fat	9-12
Sucrose	9-12
Corn syrup solids	4-6
Stabilizers/Emulsifiers	0-0.5
Total solids	36-45
Water	55-64

Source: Fox and McSweeney (2009)

2.3.2 Processing

In most countries, mix must be pasteurized to assure a pathogen-free product. Additional steps after pasteurization and cooling may include aging, flavoring and coloring. The next step is freezing and hardening of the final product. During this step mix is frozen in a ‘freezer’, cooled during the hardening stage, and is subsequently distributed to markets (Schmidt, 2004). The main steps of ice cream processing are depicted in the flowchart in Figure 2.6.

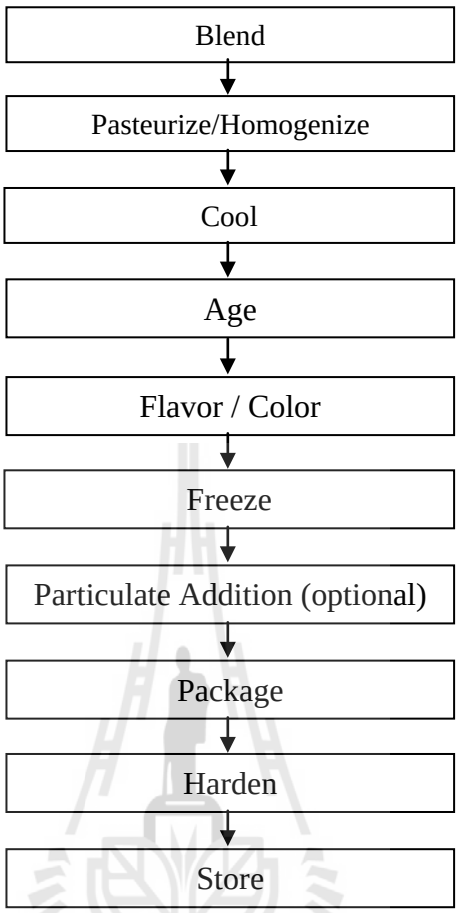


Figure 2.6 Flowchart for ice cream processing

Source: Schmidt (2004)

2.3.3 Ice cream quality

The quality of ice cream depends mainly upon the ingredients and procedures which affect ice cream structure. The ice cream structure has effects on the chemical, compositional, physical, microbiological, and sensory characteristics (Marshall et al., 2003). The specifics of transformation of ice cream are a result of these characteristics, which can deteriorate ice cream quality (Palka and Palich, 2007).

2.3.3.1 Physical properties

In particular, fat appears to contribute largely to the properties of ice cream during freezing and whipping, especially through the partially coalesced/agglomerated continuous three-dimensional network of homogenized globules (Goff, 1997). The structure of ice cream is characterized mostly by examining the state of dispersion of the fat as well as the sizes of the ice crystals and air bubbles. These affect the hardness, apparent viscosity, fat globule size, melting rate, shape retention, freezing behavior, overrun, and volume. Normally, the melting point and overruns of ice cream decrease when total soluble solids in ice cream increase, resulting in reduced hardness. However, the hardness of ice cream also depends on other factors or ingredients such as stabilizers or types of fat.

2.3.3.2 Microbiological contamination

Although ice cream is not a sterilized product, it contains no harmful microorganisms when it is produced by an approved process under hygienic conditions. Freezing can destroy many bacteria, and the frozen condition of the product prevents growth of microorganisms. Except for a large outbreak of salmonellosis, only a few cases of food-borne illness from commercially manufactured ice cream have been reported. And these instances were the result of contaminated raw eggs. Most regulatory agencies require that ice cream mixes should be pasteurized. The primary area of concern about microorganisms in frozen desserts is contamination of the frozen product during the addition of fruits, nuts and flavorings as well as during filling of containers (Marshall et al., 2003).

2.3.3.3 Sensory characteristics

Ice cream has many attributes that make it a favored food of most people. Among these are a rich sweet flavor, a smooth and resistant texture, and a cold sensation that contrasts to the warmth of most other foods. The chemical and physical properties imparted by the ingredients and the processes used in manufacture and handling largely determine the sensory properties. The chemical, compositional, and physical properties affect ice cream structure by determining several important sensorial parameters in the final product, such as stiffness, dryness, melt resistance and texture (Goff, 1997). High quality unflavored ice cream tastes sweet, heated (cooked), nutty, and creamy and/or buttery. Depending on the type and amount of flavoring added, all of these flavors except sweet may become imperceptible to all but the expert evaluator (Marshall et al., 2003). Additionally, defects in flavor of frozen desserts are conveniently grouped in the following categories: 1) Dairy products of poor quality - sour (acid), oxidized, stale, lipolyzed, unclean, and excessively cooked or scorched; 2) Sweetener(s) - unnatural, excessive, or deficient; 3) Flavoring - unnatural, excessive, or deficient; 4) Blend - unpleasant balance of ingredients; 5) Storage - stale or absorbed flavor (Marshall et al., 2003).

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CHAPTER III

PRODUTION OF RECOMBINANT β-GALACTOSIDASE IN *LACTOBACILLUS PLANTARUM*, USING A pSIP-BASED FOOD-GRADE EXPRESSION SYSTEM

3.1 Abstract

Food-grade expression systems based on using food-grade microorganisms have been developed for the production of recombinant enzymes and could be used in food applications. Lactic acid bacteria (LAB), especially Lactobacilli, have been widely used for various purposes in food and recognized as a promising host for food-grade enzyme production. In this study, the pSIP409 vectors, originally containing the *erm* selective gene and expressing the β-galactosidase gene, were used and replaced by the *alr* gene, resulting in the production of the pSIP609 expression vector in *L. plantarum*. This vector can express high amounts of β-galactosidases, showing both high volumetric and specific enzymatic activity. Thus, the food-grade β-galactosidases recombinant enzyme production in *L. planatarum* harboring pSIP609 was very fruitful, and, therefore, has a potential for the commercial food industries.

Keywords: Food-grade expression systems, pSIP system, Lactic acid bacteria, β-galactosidases, Alanine racemase gene

3.2 Introduction

Lactic acid bacteria (LAB) play important roles in foods because they have been recognized as a food-grade additive. Several LAB, especially Lactobacilli, have been developed as cell factories relating to inducible gene expression for food applications in which they are used for the production of a range of interesting proteins. Such enzyme production can be obtained using the Nisin-Controlled Expression (NICE) or pheromone-inducible (pSIP) systems, which are well-known overexpression systems for LAB (Sorvig et al., 2003; Sorvig et al., 2005; Halbmayr et al., 2008; Mathiesen et al., 2008; Straume et al., 2006). Furthermore, the vectors in the pSIP system permit all parts of the plasmid to be easily modified and used among different LAB, especially *Lactobacillus* spp., while plasmids in the NICE system are suitable mainly for *Lactococcus* spp. (Sorvig et al., 2003; Maischberger et al., 2010). Moreover, it was shown that the pSIP system resulted in higher levels of over-expressed enzyme than the NICE system in some instances (Nguyen et al., 2011[b]). Thus, recently, the use of the pSIP system has been increasingly attractive in the food industry. However, the pSIP system still has a major limitation when it is applied in foods. In the original system, the erythromycin antibiotic resistance gene (*erm*) is used as a selective marker for the cell selection stage of enzyme production, which might affect the microflora in the human body. In addition, the *erm* gene may be transferred to other organisms resulting in resistance to this antibiotic. To avoid this undesirable effect, the alanine racemase gene (*alr*) is used as a selection marker, instead of the *erm* gene, which is safer in terms of human food consumption. The alanine racemase enzyme is important for cell wall biosynthesis, especially in LAB. It converts L-alanine to D-alanine, which is an essential component for growth and cell

wall biosynthesis of prokaryotic cells (Hols et al., 1997). Moreover, pSIP vectors carrying the *alr* gene as a selection marker have been successfully applied in complementation approaches both in Lactococci and Lactobacilli (Nguyen et al., 2011[a]). Thus, expression of the *lacZ* gene, coding for β-galactosidase, using the *alr* gene as a selection marker can provide a safer new method to produce recombinant β-galactosidase in a completely food-grade manner.

Beta-galactosidase (lactase, EC 3.2.1.23) is an important enzyme in the dairy industry (Nguyen et al., 2012). This enzyme hydrolyzes lactose into glucose and galactose. It can be used to prevent lactose crystallization in dairy products, to increase the solubility of milk products, and to produce lactose-free food products (Sani et al., 1999). Moreover, β-galactosidase can produce galacto-oligosaccharides (GOS), which occurs simutaneously during lactose hydrolysis (Nakayama et al., 1999). Even though use of the pSIP vectors with the *erm* gene in Lactobacilli as a host can express high yields of β-galactosidase (Nguyen et al., 2012; Nguyen et al., 2011[b]), there remains a problem of antibiotic resistance in such systems. To date, only the work of Nguyen and coworkers (2011)[a] has dealt with this problem by using an expression vector carrying the *alr* gene with a suitable *alr*-deletion strain to overexpress a β-galactosidase of the *LacLM* type (Hols et al., 1997). This *lacLM* protein is heterodimeric and is encoded by two partially overlapping genes, which might be more complicated to express and study as a model system. Thus, the expression of homodimeric *lacZ* β-galactosidase encoded by the *lacZ* gene with the *alr* gene as a selective marker in a pSIP vector, known as a recombinant system, might be more beneficial, in terms of cost, time, and safety, than the heterodimeric system. The objective of this study was to construct and express the food-grade

β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* in *L. plantarum* for a potential use.

3.3 Materials and methods

3.3.1 Culture of bacterial strains

Escherichia coli Top10, used as cloning hosts, was purchased from Invitrogen (CA, USA). They were grown in Luria-Bertani (LB) medium either on solid agar plates or in liquid medium, and incubated for overnight at 37ºC in a cabinet (Memmert, BE 500, WTB Binder BD115, Memmert GmbH + Co. KG, Schwabach, Germany) for plates or in a shaker incubator (Innova 4230 refrigerated incubator shaker, New Brunswick Scientific, USA) for liquid cultures. Erythromycin (800 μg/mL) was added to the medium to select for growth of *E. coli* Top10 strains containing the plasmids.

Escherichia coli MB2159 (D-alanine auxotroph) was used as cloning host for food-grade expression vectors, as described by Strych and co-workers (2001). The bacterium was grown in Luria-Bertani (LB) medium supplemented with 200 μg/mL of D-alanine and incubated for overnight at 37ºC in a cabinet (Memmert, BE 500, WTB Binder BD115, Memmert GmbH + Co. KG, Schwabach, Germany) for plates or in a shaker incubator (Innova 4230 refrigerated incubator shaker, New Brunswick Scientific, USA) for liquid cultures.

Lactobacillus plantarum WCFS1 (wild type) was used as described by Kleebezem and co-workers (2003). They were grown either in Man-Rogosa-Sharpe (MRS) medium or on solid MRS-agar plates. *L. plantarum* cultures were incubated under facultative aerobic condition at 37°C without agitation for 18-24

hours in a cabinet for plates (Memmert, BE 500, WTB Binder BD115, Memmert GmbH + Co. KG, Schwabach, Germany). *L. plantarum* WCFS1 cells containing plasmids harboring an antibiotic resistance gene were grown in agar plates and liquid medium containing 5 μg/mL of erythromycin concentration.

Lactobacillus plantarum TLG02 (Δ*alr*, D-alanine auxotroph), used as expression host for food-grade expression vectors, was used as described by Nguyen and co-workers (2011)[a]. The cells were grown in in MRS medium supplemented with 200 μg/mL D-alanine and incubated for overnight at 37ºC in a cabinet (Memmert, BE 500, WTB Binder BD115, Memmert GmbH + Co. KG, Schwabach, Germany) for plates or in a shaker incubator (Innova 4230 refrigerated incubator shaker, New Brunswick Scientific, USA) for liquid cultures.

3.3.2 Construction of β-galactosidase Expression Vectors

The food-grade expression vector was constructed in the pSIP vectors (Figure 3.1) by replacing the erythromycin resistance gene (*erm*) with the alanine racemase gene (*alr*) from the *L. plantarum* genome as described by Nguyen and co-workers (2011)[a]. This modification changes the plasmid from pSIP409(*erm*) to pSIP609(*alr*).

3.3.3 Expression and Confirmation of β-galactosidases with *alr*-Based Vectors

Overnight cultures of *L. plantarum* TLG02 harboring pSIP609(*alr*) were diluted in 300 ml of fresh pre-warmed MRS medium (for *erm*-based systems, 5 μg/mL of erythromycin was added) to get a cell concentration OD_{600nm} of ~0.1 by determining the absorbance at 600 nm using a spectrophotometer (Ultrospec 2000, Pharmacia biotech, Cambridge, UK). The mix was then incubated at 30°C until

OD_{600nm} of ~0.3 was reached. Peptide pheromone at 25 ηg/mL (IP-673) was added to the mix, and the cultivation was continued until a final cell culture concentration of OD_{600nm} at 1.8 to 2.0 was reached. Ten mL of cell culture solutions were centrifuged by the centrifuge machine (Eppendrof centrifuge 5810 R, Eppendrof, USA) at 3500 g for 10 minutes at 4°C. The pelleted cells were collected, washed with buffer P (50 mM sodium phosphate buffer, pH 6.5, 20% of glycerol and 1 mM DTT), and resuspended in 500 μL of buffer P. The pelleted cells were disrupted by sonicator (40% power, 5 sec on and 6 sec off for 5 minutes 2 times; Waken GE100 Ultrasonic processor, Japan) to get the cell lysate. Finally, this suspension was centrifuged by the centrifuge machine (Thermoscientific, Sorvall legend XTR centrifuge, USA) at 9000g at 4°C for 5 minutes to get the cell-free extracts used for activity assays and protein concentration determination using the denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.3.4 Enzyme Assay and Protein Determination

3.3.4.1 Gel electrophoresis analysis

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in accordance with Laemmli (1970). Protein samples were heated at 100°C for 5 minutes in the loading buffer. Protein samples were loaded onto 10% acrylamide gel. Gels were run at a constant voltage at 90V. Protein bands were stained with 0.125% Coomassie brilliant blue R 250 and destained in a solution containing 25% ethanol and 10% acetic acid). A protein ladder (10–200 kDa) from Bio-Rad Laboratories (CA, USA) was used for protein maker.

3.3.4.2 Protein determination

Proteins concentration was determined by Quick start™ Bradford protein assay (Bio-Rad Laboratories, CA, USA)) using bovine serum albumin as standard. The dye reagent was mixed 1 to 4 with DI water and filtered. The properly diluted samples (60 µl) were mixed with 200 µl of dye reagent and stand at room temperature for 2 minutes. The absorbance at 595 nm was measured by ELISA reader (Sunrise, TECAN, Austria). The protein concentration was calculated from the following relationship (Bradford et al., 1976):

$$\text{Protein concentration (mg/mL)} = \frac{OD_{595}}{k} \times \text{Dil}$$

Where OD_{595} is the sample-measured absorption at 595 nm, k is the slope of the BSA standard curve, and Dil is the dilution factor, after correcting for the blank.

3.3.4.3 Beta-galactosidase activity assay

Ortho-nitrophenyl-β-D-galactopyranoside *(o*-NPG) from Sigma -Aldrich (CA, USA) was the chromogenic substrate to assay the β-galactosidase activity. Twenty two mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) was prepared as the substrate solution. The β-galactosidase activity assay performed by 20 µL of enzyme solution was added into 480 µL of the substrate solution and incubated at 30°C for 10 minutes with 600 rpm speed agitation in a dry bath incubator (HB1, Wealtee Corp., USA). After reaching the incubation time, the reaction was stopped by adding 750 µL of 0.4 M Na_2CO_3. The release of ortho-nitrophenol (*o*NP) was assessed by determining the absorbance at 420 nm by spectrophotometer (Ultrospec 2000, Pharmacia biotech, Cambridge, UK). 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. The β-galactosidase activity or the volumetric activity was calculated from the following relationship (Nguyen et al., 2006):

$$\text{β-galactosidase activity or Volumetric activity (U/mL)} = \frac{OD_{420}}{k} \times \frac{1}{t} \times \frac{V_{oNPG} + V_{enzyme}}{V_{enzyme}} \times Dil$$

Where OD_{420} is the sample-measured absorption at 420 nm, k is the slope of the *o*NP standard curve, t is the reaction time (10 minutes), V_{oNPG} is the volume of *o*NPG (480 µL), V_{enzyme} is the volume of enzyme sample (20 µL), and Dil is the dilution factor, after correcting for the blank.

The specific activity was calculated from the following relationship:

$$\text{Specific activity (U/mg protein)} = \frac{\text{β-galactosidase activity (U/mL)}}{\text{Protein concentration (mg /mL)}}$$

3.3.5 Statistical analyses

All experiments and measurements were performed at least in duplicate. Analysis of variance (ANOVA) was determined using SPSS program (SPSS version 16, Windows version). Duncan Multiple Range Testing (DMRT) was used to compare differences among the means at $P<0.05$.

3.4 Results and discussion

3.4.1 Preparation of vectors and insert gene for food-grade expression vector

The vectors, pSIP409-*lacZ* and pSIP409–*lacZ*-His were extracted using QIAGEN™ plasmid preparation kit (Qiagen, Hilden, Germany). The presence of the *erm* insert gene in the plasmid was confirmed by *Bam*HI-HF and *Cla*I restriction enzyme digestion (New England Biolabs, MA, USA) and analysis of the restriction pattern (Figure 3.1) by agarose gel electrophoresis (Figure 3.2). The alanine racemase gene (*alr*) of the *L. plantarum* WCFS1 genome (Nguyen et al., 2011[a]) was used as the insert gene. Then, the pSIP609*gusA* was extracted using the QIAGEN™ plasmid preparation kit to verify the restriction pattern (Figure 3.3). DNA fragments of *lacZ* gene (~2.2 kbp) and *alr* gene(~1.8-2.0 kbp) were shown after double digestion with *Bam*HI-HF and *ClaI* restriction enzymes by agarose gel electrophoresis (Figure 3.4). Ligation reactions were performed with the relevant restriction endonucleases to digest the vector. A molar ratio of linearized vector to insert was approximately 1:15. The amount of linearized vector for each ligation reaction was 100 ηg. Ligations were performed for 16 hours at 16°C in the presence of T4 DNA ligase in a final volume of 25 µL. T4 DNA ligase was heat-inactivated (65°C for 15 minutes) before transformation. The constructs were transformed into competent *E. coli* Top10 (Invitrogen, CA, USA), resulting in the plasmid pSIP409-*lacZ* and pSIP409–*lacZ*-His. The transformants were selected on LB agar containing 800 µg/mL erythromycin in the culture condition at 37°C for 16 hours.

The plasmids were extracted from positive clones of the recombinant *E. coli* harboring pSIP409-*lacZ* and pSIP409–*lacZ*-His using the QIAGEN™ plasmid

preparation kit. Then plasmids were digested with *Bam*HI-HF and *Cla*I to analyze the restriction pattern by agarose gel electrophoresis (Figure 3.5). Afterwards, pSIP409-*lacZ* and pSIP409–*lacZ*-His were transformed into competent *L. plantarum* WCFS1, which is an expression host introduced by electroporation. Electroporation condition was performed at 25% amplitude with a 5 sec pulse followed by 3 minutes on ice for 2 rounds. The transformants were selected using a MRS agar plate containing 5 µg/mL erythromycin. All strains were stored in sterilized 1.5 mL Eppendorf tubes at -80°C in MRS broth medium containing 20% (v/v) glycerol until used. The positive clones of *L. plantarum* WCFS1 harboring pSIP409-*lacZ* and pSIP409-*lacZ*-His were confirmed by the expression of β-galactosidases using SDS-PAGE analysis as shown in Figure 3.6 and 3.7. The recombinant *lacZ* showed a molecular weight of approximately 110 kDa that was the same weight of two cold-sensitive mutants of the β-galactosidase from *L. delbruckii* subsp. *bulgaricus* showing two 110 kDa subunits (Adams et al., 1994) and agreed with the study of homodimeric β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* DSM 20081, expressed in *L. plantarum* (Nguyen et al., 2012). According to the references, the expressed β-galactosidase in *L. plantarum* WCFS1 shows the molecular weight of β-galactosidase from *L. delbrueckii* subsp. *bulgaricus*.

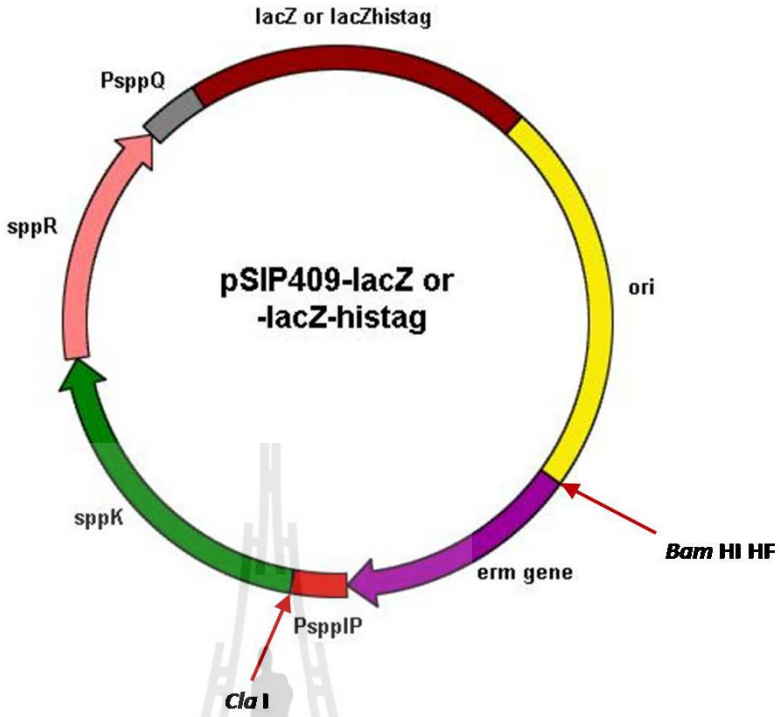
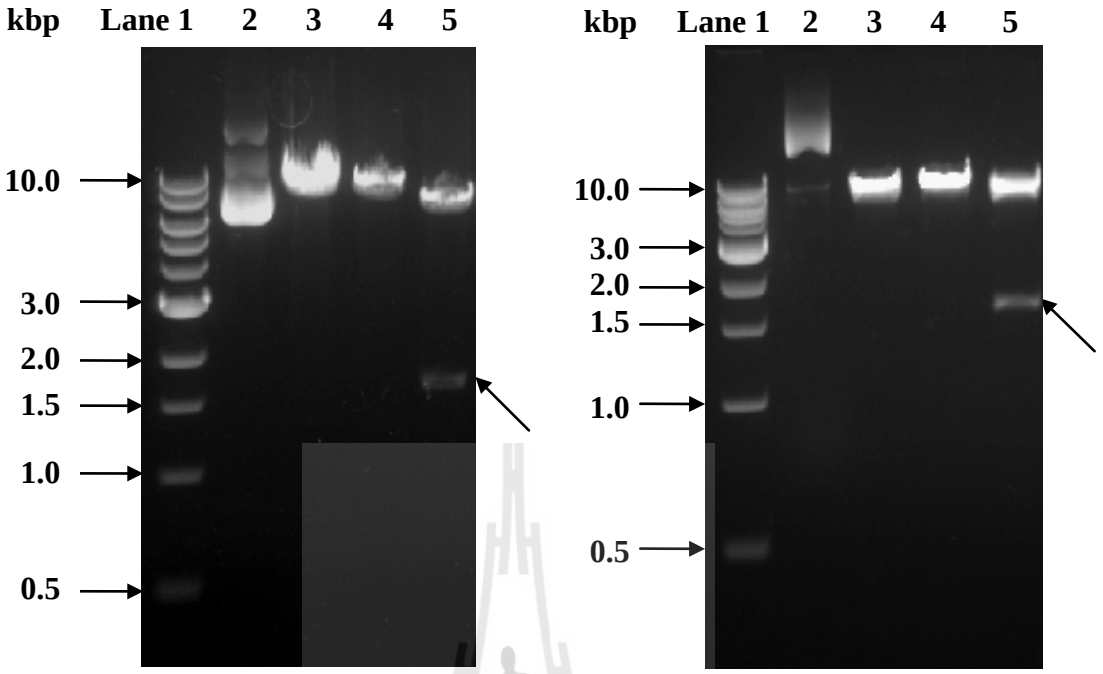


Figure 3.1 Schematic overview of food-grade expression vector (pSIP409-*lacZ* or pSIP409–*lacZ*-His) using the erythromycin resistance (*erm*) gene as a selection marker. The expression of sppK (histidine kinase) and sppR (response regulator) was regulated by PsppIP promoter. In addition, the expression of structural genes was controlled by the inducible promoter PsppQ, which was switched on by adding the peptide pheromone IP-673. The *Bam*HI-HF and *Cla*I restriction enzymes were used for cloning. The arrows indicate the gene fragment, which includes the *erm* gene.



(A) (B)

Figure 3.2 Restriction analysis of recombinant constructs. (A) pSIP409-*lacZ*, (Lane 1, 1kbp DNA ladder; Lane 2, uncut pSIP409-*lacZ* ; Lane 3, cut pSIP409-*lacZ* with *Bam*HI-HF; Lane 4, cut pSIP409-*lacZ* with *Cla*I; Lane 5, double digested pSIP409-*lacZ* with *Bam*HI-HF and *Cla*I); (B) pSIP409-*lacZ-His*, (Lane 1, 1kbp DNA ladder; Lane 2, uncut pSIP409-*lacZ*-His; Lane 3, cut pSIP409-*lacZ-His* with *Bam*HI-HF; Lane 4, cut pSIP409-*lacZ-His* with *Cla*I; Lane 5, double digested pSIP409-*lacZ*-His with *Bam*HI-HF and *Cla*I). The arrows indicate the *erm* gene.

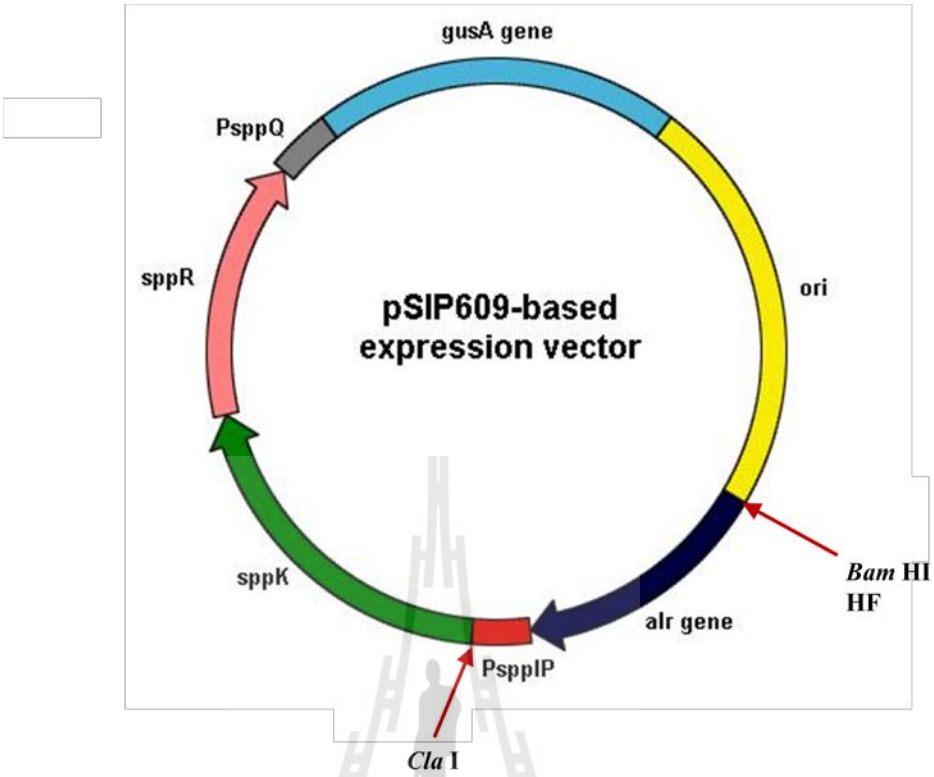


Figure 3.3 Schematic overview of pSIP609*gusA* used in this study to obtain the insert gene for β-galactosidase expression. The *Bam*HI-HF and *Cla*I restriction enzymes were used for cloning. The arrows indicate the gene fragment, which includes the *alr* gene. The fragment gene will be removed from this plasmid and put on the vector.

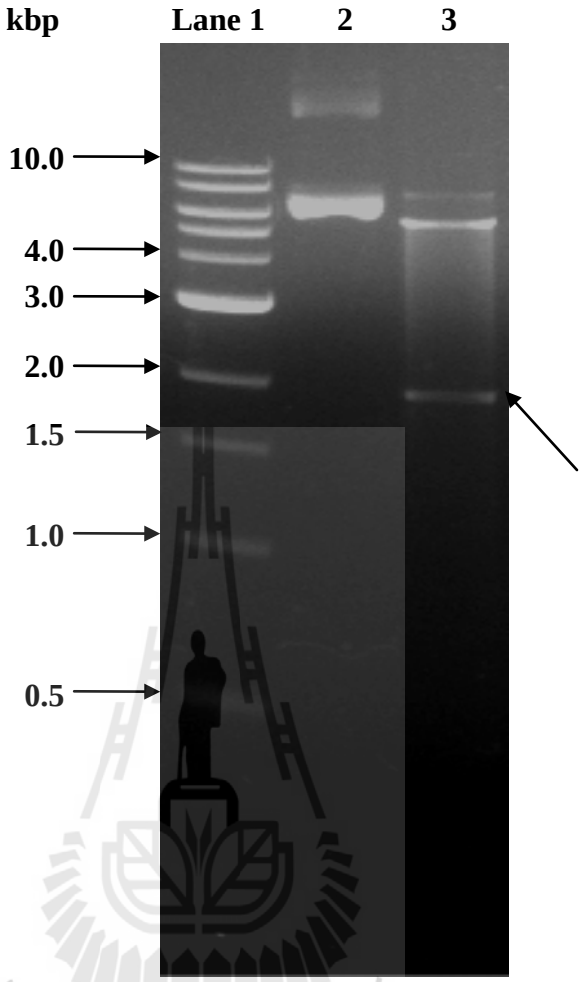


Figure 3.4 Restriction analysis of pSIP609*gusA* (Lane 1, 1kbp DNA ladder; Lane 2, uncut pSIP609*gusA* vector; Lane 3, double digested pSIP609*gusA* vector with *Bam*HI-HF and *Cla*I). The arrow indicates the *alr* gene.

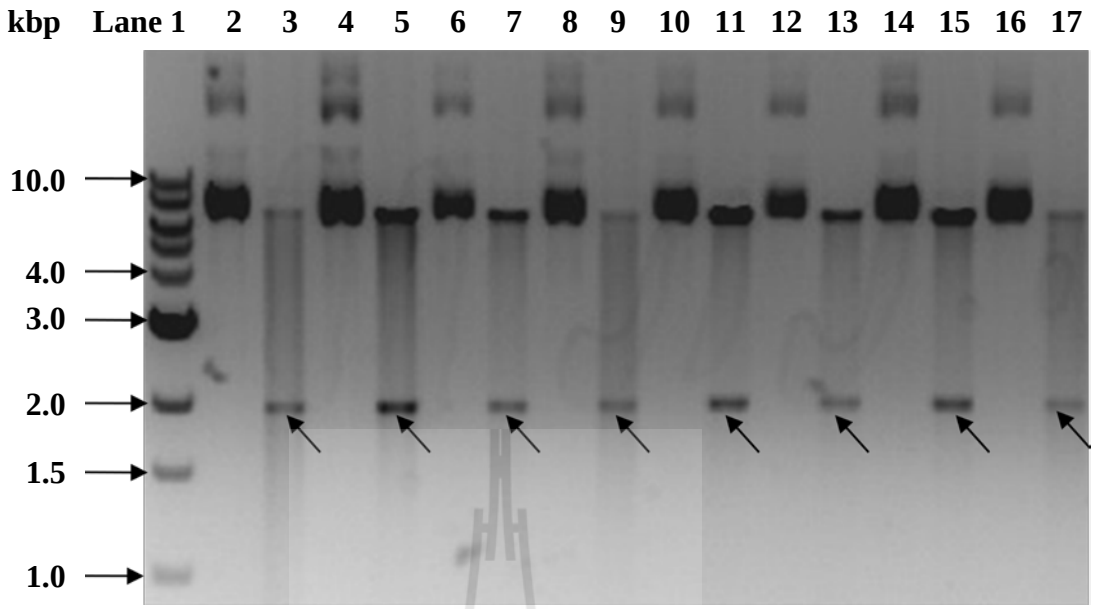


Figure 3.5 Restriction analysis of recombinant constructs; Lane 1, 1kbp DNA ladder; Lane 2-3, pSIP409-*lacZ* clone 1, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 4-5, pSIP409-*lacZ* clone 2, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 6-7, pSIP409-*lacZ* clone 3, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 8-9, pSIP409-*lacZ* clone 4, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 10-11, pSIP409-*lacZ*-His clone 1, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 12-13, pSIP409-*lacZ*-His clone 2, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 14-15, pSIP409-*lacZ*-His clone 3, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively; Lane 16-17, pSIP409-*lacZ*-His clone 4, uncut and double digested with *Bam*HI-HF and *Cla*I, respectively. The arrows indicate the *alr* gene.

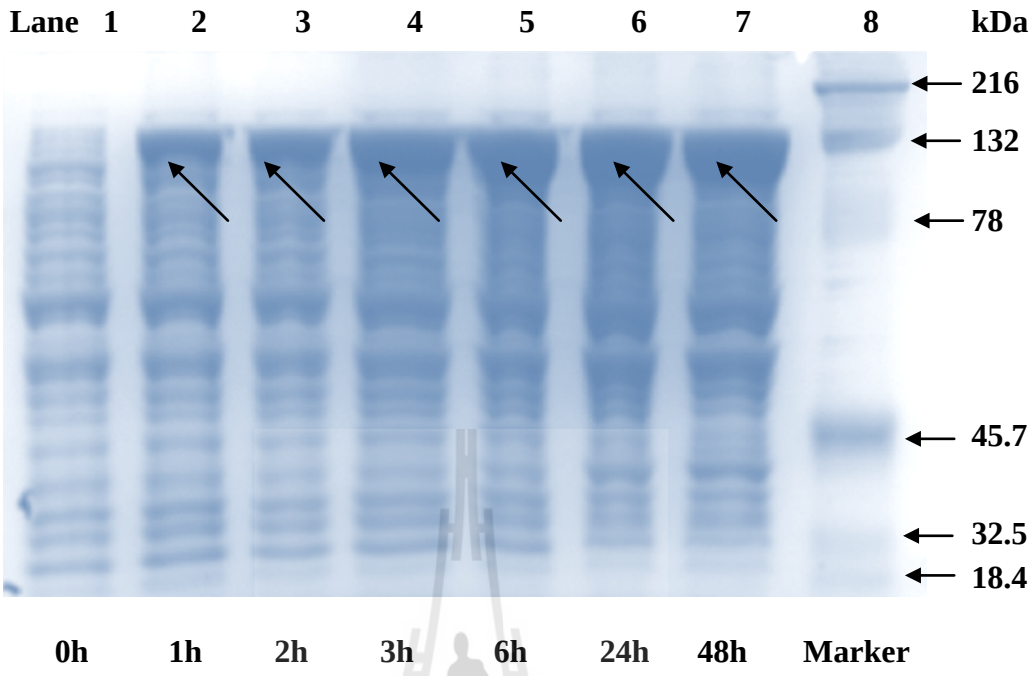


Figure 3.6 Expression of *L. plantarum* WCFS1 harboring pSIP409-*lacZ* at different time intervals. The cell-free extracts from induced cells with IP-673 were analyzed by SDS-PAGE (Lane 1, non-induced by IP-673 counted as 0 hour; Lane 2, induced for 1 hour; Lane 3, induced for 2 hours; Lane 4, induced for 3 hours; Lane 5, induced for 6 hours; Lane 6, induced for 24 hours; Lane 7, induced for 48 hours; and Lane 8, Precision plus Protein standard ladder (Bio-Rad)). The arrows indicate the bands of expressed β-galactosidase. The gel was stained with Coomassie blue.

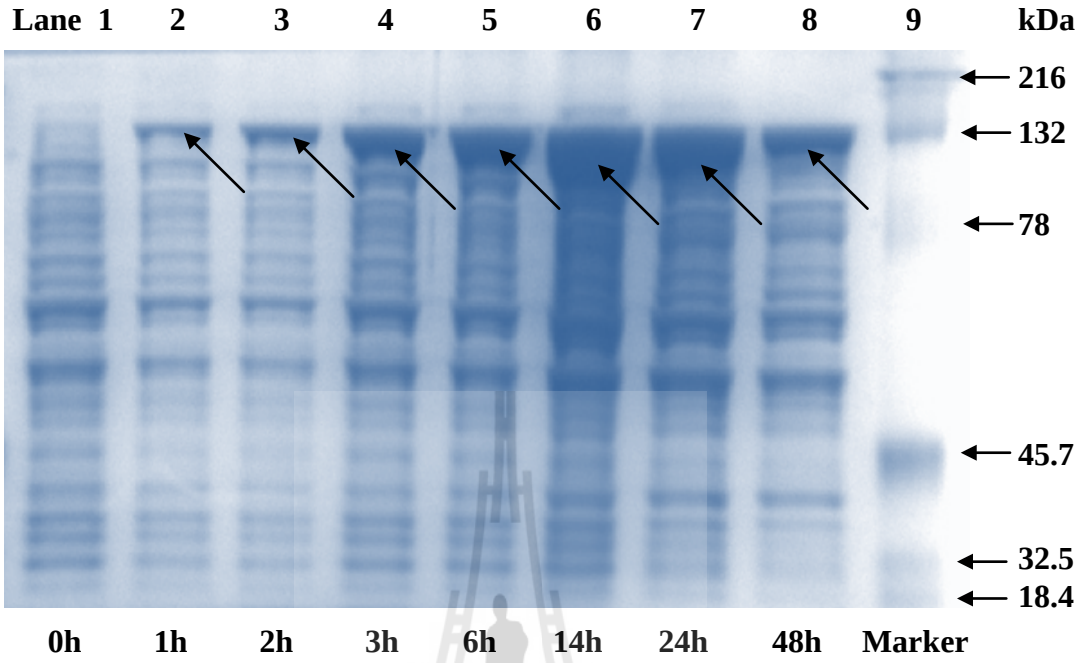


Figure 3.7 Expression of *L. plantarum* WCFS1 harboring pSIP409-*lacZ*-His at different time intervals. The cell-free extracts from induced cells with IP-673 were analyzed by SDS-PAGE (Lane 1, non-induced by IP-673 counted as 0 hour; Lane 2, induced for 1 hour; Lane 3, induced for 2 hours; Lane 4, induced for 3 hours; Lane 5, induced for 6 hours; Lane 6, induced for 14 hours; Lane 7, induced for 24 hours; Lane 8, induced for 48 hours; Lane 9, Precision plus Protein standard ladder (Bio-Rad)). The arrows indicate the bands of expressed β-galactosidase. The gel was stained with Coomassie blue.

3.4.2 Construction of food-grade expression vectors

The food-grade expression vectors (*alr*-based vectors) were constructed by replacing the erythromycin resistant (*erm*) gene of the pSIP409-*lacZ* and pSIP409-*lacZ*-His vectors with the alanine racemes gene (*alr*). The recombinant pSIP409-*lacZ* and pSIP409-*lacZ*-His in the *erm*-based vectors (Figure 3.1) were designed as pSIP609-*lacZ* and pSIP609-*lacZ*-His (Figure 3.8). The transcription of *lacZ* genes was controlled by the promoter P_{sppQ}. Selection of constructs harboring the food-grade expression vectors was made by growing the *E.coli* MB2159 (D-alanine auxotroph) in LB media without using antibiotic. The integration of pSIP609-*lacZ* and pSIP609–*lacZ*-His was confirmed by *Bam*HI-HF and *Cla*I restriction enzyme digestion.

In accordance with Nguyen and co-workers (2011)[a], the fragment gene from restriction analysis including the *alr* gene was the size band about 1.8–2.0 kbp for this study. However, the size band of *alr* gene size had been also reported at about 1.3 kbp (Bron et al., 2002). Therefore, the *alr* gene from the restriction analysis of pSIP609-*lacZ* and pSIP609-*lacZ*-His in Figure 3.9 and 3.10 showed the right band size. The food-grade vectors were then transformed into the expression host, *L. plantarum* TLG02, for the production of food-grade β-galactosidase recombinant enzyme.

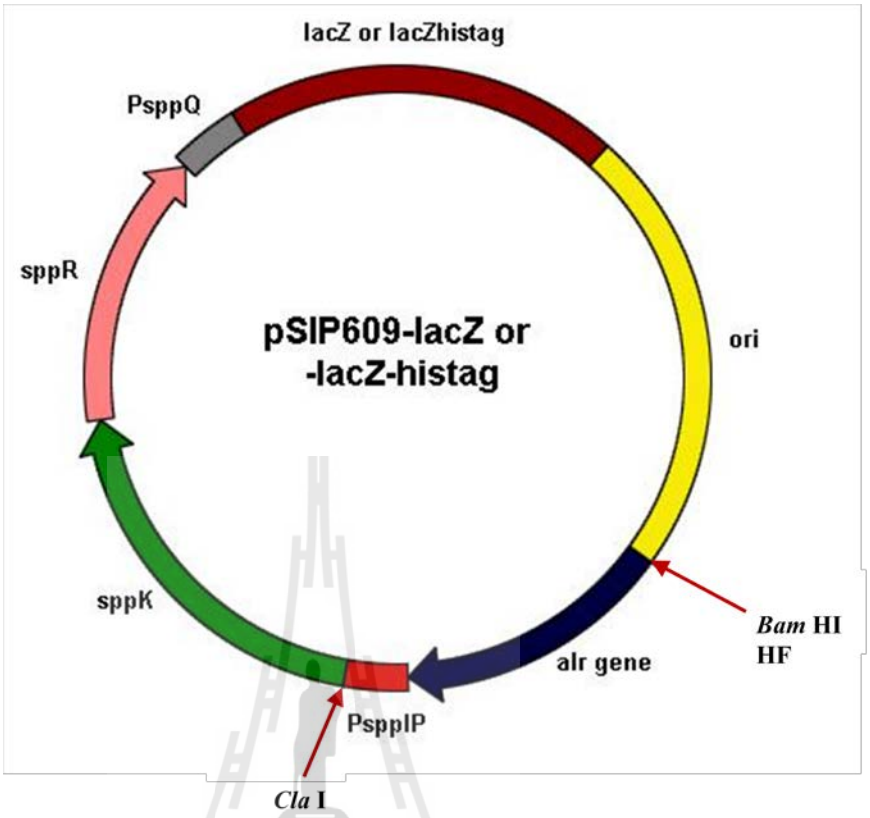


Figure 3.8 Schematic overview of food-grade expression vectors (pSIP609-*lacZ* or pSIP609-*lacZ*-His) using the alanine racemase (*alr*) gene as a selection marker. The expression of sppK (histidine kinase) and sppR (response regulator) was regulated by PsppIP promoter. In addition, the expression of structural genes was controlled by the inducible promoter PsppQ, which was switched on by adding the peptide pheromone IP-673. The *Bam*HI-HF and *Cla*I restriction enzymes were used for cloning. The arrows indicate the gene fragment, which includes the *alr* gene.

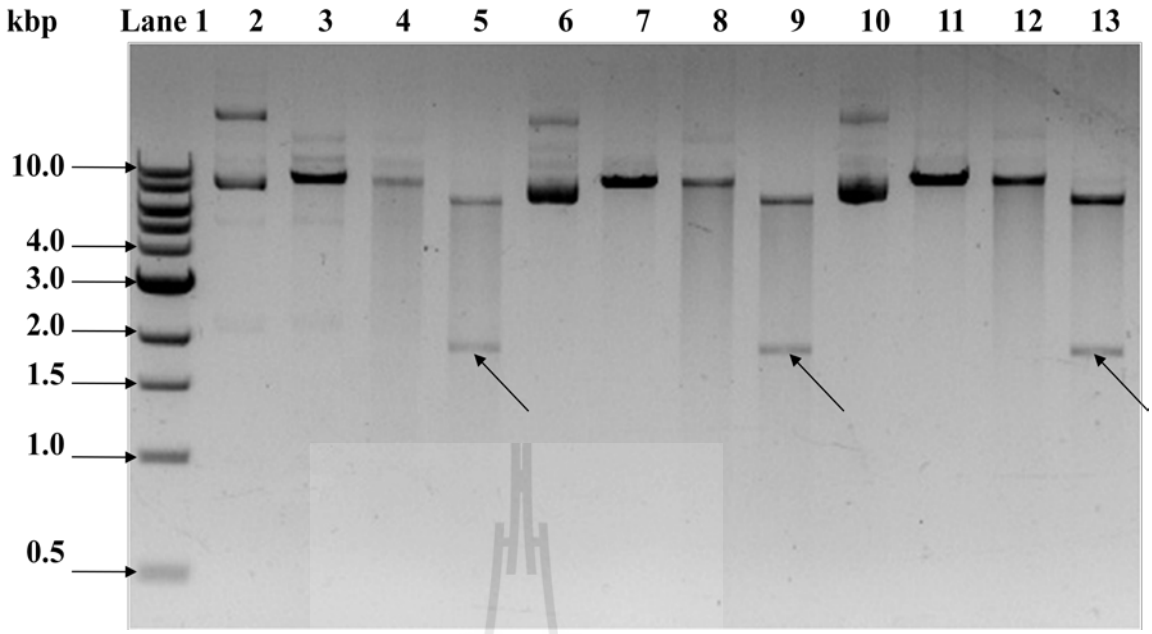


Figure 3.9 Restriction analysis of pSIP609-*lacZ*, (Lane 1, 1kbp DNA ladder; Lane 2, uncut pSIP609-*lacZ* clone 1; Lane 3, pSIP609-*lacZ* clone 1 cut with *Bam*HI-HF; Lane 4, pSIP609-*lacZ* clone 1 cut with *Cla*I; Lane 5, double digested pSIP609-*lacZ* clone 1 with *Bam*HI-HF and *Cla*I; Lane 6, uncut pSIP609-*lacZ* clone 2; Lane 7, pSIP609-*lacZ* clone 2 cut with *Bam*HI-HF; Lane 8, pSIP609-*lacZ* clone 2 cut with *Cla*I; Lane 9, double digested pSIP609-*lacZ* clone 2 with *Bam*HI-HF and *Cla*I; Lane 10, uncut pSIP609-*lacZ* vector clone 3; Lane 11, pSIP609-*lacZ* clone 3 cut with *Bam*HI-HF; Lane 12, pSIP609-*lacZ* clone 3 cut with *Cla*I; Lane 13, double digested pSIP609-*lacZ* clone 3 with *Bam*HI-HF and *Cla*I. The arrows indicate the DNA fragments of the *alr* gene.

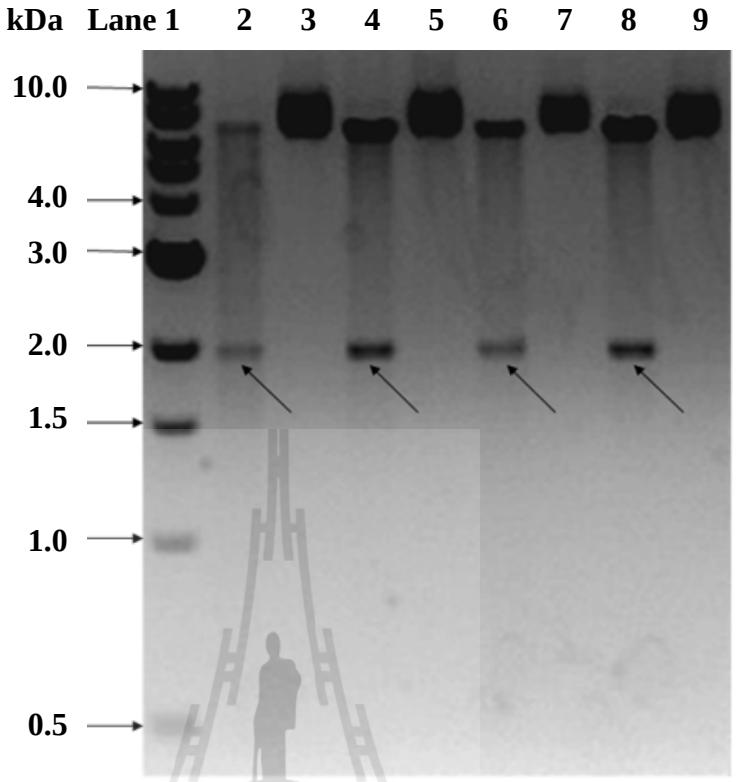


Figure 3.10 Restriction analysis of pSIP609-*lacZ*-His, (Lane 1, 1kbp DNA ladder; Lane 2, double digested pSIP609-*lacZ*-His clone 1 with *Bam*HI-HF and *Cla*I; Lane 3, uncut pSIP609-*lacZ*-His clone 1; Lane 4, double digested *lacZ*-His clone 2 with *Bam*HI-HF and *Cla*I; Lane 5, uncut pSIP609-*lacZ*-His clone 2; Lane 6, double digested pSIP609-*lacZ*-His clone 3 with *Bam*HI-HF and *Cla*I; Lane 7, uncut pSIP609-*lacZ*-His clone 3; Lane 8, double digested pSIP609-*lacZ*-His clone 4 with *Bam*HI-HF and *Cla*I; Lane 9, uncut pSIP609-*lacZ*-His clone 4.The arrows indicate the DNA fragments of the *alr* gene.

3.4.3 Expression of β-galactosidase in the food-grade expression vectors

The constructed plasmids (pSIP609-*lacZ* and pSIP60-*lacZ*-His) were transformed into D-alanine auxotroph *L. plantarum* TLG02 by electroporation. The organisms harboring *alr*-based vectors were then cultivated for β-galactosidase production. The results showed that expression of *alr*-based vectors obtained at different induction times and concentrations yielded high levels of β-galactosidases in a dose response manner as shown in Figure 3.11, 3.12 and 3.13. Also, the SDS-PAGE analysis of food-grade β-galactosidases from *L. delbrueckii* subsp. *bulgaricus* in *L. plantarum* TLG02 showed the molecular weight about 110 kDA of *L. delbrueckii* subsp. *bulgaricus* correctly (Adams et al., 1994; Nguyen et al., 2012). These expression levels were also confirmed by measuring the β-galactosidase activity and protein content, resulting in the specific activity as Table 3.1. The volumetric activity of crude enzymes of *L. plantarum* TLG02, harboring pSIP609-*lacZ* and pSIP609-*lacZ*-His were approximately 307±8 and 81±2 U/mL of fermentation broth, respectively indicating that the specific activity of *LacZ*-His was obviously lower than of *LacZ*. Then, the enzyme activity of *LacZ*-His was always significantly lower than *LacZ* by approximately 20-30% that caused the C-terminal His-tag (Nguyen et al., 2012). Resulting in a poly-histidine tag can adversely affect the biochemical properties change the binding characteristics, alter protein structure conformation, and prompt protein oligomerization (Gaberc-Porekar et al, 1999; Chant et al,2005; Amor-Mahjoub, 2006; Horchani et al, 2009; Kuo, W.H.K. and Chase. H.A., 2011).

The volumetric activity of β-galactosidase from *L. bulgaricus* in *L. plantarum* with the *erm*-based vector, noninduced peptide pheromone (IP-673) was 0.63 U/mL of fermentation broth for *LacZ*, and 0.51±0.04 U/mL of fermentation

broth for *LacZ*-His (Nguyen et al., 2011[a]) that these specific activities were lower than both the volumetric activity of β-galactosidase from cell-free extract of nontagged (307 U/ml) and of his-tagged (81 U/ml). This confirmed that there was the overexpression of β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* certainly. Additionally, the volumetric activity of crude enzyme in this study was similar to the volumetric activity of crude enzyme of the same stain in the *erm*-based system that was 193 U/mg protein in *LacZ* and 168 U/mg protein in *LacZ*-His. In terms of the specific activity of β-galactosidase from the crude enzyme of *L. plantarum* TLG02 with the *alr*-based system in this study (30 ml cell culture) was 22.3 U/mg protein of pSIP609-*lacZ* and 26.4 U/mg of pSIP60-*lacZ*-His. This result opposed a work of Nguyen and co-workers (2012) that reported the specific activity of β-galactosidase from the crude enzyme of *L. plantarum* WCFS1, harboring *LacZ* from *L. bulgaricus* with the *erm*-based system (15 mL cell culture) was 193 U/mg protein and 168 U/mg protein of *LacZ* and *LacZ*-His, respectively. The specific activity obtained the result of the β-galactosidase activity divided by the protein concentration, then, the volumetric activity of β-galactosidase with the *alr*-based system in this study was approximate the volumetric activity of β-galactosidase with the *erm*-based system, a work of Nguyen and co-workers (2012). Therefore, the different specific value was a result of the protein concentration that made the changing of specific activity. The presenting protein concentration in the crude enzyme of *L. plantarum* TLG02 from recombinant β-galactosidase with the *alr*-based system was from the overexpression of β-galactosidase as well as other protein, which expressed during the fermentation. The other protein expression could observe the dark bands that was not in the molecular weight range about 100-115 kDa. However, the volumetric activity, the

protein concentration and the specific activity in this chapter is the indication of β-galactosidase overexpression in a food-grade expression vectors, they must be studied again after the purification for further work.

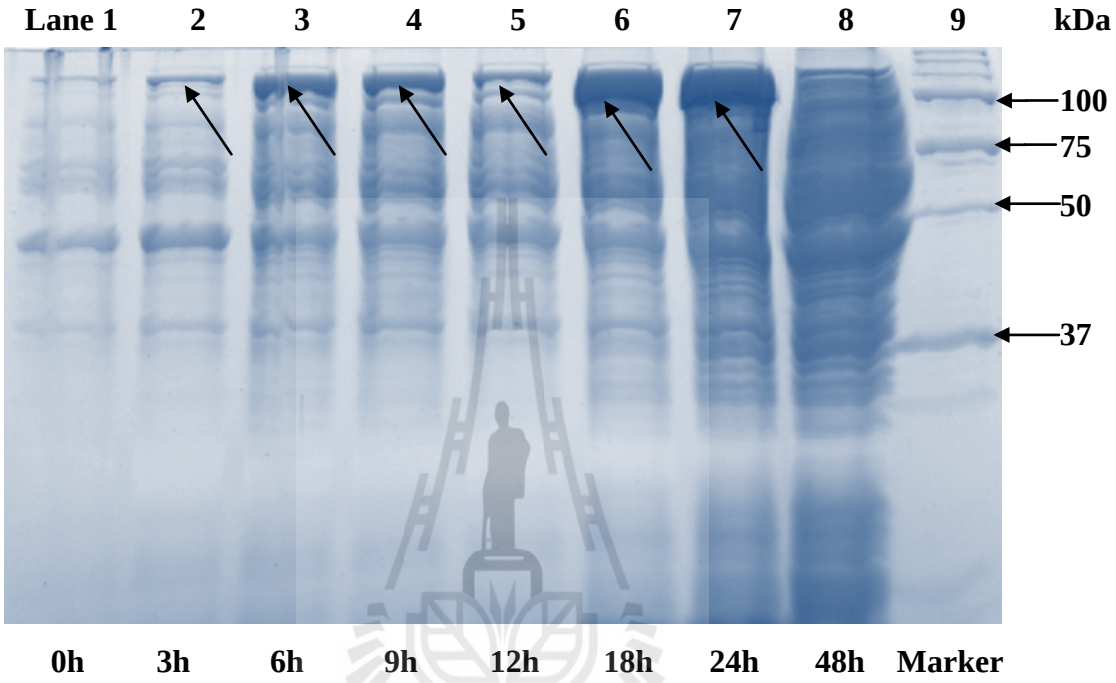


Figure 3.11 Expression of *L. plantarum* TLG02 harboring pSIP609-*lacZ* at different time intervals. The cell-free extracts from IP-673induced cells were analyzed by SDS-PAGE (Lane 1, non-induced by IP-673 counted as 0 hour; Lane 2, induced for 3 hours; Lane 3, induced for 6 hours; Lane 4, induced for 9 hours; Lane 5, induced for 12 hours; Lane 6, induced for 18 hours; Lane 7, induced for 24 hours; Lane 8, induced for 48 hours; Lane 9, Precision plus Protein standard ladder (Bio-Rad)). The arrows indicate the bands of expressed β-galactosidase. The gel was stained with Coomassie blue.

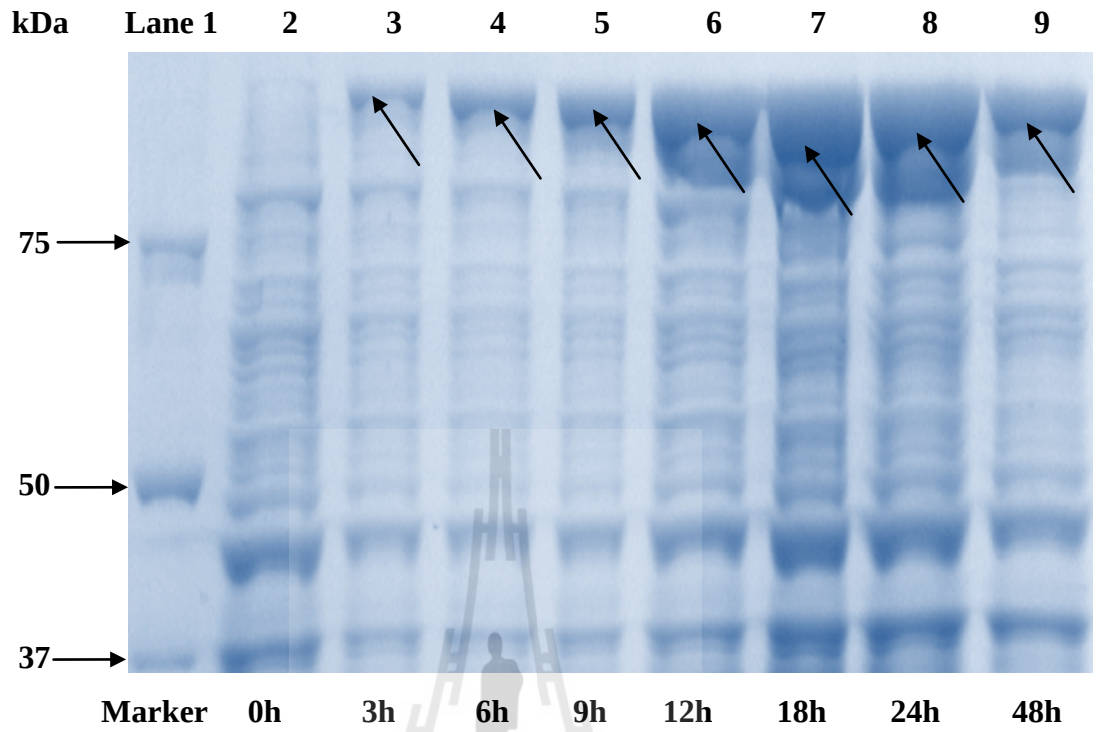


Figure 3.12 Expression of *L. plantarum* TLG02 harboring pSIP609-*lacZ*-His at different time intervals. The cell-free extracts fromIP-673 induced cells were analyzed by SDS-PAGE (Lane 1, Precision plus Protein standard ladder (Bio-Rad); Lane 2, non-induced by IP-673 counted as 0 hour; Lane 3, induced for 3 hours; Lane 4, induced for 6 hours; Lane 5, induced for 9 hours; Lane 6, induced for 12 hours; Lane 7, induced for 18 hours; Lane 8, induced for 24 hours; Lane 9, induced for 48 hours). The arrows indicate the bands of expressed β-galactosidase. The gel was stained with Coomassie blue.

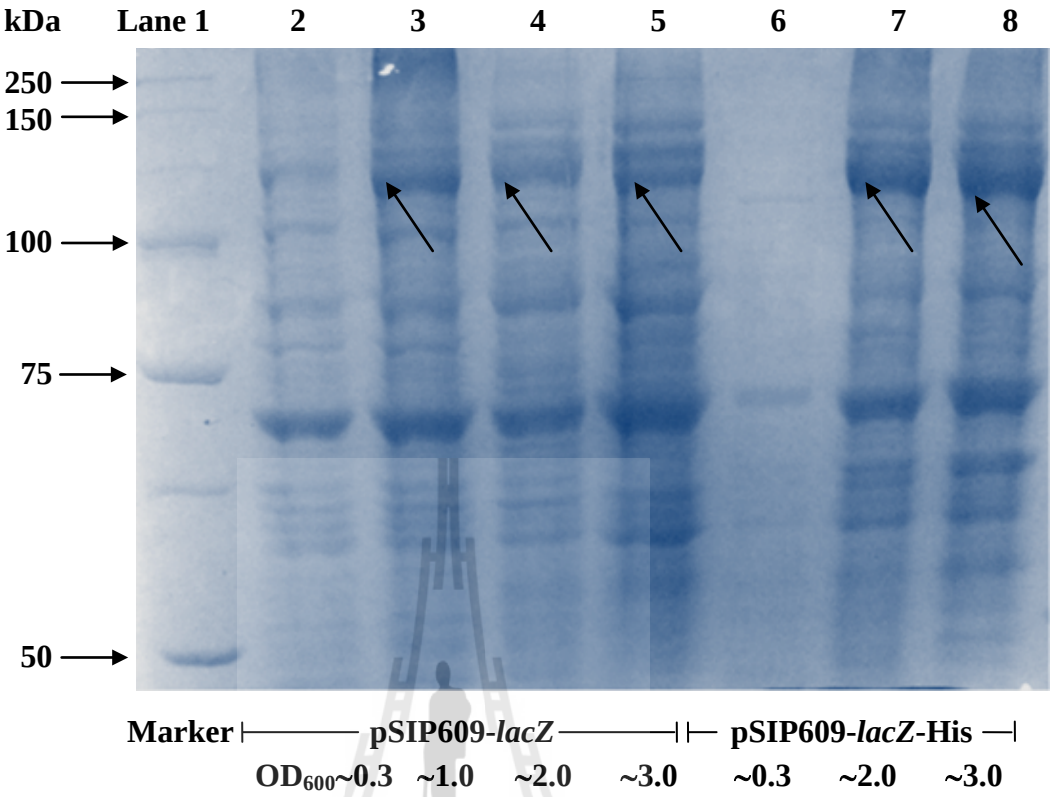


Figure 3.13 SDS-PAGE analysis of crude enzyme from different cell concentrations (OD_{600nm}~0.3, 1.0, 2.0, and 3.0) of *L. plantarum* TLG02 harboring pSIP609-*lacZ* (Lanes 2, 3, 4, 5), and pSIP609-*lacZ*-His (OD_{600nm}~0.3, 2.0, and 3.0; Lanes 6, 7, 8). Lane 1 shows the Precision plus Protein standard ladder (Bio-Rad). The arrows indicate the bands of expressed β-galactosidase. The gel was stained with Coomassie blue.

Table 3.1 Beta-galactosidase activity in crude enzymes of induced *L. plantarum* TLG02 harboring pSIP609-*lacZ* and pSIP609-*lacZ*-His by *o*NPG as a substrate.

Plasmid	β-galactosidase activity (U/mL)	protein concentration (mg/mL)	Specific activity (U/mg protein)
pSIP609-*lacZ*	307 ± 8	13.8 ± 0.9	22.3 ± 0.6
pSIP609-*lacZ*-His	81 ± 2	3.07 ± 0.1	26.4 ± 0.7

Note: Three hundred mL of cultured medium was a sample for β-galactosidase purification to determine the volumetric activity and the protein concentration. Experiments were performed in duplicate, and the standard deviation was <5%.

3.5 Conclusions

The expression plasmids pSIP609-*lacZ* and -*lacZ*-His were successfully constructed by replacing the *erm* gene with the *alr* gene from *L. plantarum*. This gene is derived from a GRAS organism and is an essential step towards food-grade production of proteins in *L. plantarum*. These constructed plasmids resulted in efficient overproduction of β-galactosidases when using the *alr*-based vectors. The expression of these crude enzymes from pSIP609-*lacZ* and -*lacZ*-His showed a high potential for applying to food industries for reduced-lactose dairy products.

3.6 References

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CHAPTER IV

BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT β-GALACTOSIDASE FROM *LACTOBACILLUS PLANTARUM*

4.1 Abstract

The recombinant β-galactosidase from *Lactobacillus delbruekii* subsp. *bulgaricus* DSM 20081 expressed in *L. plantarum* TLG02 as a food-grade host was determined for the overexpression, characterization, and lactose hydrolysis. The results showed that the overexpression of β-galactosidase had a molecular mass of ~110 kDa. The specific activity of the enzyme, using *o*NPG and lactose as substrates, was 212 and 165 U per mg protein, respectively. The volumetric activity of β-galactosidase from pSIP609-*lacZ* and pSIP609-*lacZ*-His was ~44,800 and ~45,800 U of fermentation medium, respectively. The β-galactosidase yield was ~240 mg/L of pSIP609-*lacZ* and ~277 mg/L of pSIP609-*lacZ*-His under the same cultivations (400 mL flask cultures). The optimal temperature of β-galactosidase when using the lactose and *o*NPG as the substrates was 55–60 and 50°C, respectively. The kinetic parameters at various temperatures (4-65°C) was determined in the milk buffer. The higest value of Michaelis-Menten constant (K_m) was approximately 5.6 mM at 30°C which the maximal velocity ($V_{max,Glc}$) at this temperature was

approximately 153 μmol·min^{-1}·mg^{-1}. The presence of Mg^{2+} in the buffer could extend the catalytic stability (half-life time) of the enzyme; however, its stability could rapidly lose activity beyond 45°C with or without Mg^{2+}.

Keywords: overexpression, characterization, the K_m, the catalytic efficiency, the half-life time, milk buffer

4.2 Introduction

Beta-galactosidases (lactase, EC 3.2.1.23) are important enzymes for dairy industry applications (Nakayama and Arnachi, 1999; Panesar et al., 2010; Nguyen et al., 2006, Splechtna et al., 2007; Nguyen et al., 2011[b]). The ability of β-galactosidases to convert lactose into galactose and glucose is used to prevent the crystallization of lactose, to improve sweetness, to increase the solubility of milk products, and to produce lactose-free food products (Sani et al., 1999; Nguyen et al., 2011[b]). Lactose hydrolysis is 75-80% efficient in industrial applications and about 90% efficient (or 5 g/L of lactose in lactose-hydrolyzed milk product) when ingested in pill form for lactose product consumption by lactose intolerant individuals (Prenosil et al., 1987; Hernandez and Asenjo, 1982; Matioli et al., 2003).

Beta-galactosidases from *Lactobacillus* spp. can be divided into two families, GH2 and GH42 (Cantarel et al., 2009; Nguyen et al., 2012). Beta-galactosidase, produced from *L. reuteri, L. acidophilus, L. pentosus, L. plantarum,* and *L. sakei*; have been classified in the GH2 family. Beta-galactosidases in this family from these microorganisms consists of two different subunits encoded by two overlapping genes *lacL* (large subunit) and *lacM* (small subunit) which are the β-galactosidases from

L. reuteri, *L. acidophilus*, *L. pentosus*, *L. plantarum*, and *L. sakei* (Nguyen et al., 2006; Nguyen et al., 2007; Iqbal et al., 2010; Maischberger et al., 2010; Iqbal et al., 2011; Nguyen et al., 2012). However, β-galactosidase from *L. bulgaricus* are encoded by a single gene, which is absent in lactobacilli, and mostly present in other lactic acid bacteria such as *Streptococcus* spp. or bifidobacteria such as *Bifidobacterium* spp. (Hung et al., 2001; Vaillancourt et al., 2002; Goulas et al., 2007; Nguyen et al., 2012).

Since β-galactosidases can be produced from various microorganisms, they typically have different biochemical properties, resulting in different rates of hydrolysis for specific glycosidic linkages (Nguyen et al., 2006). Differences in biochemical properties are also dependent upon substrate selection and hydrolysis reaction conditions. The biochemical properties of β-galactosidase from different *Lactobacillus* spp., including optimum temperature, metal ion requirement, and kinetic parameters (such as the Michealis-Menten constant; K_m, the maximal velocity; V_{max}, the turnover number; k_{cat}), are summarized in Table 4.1. The goals of this study were to overexpress and characterize the recombinant β-galactosidase from *L. delbruekii* subsp. *bulgaricus* DSM 20081 expressed in *L. plantarum* TLG02.

Table 4.1 Biochemical properties of β-galactosidases from *Lactobacillus* spp.

Source	Optimum temperature	kinetic parameter for lactose hydrolysis			Metal ion requirement	References
		K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})		
β-galactosidases from *L. reuteri* L103	50°C for *o*NPG hydrolysis 45°C for lactose hydrolysis	13±2	34	60	Na^+, K^+, Mg^{2+}, Mn^{2+}	Nguyen et al., 2006
β-galactosidases from *L. reuteri* L461	50°C for *o*NPG hydrolysis 50°C for lactose hydrolysis	31±5	33	58	Na^+, K^+, Mg^{2+}, Mn^{2+}	Nguyen et al., 2006
β-galactosidases from *L. acidophilus* R22	55°C (both lactose and *o*NPG hydrolysis)	4.0±0.3	28.8 ± 0.2	50.4	Mg^{2+}	Nguyen et al., 2007
β-galactosidases from *L. pentosus* KUB-ST10-1	55°C for *o*NPG hydrolysis 60-65°C for lactose hydrolysis	38	11.3 ± 0.8	20	Mg^{2+}	Maischberger et al., 2010
β-galactosidases from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, expressed in *E. coli* ER2566	30-50°C (substrate not specified)	0.98 (at pH 6.5)	57.5	45.7 (at pH 6.5)	Mn^{2+}, Co^{2+}	Rhimi et al., 2009

Table 4.1 Biochemical properties of β-galactosidases from *Lactobacillus* spp. (continued)

Source	Optimum temperature	kinetic parameter for lactose hydrolysis			Metal ion requirement	Reference
		K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})		
β-galactosidases from *L. plantarum* WCFS1, expressed in *L. plantarum*	55°C for *o*NPG hydrolysis 60°C for lactose hydrolysis	29±1	12.9 ± 0.5	98	Na^+, K^+, Mg^{2+}, Mn^{2+}	Iqbal et al., 2010
His-tagged β-galactosidases from *L. sakei* Lb790, expressed in *E. coli*	55°C (both *o*NPG and lactose hydrolysis)	20±2	24±1	43±1	Mg^{2+}	Iqbal et al., 2011
His-tagged β-galactosidases from *L. delbrueckii* subsp. *bulgaricus* DSM 20081, expressed in *L. plantarum* WCFSl with the *erm*-based vectors	55°C (both *o*NPG and lactose hydrolysis)	19.9±4	111±4	211±10	Na^+, K^+, Mg^{2+}	Nguyen et al., 2012
β-galactosidases from *L. kefiranofaciens* ZW3, expressed in *E. coli* BL21(DE3)	50°C for *LacLM* for *o*NPG hydrolysis	1.61±0.07	2.44±0.11	2.96	Mg^{2+}	He et al., 2016

4.3 Materials and methods

4.3.1 Bacterial strains and media

L. plantarum TLG02 harboring pSIP609(*alr*)-*lacZ* and pSIP609(*alr)*-*lacZ*-His were grown in MRS medium (Oxoid, Basingstoke, U.K.) at 37°C in incubator (Memmert, BE 500, WTB Binder BD115) without agitation.

4.3.2 Expression of β-galactosidases

Overnight cultures of *L. plantarum* harboring pSIP609(*alr*)-*lacZ* and pSIP609(*alr*)-*lacZ*-His were diluted in fresh, pre-warmed MRS medium to reach a cell concentration of OD_{600nm}~0.1, determined the absorbance at 600 nm by spectrophotometer (Ultrospec 2000, Pharmacia biotech, Cambridge, UK). The cultured medium was then incubated at 30°C in incubator (Memmert, BE 500, WTB Binder BD115) until the OD_{600nm} reached at ~0.3. Then, expression of recombinant protein was induced by adding 25 ηg/mL peptide pheromone IP673 (supplied by Food Biotechnology Laboratory, Department of Food Sciences and Technology, BOKU University of Natural Resources and Life Sciences, Vienna, Austria). Forty mL of induced cells were harvested periodically (0, 3, 6, 9, 12, 15, 18, 21 and 24 hours) until a final cell culture concentration of OD_{600nm} at 3.0 was reached. The harvested cells were pelleted by the centrifuge machine (Eppendorf centrifuge 5810R, Eppendorf, USA) at 3500 g for 10 minutes at 4°C. They were washed with 50 mM sodium phosphate buffer (pH 6.5) and resuspended in 500 μL of the same buffer. The cells were disrupted to obtain the cell lysate as described by Nguyen and co-workers (2011)[a]. The lysate was centrifuged by the centrifuge machine (Thermoscientific, Sorvall legend XTR centrifuge, USA) at 9000 g (4°C) for 5 minutes to get the crude enzymes used for determination of activity assays and

protein concentration using the denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.3.3 Fermentation and protein purification

L. plantarum TLG02 harboring pSIP609-*lacZ*-His was cultivated in 400 mL of fermentation medium in order to obtain sufficient material for purification of *LacZ*. The bacteria were grown in MRS medium in a 500 mL reagent bottle loosely closed with screw-cap at 30°C in incubator (Memmert, BE 500, WTB Binder BD115). Precultured bacteria were grown overnight in 40 mL MRS medium and inoculated into 400 mL fresh pre-warmed MRS medium at the cell concentration (OD_{600}) of 0.1 until the OD_{600nm} reached at ~0.3. Then, expression of recombinant protein was induced by adding 25 ηg/mL peptide pheromone IP673. The cultures were incubated until a final cell culture concentration of OD_{600nm} was about 2.0 - 3.0. The induced cells were harvested and washed twice with 50 mM sodium phosphate buffer (pH 6.5). After centrifugation, cells were disrupted by using a French press (Aminco, Silver Spring, MD), and debris was removed by the centrifugation (Thermoscientific, Sorvall legend XTR centrifuge, USA) at 12000 g, 30 minutes, 4°C. The recombinant enzyme was purified by immobilized metal affinity chromatography using a Ni-Sepharose column (GE Healthcare, Uppsala, Sweden; supplied by Food Biotechnology Laboratory, Department of Food Sciences and Technology, BOKU University of Natural Resources and Life Sciences, Vienna, Austria) as Nguyen and co-workers (2006). Purified enzymes were then eluted in 50 mM sodium phosphate buffer, pH 6.5, at 4°C.

4.3.4 Protein determination

Proteins concentration was determined by Quick start™ Bradford protein assay (Bio-Rad Laboratories, CA, USA) using bovine serum albumin as a standard. The dye reagent was mixed at 1 to 4 with DI water and filtered. The properly diluted samples (60 µL) were mixed with 200 µl of dye reagent and stood at room temperature for 2 minutes. The absorbance at 595 nm was measured by ELISA reader (Sunrise, TECAN, Austria). The protein concentration was calculated from the following relationship (Bradford et al., 1976):

$$\text{Protein concentration (mg/mL)} = \frac{OD_{595}}{k} \times \text{Dil}$$

Where OD_{595} is the sample-measured absorption at 595 nm, k is the slope of the BSA standard curve, and Dil is the dilution factor, after correcting for the blank.

4.3.5 Enzyme Assays

4.3.5.1 Assay with chromogenic glycoside

ortho-Nitrophenyl-β-D-galactopyranoside (*o*NPG) from Sigma-Aldrich (CA, USA) was used as the chromogenic substrate. To assay the β-galactosidase activity, the substrate solution was prepared from mixing 22 mM *o*NPG into 50 mM sodium phosphate buffer (pH 6.5). Twenty µL of enzyme solution was added into 480 µL of the substrate solution. Then, the mixture was incubated at 30°C for 10 minutes with 600 rpm speed agitation in dry bath shaking incubator (HB1, Wealtee Corp., USA). After the incubation time, the reaction was stopped by adding 750 µL of 0.4 M Na_2CO_3. The release of *ortho*-nitrophenol (*o*NP) was assessed by determining the absorbance at 420 nm by spectrophotometer (Ultrospec

2000, Pharmacia biotech, Cambridge, UK). 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. The β-galactosidase activity or the volumetric activity was calculated from the following relationship (Nguyen et al., 2006):

$$\text{β-galactosidase activity or Volumetric activity (U/mL)} = \frac{OD_{420}}{k} \times \frac{1}{t} \times \frac{V_{oNPG} + V_{enzyme}}{V_{enzyme}} \times \text{Dil}$$

Where OD_{420} is the sample-measured absorption at 420 nm, k is the slope of the *o*NP standard curve, t is the reaction time (10 minutes), V_{oNPG} is the volume of *o*NPG (480 µL), V_{enzyme} is the volume of enzyme sample (20 µL), and Dil is the dilution factor, after correcting for the blank.

4.3.5.1 Assay with Lactose as substrate

Lactose was used as the substrate in this assay. Twenty µL of enzyme solution was added to 480 µL of a 600 mM lactose solution in 50 mM sodium phosphate buffer (pH 6.5). This reaction mixture was incubated at 30°C for 10 minutes and shaking at 600 rpm in dry bath incubator (HB1, Wealtee Corp., USA). After 10 minutes, the reaction was heat-inactivated at 99°C for 5 minutes. The sample had been cooled to room temperature and then, the release of D-glucose was assessed colorimetrically using the enzymatic assay of glucose oxidase (GOD/POD assay) following a wok of Kunst and co-workers (1988) by adding 60 µL of reaction mixture into 600 µL of a solution containing GOD (94 µg/mL; Sigma-Aldrich, CA, USA), POD (6.1 µg/mL; Sigma-Aldrich, CA, USA), 4-aminoantipyrine (157 µg/mL; Sigma-Aldrich, CA, USA), and phenol (1.95% v/v; Merck Ltd.) in 50 mM sodium phosphate buffer (pH 6.5). This assay mixture (660 µL) was incubated in a dark room at ambient

temperature for 40 minutes, and the absorbance at 505 nm was measured by spectrophotometer (Ultrospec 2000, Pharmacia biotech, Cambridge, UK). One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. All measurements and experiments were performed at least in duplicate, and the experimental error was less than 5%. The glucose concentration (*C*) was defined as follows (Kunst *et al*., 1988):

$$C\,[\mathrm{g/l}] = \frac{OD_{505}}{k} \times (\text{sample dilution factor})$$

$$C\,[\mathrm{mM}] = \frac{C\,(\mathrm{g/l})}{Mr_{glucose}} \times 1000$$

Where OD_{505} is the sample-measured absorption at 505 nm, k is the slope of the glucose standard curve, and $Mr_{glucose}$ is the molecular mass of glucose (180.16 g/mol), after correcting for the blank.

The β-galactosidase activity was calculated from the following relationship (Nguyen et al., 2006):

$$\begin{matrix}\text{β-galactosidase activity}\\ \text{or}\\ \text{volumetric activity (U/mL)}\end{matrix} = C\,[\mathrm{mM}] \times \frac{1}{t} \times \frac{(V_{lactose} + V_{enzyme})}{V_{enzyme}} \times \mathrm{Dil}$$

Where *C* [mM] is the glucose concentration (mM), t is the reaction time (10 minutes), $V_{lactose}$ is the volume of lactose (480 µL), V_{enzyme} is the volume of enzyme sample (20 µL), and Dil is the enzyme dilution factor, after correcting for the blank.

The specific activity was calculated from the following relationship (Nguyen et al., 2006):

$$\text{Specific activity (U/mg protein)} = \frac{\text{β-galactosidase activity (U/mL)}}{\text{Protein concentration (mg protein/mL)}}$$

4.3.6 Characterization of recombinant β-galactosidase

4.3.6.1 Optimal temperature

The temperature dependence of β-galactosidase activity was assessed by measuring activity in the range of 20−90°C for 10 minutes in order to achieve the optimal temperature of β-galactosidase. Twenty two mM of *o*NPG and 600 mM of lactose were used as a substrate for detecting the enzyme activity. This was performed in a sodium phosphate buffer (pH6.5) or milk buffer (pH 6.5). Milk buffer consists of KCL + $MgSO_4 \cdot 7H_2O$ + $NaH_2PO_4 \cdot H_2O$ + $CaSO_4 \cdot 2H_2O$ + $CaCl_2 \cdot 2H_2O$ + Citric acid at 1.10 + 0.71 + 1.87 + 1.00 + 0.99 + 2.00 g/L, respectively (Gutierrez et al., 2002). The measured activities were compared with the blank standard reaction.

4.3.6.2 Steady-state kinetic measurements

The steady-state kinetic measurements were studied at 8, 30, 37, 45, 50, 55, 60 and 65°C in milk buffer, pH 6.5 (Gutierrez et al., 2002) with the lactose concentrations ranging from 0 to 600 mM of lactose. The enzyme activity of lactose hydrolysis was calculated by the SigmaPlot (SPSS, Chicago, IL) following the Michaelis-Menten equation, consequencing in the V_{max} and K_m. These values were calculated to provide the k_{cat} and the catalytic efficiency (k_{cat}/K_m).

4.3.6.3 Catalytic stability

The catalytic stability of β-galactosidase using *o*NPG and lactose as the substrates was determined by incubating the enzyme in a milk buffer (Gutierrez et al., 2002) at various temperatures (8, 30, 37, 45, 50 and 55°C) and by subsequent measurements of the remaining enzyme activity *(A)* at various time points *(t)*. Residual activities (A_t/A_0, where A_t is the activity measured at time t and A_0 is the initial activity) were plotted versus the incubation time. The inactivation constants k_{in} were obtained by line arregression of ln (activity) versus time. The half-life values of thermal inactivation $\tau_{1/2}$ were calculated using $\tau_{1/2} = \ln 2/k_{in}$ (Nguyen et al., 2012).

To study the effect of various cations on β-galactosidase activity, the enzyme was assayed with 22 mM *o*NPG and 600 mM lactose in 50 mM sodium phosphate buffer, containing different Mg^{2+} concentrations (0, 1, and 10 mM of Mg^{2+}) and in milk buffer as the substrates. The measured activities were compared with the blank standard reaction, which shared identical conditions but without cations.

4.3.7 Statistical analyses

All experiments and measurements were performed at least in duplicate. Analysis of variance (ANOVA) was determined using SPSS program (SPSS version 16, Windows version). Duncan Multiple Range Testing (DMRT) was used to compare the differences among the means at $P < 0.05$.

4.4 Results and discussion

4.4.6 Fermentation of β-galactosidase

The cell concentration of *L. plantarum* TLG02, harboring pSIP609-*lacZ* and pSIP609-*lacZ*-His from *L. delbrueckii* subsp. *bulgaricus* DSM 20081, was measured over a period of 0 to 24 hours. The cell pellet samples in each period time were prepared for the crude enzymes. These crude enzymes were analyzed by SDS-PAGE and their specific bands were about 110 kDa in both pSIP609-*lacZ* (Figure 4.1) and pSIP609-*lacZ*-His (Figure 4.2).

Overexpression of recombinant *β*-galactosidase at ~110 kDa in this study was consistent with those of three other studies conducted via different expression systems. These studies included cold-sensitive mutants of β-galactosidase from *L. delbruckii* subsp. *bulgaricus* (Adams et al., 1994), β-galactosidase from *L. bulgaricus* expressed in *E.coli* ER2566 (Rhimi et al., 2009), and β-galactosidase from *L. bulgaricus* DSM 20081 expressed in *L. plantarum* WCFS1 as a food grade host (Nguyen et al., 2012).

The relationship of volumetric activity and cell concentration of *L. plantarum* TLG02, harboring pSIP609–*lacZ* and pSIP609-*lacZ*-His, was also studied at different time intervals. This microorganism exhibited the highest volumetric activity at 18 hours. However, their volumetric activity decreased rapidly after 18 hour in both pSIP609–*lacZ* and pSIP609-*lacZ*-His (Figure 4.3 and 4.4). Moreover, at 24 hours, this microorganism showed the less value of the volumetric activity, indicating the cells could not grow after 24 hours. Consistent with this study, Nguyen and co-workers (2011)[a] reported that *L. plantarum*, harboring *LacLM* from *L. reuteri* with the *alr*-based vectors, was unable to grow after 24 hours at 37°C.

The results of cell concentration, pH, volumetric activity and protein concentrations from the expression of β-galactosidase with different genes are summarized in Table 4.2 and 4.3. The *L. plantarum* TLG02 in this study could provide the highest protein concentration at 12 hours. This result was consistent with the cell concentration as a function of the increasing time. The pH values of cultured medium were rapidly decreased from pH 5.3–pH 4.7 within 9 hours. However, the pH had gradually decreased to 4.3 at the end of fermentation process. The decreasing of pH values was found in both pSIP609–*lacZ* and pSIP609-*lacZ*-His gene. The changing of cell concentration, volumetric activity, protein concentrations, and pH can be explained by the behavior of lactic acid bacteria. The more lactic acid bacteria grew, the less pH was found. The pH of the cultured medium decreased as the accumulation of organic acids. Not only the decrease of pH could slow down the growth of such bacteria growth but also the cells of bacteria could be destroyed, consequencing in the loss of cell viability (Hutkins and Nannen, 1993).

For the volumetric activity in terms of enzyme production of crude enzyme incubated 400 mL of cultured medium for 18 hours, it showed that the volumetric activity of crude enzyme from pSIP609-*lacZ* and pSIP609-*lacZ*-His was ~44,800±1,700 and 45,800±1,200 U of β-galactosidase activity per liter of medium, respectively (Table 4.4). The β-galactosidase yield was 240±6 mg/L of pSIP609-*lacZ* and ~277±4 mg/L of pSIP609-*lacZ*-His under the same cultivations. A work of Nguyen and co-workers (2012) reported that the volumetric activity of β-galactosidase from *L. bulgaricus* DSM 20081 with the *erm*-based was ~53,000±2,000 U of β-galactosidase activity per liter of medium and ~170 mg of recombinant protein per liter. While Halbmayr and co-workers (2008) showed that β-galactosidase from

L. plantarum WCFS1 harboring the plasmid pEH3R containing the *lacLM* gene from *L. reuteri* L103 was ~23,000 U of β-galactosidase activity per liter of medium and 100 mg of recombinant protein per liter. Whereas, β-galactosidase from *L. plantarum* WCFS1 harboring the plasmid pEH9R containing the *lacLM* gene from *L. reuteri* L103 was ~35,000-40,000 U of β-galactosidase activity per liter of medium and 200 mg of recombinant protein per liter (Nguyen et al., 2015).

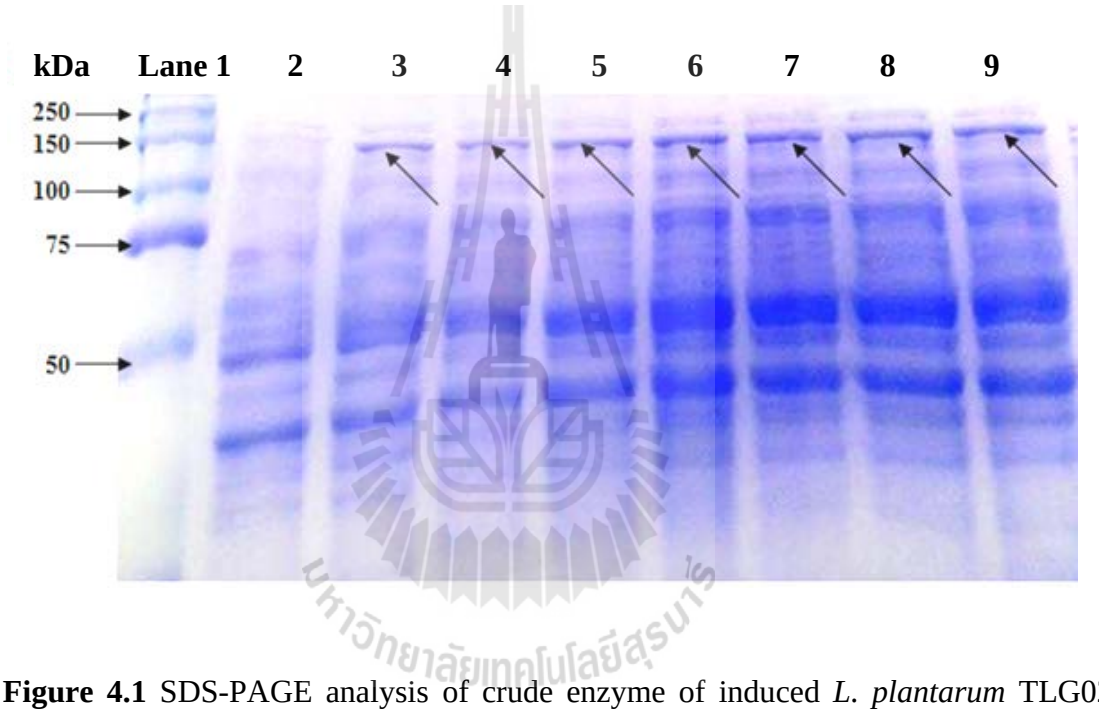


Figure 4.1 SDS-PAGE analysis of crude enzyme of induced *L. plantarum* TLG02 harboring pSIP609-*lacZ* from 40 mL of cell cultured (Lane 1, Precision plus Protein standard ladder (Bio-Rad); Lane 2, non-induced by IP-673 counted as 3 h; Lane 3, induced for 6 h; Lane 4, induced for 9 h; Lane 5, induced for 12 h; Lane 6, induced for 15 h; Lane 7, induced for 18 h; Lane 8, induced for 21 h; Lane 9, induced for 24 h).

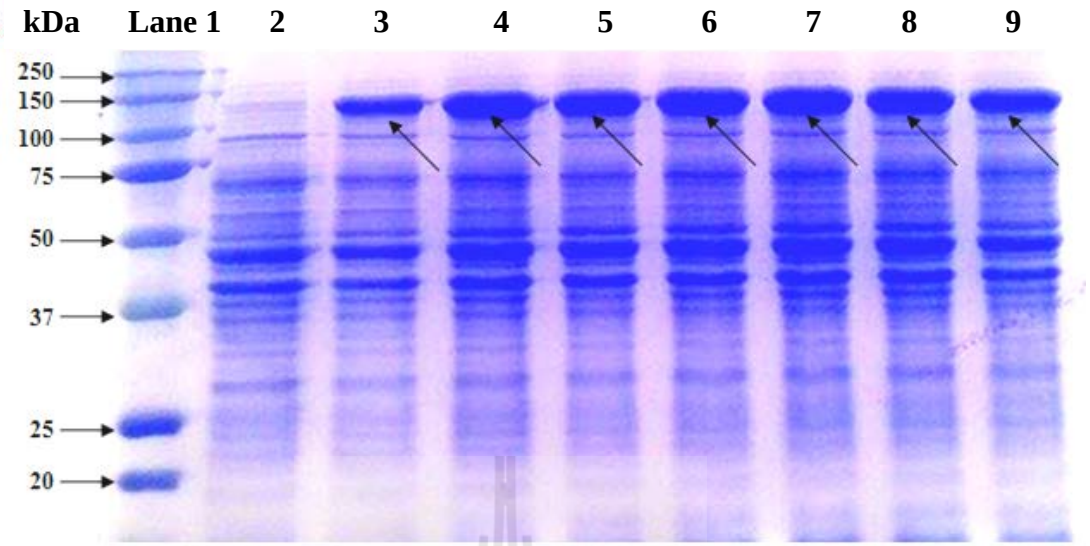


Figure 4.2 SDS-PAGE analysis of crude enzyme of induced *L. plantarum* TLG02 harboring pSIP609-*lacZ*-His from 40 mL of cell cultured (Lane 1, Precision plus Protein standard ladder (Bio-Rad); Lane 2, non-induced by IP-673 counted as 0 h; Lane 3, induced for 6 h; Lane 4, induced for 9 h; Lane 5, induced for 12 h; Lane 6, induced for 15 h; Lane 7, induced for 18 h; Lane 8, induced for 21 h; Lane 9, induced for 24 h)**.**

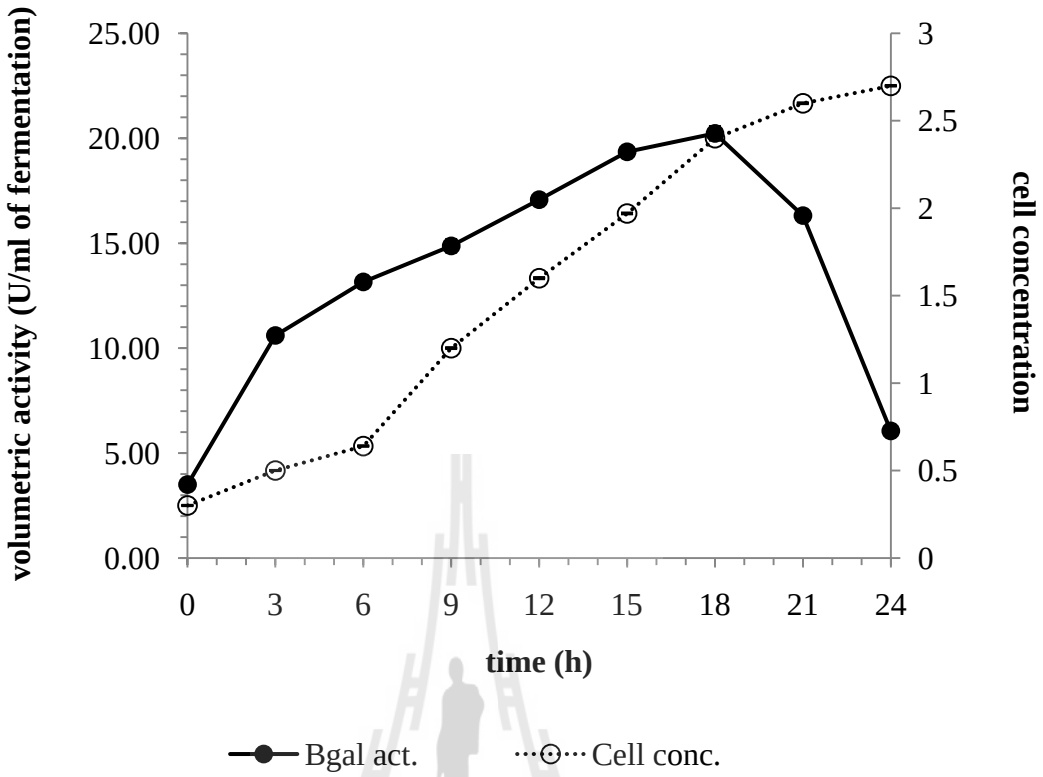


Figure 4.3 The relationship between the volumetric activity and the concentration of induced *L. plantarum* TLG02 harboring pSIP609-*lacZ* (U/L of fermentation): (O) cell concentration (OD_{600}); (●) volumetric activity (U/L of fermentation). Lactose was used as a substrate.

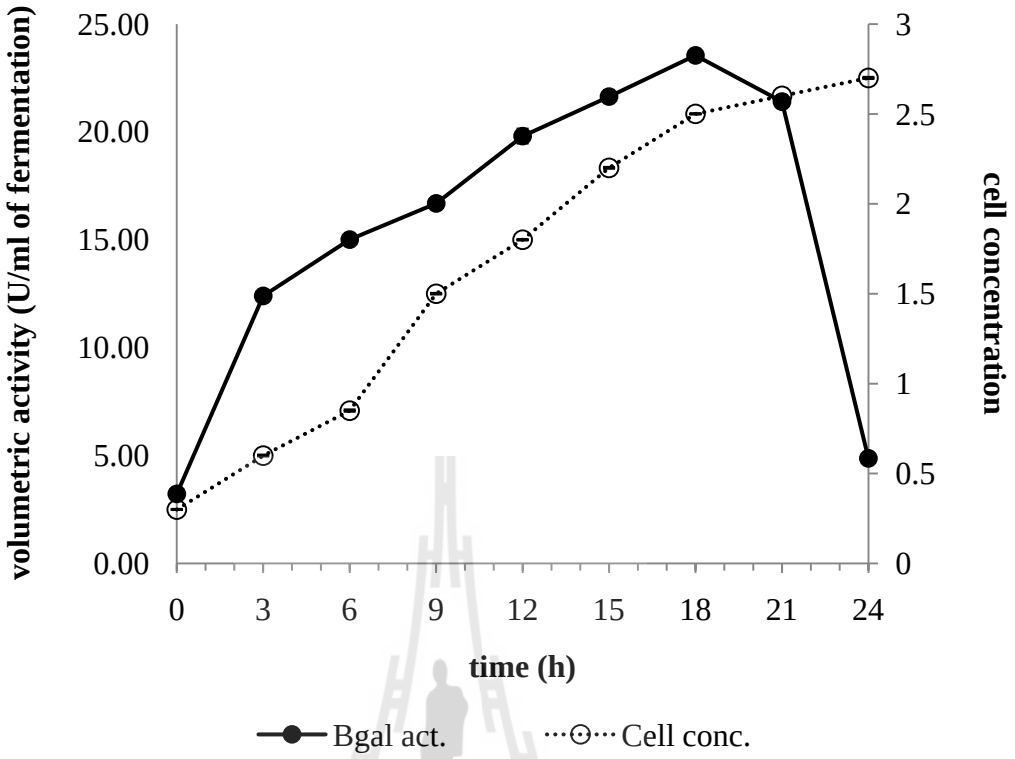


Figure 4.4 The relationship between the volumetric activity and the concentration of induced *L. plantarum* TLG02 harboring pSIP609-*lacZ*-His (U/L of fermentation): (O) cell concentration (OD_{600}); (●) volumetric activity (U/L of fermentation). Lactose was used as a substrate.

Table 4.2 The variation of induced *L. plantarum* TLG02 harboring pSIP609-*lacZ* concentration, pH, volumetric activity and protein concentration at different times

Time (h)	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Cell concentration (OD_{600})	0.30±2E-03^{a}	0.50±2E-03^{b}	0.64±3E-03^{c}	1.2±4E-03^{d}	1.6±4E-03^{e}	1.97±3E-03^{f}	2.4±5E-03^{g}	2.6±3E-03^{h}	2.7±3E-03^{i}
volumetric activity (U/mL of fermentation medium)	3.50±0.06^{a}	10.6±0.22^{c}	13.2±0.14^{d}	14.9±0.30^{e}	17.1±0.06^{f}	19.4±0.03^{g}	20.2±0.33^{h}	16.32± 0.19^{i}	6.06±0.18^{b}
Protein concentration (mg/mL)	0.72±5E-03^{c}	0.72±5E-03^{c}	0.70±4E-03^{c}	0.83±1E-03^{d}	1.1±9E-03^{f}	0.93±7E-03^{e}	0.92±6E-03^{e}	0.56±1E-02^{b}	0.36±1E-03^{a}
pH	5.31^{g}	5.13^{f}	4.98^{e}	4.74^{d}	4.65^{c}	4.57^{c}	4.38^{b}	4.33^{a}	4.32^{a}

Note: Forty mL of cultured medium was a sample for determining the volumetric activity and the protein concentration. Experiments were performed in duplicate, and the standard deviation was <5%. Values in each time period marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

Table 4.3 The variation of *L. plantarum* TLG02 harboring pSIP609-*lacZ*-His concentration, pH, volumetric activity and protein concentration at different times.

Time (h)	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Cell concentration (OD_{600})	0.3±2E-03[a]	0.6±4 E-03[b]	0.85±5 E-3[c]	1.5±3 E-03[d]	1.8±3 E-03[e]	2.2±3E-03[f]	2.5±2 E-03[g]	2.6±3E-03[g]	2.7±4E-03[h]
Volumetric activity (U/mL of fermentation medium)	3.22±0.04[a]	12.4±0.2[c]	15.0±0.2[d]	16.7±0.1[e]	19.8±0.3[g]	21.6±0.3[h]	23.6±0.1[i]	21.4±0.3[h]	4.87±0.2[b]
Protein concentration (mg/mL)	0.61±1 E-02[c]	0.53±4 E-01[b]	0.85±1 E-2[df]	0.80±3 E-2[df]	0.88±3 E-03[f]	0.79±7 E-03[d]	0.77±1 E-02[d]	0.78±3 E-02[d]	0.07±9 E-03[a]
pH	5.28[e]	5.1[e]	4.87[e]	4.66[d]	4.64[d]	4.61[d]	4.37[c]	4.32[b]	4.32[a]

Note : Forty mL of cultured medium was a sample for determining the volumetric activity and the protein concentration. Experiments were performed in duplicate, and the standard deviation was <5%. Values in each time period marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

Table 4.4 Comparative study of volumetric activity (*o*NPG assay) and protein concentration of crude enzyme from *L. plantarum* TLG02 harboring pSIP609-*lacZ* and pSIP609-*lacZ*-His detecting at the end of fermentation at 18 hours with other β-galactosidases from *Lactobacillus* spp.

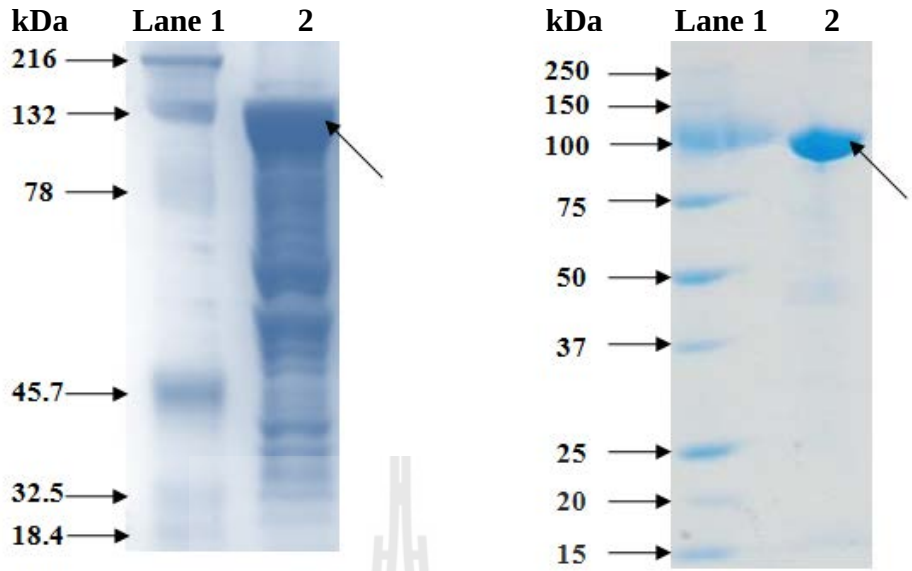
Vector	**Volumetric activity (U of β-galactosidase activity per liter of medium)**	**β-galactosidase yield (mg/L)**	**Reference**
pEH3R-*lacLM* (*E.coli* as a host strain)	~23,000	100	Halbmayr et al., 2008
pSIP409-lacZ (*erm*-based vector, *L. plantarum* WCFS1 as a host strain)	~53,000±2,000	~170	Nguyen et al., 2012
pEH9R-*lacLM* (*E.coli* as a host strain)	~35,000-40,000	200	Nguyen et al., 2015
pSIP609-*lacZ* (*alr*-based vector, *L. plantarum* TLG02 as a host strain)	44,800±1,700	240±6	this study
pSIP609-*lacZ*-His (*erm*-based vector, *L. plantarum* TLG02 as a host strain)	45,800±1,200	277±4	this study

Note: Four hundred mL of cultured medium was a sample for β-galactosidase purification to determine the volumetric activity and the protein concentration. Experiments were performed in duplicate, and the standard deviation was <5%. Values in each category (volumetric activity and protein concentration) marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

4.4.2 The purification of *L. plantarum* TLG02 harboring pSIP609-*lacZ* and pSIP609-*lacZ*-His

The crude enzyme from *L. plantarum* TLG02 harboring pSIP609-*lacZ* and the purified enzyme from *L. plantarum* TLG02 harboring pSIP609-*lacZ*-His were analyzed by SDS-PAGE and showed specific bands of ~110 kDa as shown in Figure 4.5 and Table 4.5. This finding is consistent with the works of Nguyen and co-workers (2012), Rhimi and co-workers (2009), and Adams and co-workers (1994).

The β-galactosidase activity of crude and purified enzymes using *o*NPG as a substrate was about 560±7 and 304±4 U/mL, respectively. The protein concentration was about 3.67±0.14 mg/mL of crude enzyme and 1.37±0.03 mg/mL of purified enzyme. The specific activity of crude and purified enzymes was about 78±2 U/mg and 218±8 U/mg when *o*NPG was used as a substrate. This means that the specific activity of purified enzyme was higher than that of crude enzyme around 2.72 fold (Table 4.5).



(A) (B)

Figure 4.5 SDS-PAGE analysis of crude enzyme of induced *L. plantarum* TLG02 harboring pSIP609 (A) -*LacZ*-His (Lane 1, Precision plus Protein standard ladder (Bio-Rad); Lane 2, crude enzyme); and (B) the purified recombinant β-galactosidase (Lane 1, Precision plus Protein standard ladder (Bio-Rad); Lane 2, β-galactosidase).

Table 4.5 Purification of β-galactosidase from *L. plantarum* TLG02 harboring pSIP609-*lacZ-His* when *o*NPG and lactose were used as the substrates.

Sample	**Volume of fermentation medium (mL)**	**Total activity (U/mL)**	**Total protein (mg/mL)**	**specific activity (U/mg)**	**Purified factor**	**Recovery (%)**
Crude enzyme	400	560± 7 (release of *o*NP)^{c}	3.67 ± 0.14^{b}	78 ± 2^{a}	1	100
Purified enzyme	5	304 ± 4 (release of *o*NP)^{b}	1.37 ± 0.03^{a}	212 ± 8^{b}	2.72	1.25
		226 ± 3 (release of D-glucose)	1.37 ± 0.03^{a}	165 ± 5	-	-

Note : Four hundred mL of cultured medium was a sample for β-galactosidase purification to determine the volumetric activity and the protein concentration. Data are shown as the average ± standard deviation of duplicate independent cultivations. Values in each category (volumetric activity and protein concentration) marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

4.4.3 The characterization of recombinant β-galactosidase

4.4.3.1 Optimal temperatures of a recombinant β-galactosidase using different buffers and substrates

The optimal temperature of recombinant β-galactosidase was studied in different buffers (milk buffer and sodium phosphate buffer) both in *o*NPG and lactose as the substrates (Figure 4.6 and 4.7). The optimal temperature was 50°C when using *o*NPG as a substrate both in milk buffer and in sodium phosphate buffer. When lactose was used as a substrate, the optimal temperature was at 60°C in milk buffer and 55°C in sodium phosphate buffer.

This result agrees with Nguyen and co-workers (2006), reporting that the optimal temperature of β-galactosidase from *L. reuteri* for *o*NPG hydrolysis was ~50°C. Similarly, the optimal temperatures of β-galactosidases for lactose hydrolysis from native cells of *L. pentosus* KUB-ST10-1 and the recombinant β-galactosidases from *L. plantarum* WCFS1 expressed in *L. plantarum* were 60°C (Maischberger et al, 2010; Iqbal et al., 2010). Also, the optimal temperatures of β-galactosidases for lactose hydrolysis from *L. acidophilus* R22, recombinant β-galactosidases from *L. sakei* Lb790 in *E. coli* expression system, and recombinant β-galactosidases from *L. delbrueckii* subsp. *bulgaricus* DSM 20081 in *erm*-based vectors were 55°C (Nguyen et al, 2007; Iqbal et al., 2011; Nguyen et al, 2012). The optimal temperature of β-galactosidases from *Lactobacillus* spp. was 50-60°C regardless of different substrates, different strains of *Lactobacillus* spp., native cells, recombinant cells, or different expression systems. However, each of these studies was conducted using only a pH of 6.5 to imitate the pH of milk during the lactose-

hydrolyzing process. Thus, the using of milk buffer did not affect the optimal temperature of β-galactosidase from *L. bulgaricus* with *alr*-based vectors in this study.

The optimal temperatures of β-galactosidase in both *o*NPG and lactose substrates from literatures showed slightly different values. This variance can be explained by the different products and their respective methods of measurement between these two hydrolysis reactions. *o*NP, a product from *o*NPG hydrolysis, can only be absorbed at 420 nm. However, D-glucose, a product of lactose hydrolysis, is measured by glucose oxidase and peroxidase assay (GOD/POD assay). A colored product from GOD/POD assay is absorbed at a wavelength between 470-550 nm, a broader range of wavelengths than that of *o*NP absorption (Kunst et al., 1988), resulting in higher values for measured β-galactosidase activity in lactose hydrolysis.

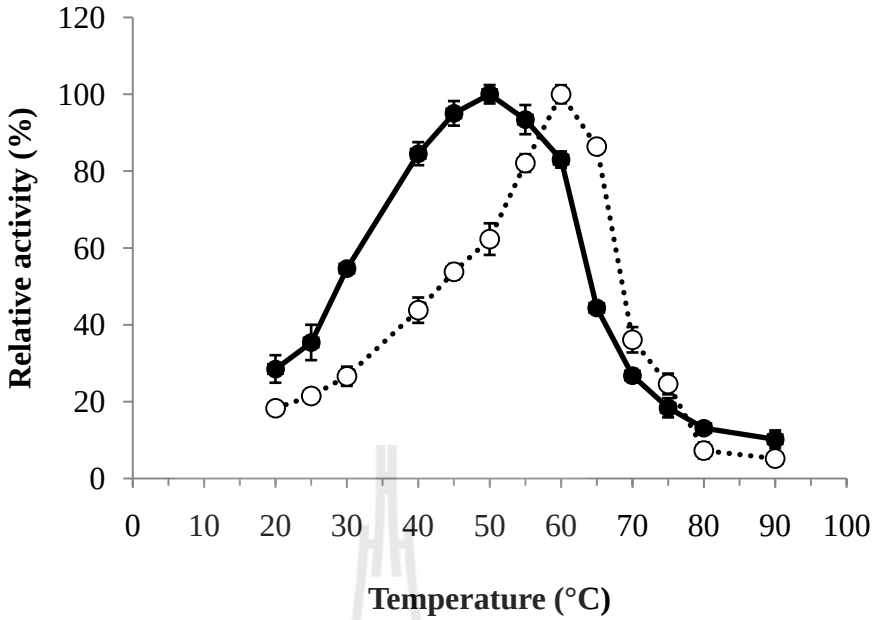


Figure 4.6 Optimal temperature of the activity of recombinant β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* DSM 20081 in milk buffer: (O) lactose as substrate; (●) *o*NPG as substrate. Relative activities are given in comparison with maximum activities calculated under optimal conditions (100%), which were 510 and 346 U/mL with *o*NPG and lactose used as substrates.

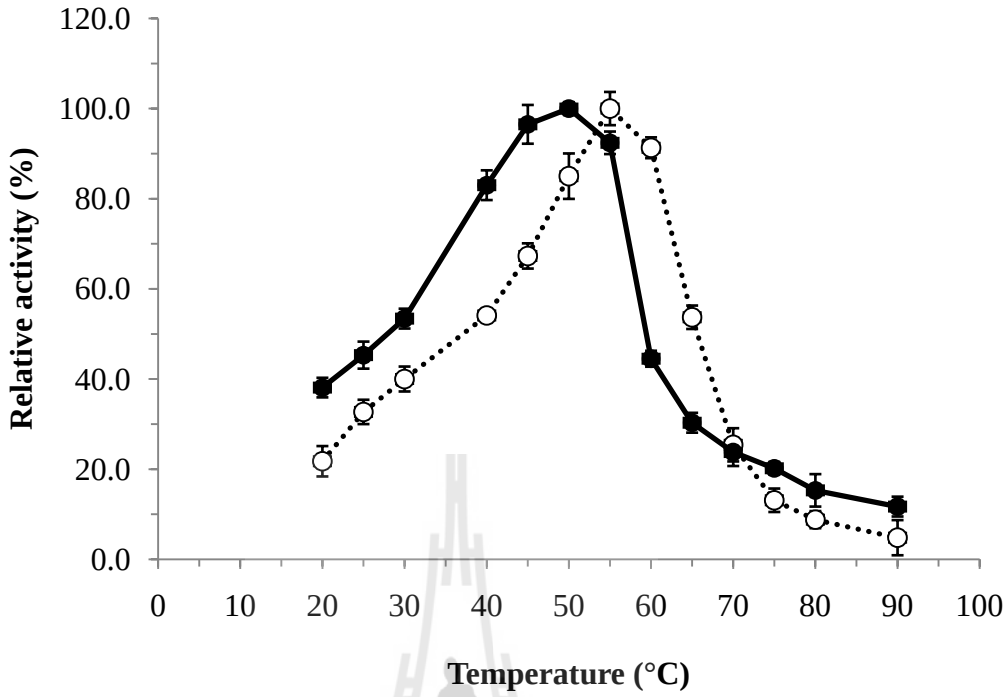


Figure 4.7 Optimal temperature of the activity of recombinant β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* DSM 20081 in sodium phosphate buffer: (O) lactose as substrate; (●) *o*NPG as substrate. Relative activities are given in comparison with maximum activities calculated under optimal conditions (100%), which were 691 and 390 U/mL with *o*NPG and lactose used as substrates.

4.4.3.2 Determination of kinetic parameters at different temperatures in the milk buffer

A recombinant β-galactosidase from *L. bulgaricus* with an *alr*-based system was determined in the milk buffer for the kinetic parameters (maximal velocity, Michaelis-Menten constant, turnover number, and catalytic efficiency), in the temperature range of 4-65°C.

The maximal velocity ($V_{max,Glc}$) of a recombinant β-galactosidase at 60°C showed the highest values about 308 μmol·min^{-1}·mg^{-1} (Table 4.6). This high value of $V_{max,Glc}$ at high temperature could be explained by the collision theory of Max Trautz in 1916 and William Lewis in 1918 (Arnaut et al., 2007). As temperature increased, molecules gained energy and moved continually faster, resulting in the increase of reaction rate. However, the enzyme might be denatured at high temperature (Daniel et al., 1996).

The best value of the Michaelis-Menten constant (K_m) of a recombinant β-galactosidase from this study was about 5.6 mM at 30°C. This value was the highest affinity for lactose. The best value of turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) was about 1054 S^{-1} at 60°C and 109000 $M^{-1}s^{-1}$ at 55°C, respectively. This agrees with Nguyen and co-workers (2012) which reported that a K_m value of β-galactosidase from *L. bulgaricus* DSM 20081 with an *erm*-based system was 19.9 mM at 30°C (standard assay). This K_m value was larger than that of β-galactosidase from *L. bulgaricus* DSM 20081with an *alr*-based system in this study (5.6 mM). This finding indicates that β-galactosidase in *alr*-based systems has a higher affinity for lactose than the β-galactosidase from *L. bulgaricus* DSM 20081 in *erm*-based systems. The small K_m value will approach V_{max} faster than the high K_m value

that enzyme has a very high affinity with the substrate (Berg et. Al., 2002). Regarding the K_m value, it found that the purified enzyme produced from this study could effectively be used for a production of low- or free-lactose milk.

In comparison with the common commercial enzymes which obtain from yeast and fungi, the K_m value was 15–52 mM for *K. fragilis*, 35 mM for *K. lactis*, 54–99 mM for *A. niger*, and 36–180 mM for *A. oryza* (Jurado et al., 2004; de Roos, 2004; Nguyen et al., 2006). These K_m values from commercial enzymes are higher than the K_m value of β-galactosidase in this study, showing that β-galactosidase with the *alr*-based system has the higher affinity for lactose than those commercial enzymes.

Table 4.6 Kinetic parameters for lactose hydrolysis for a recombinant β-galactosidase from *L. bulgaricus* at different temperatures in the milk buffer.

Temperature	Kinetic Parameters			
	$V_{max,Glc}$ (µmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
4°C	66±1[a]	7.6±0.6 [a]	226±3[a]	30,000±500[b]
30°C	153±3[c]	5.6±0.7[a]	524±10[abc]	94,000±2,000[f]
37°C	166±3[c]	6.5±0.7 [a]	568±10[abc]	87,000±2,000[d]
45°C	206±5[d]	6.9±0.7[a]	705±17[bc]	102,000±2,000[g]
55°C	259±4[e]	8.1±0.5[a]	886±14[cd]	109,000±2,000[h]
60°C	308±20[f]	32±4[b]	1054±68[d]	33,000±2,000[c]
65°C	109±5[b]	43±4[c]	373±17[ab]	9,000±400[a]

The molecular weight at 115 kDa of *LacZ*-His was used to calculate the k_{cat} from ν_{max}.

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

4.4.3.3 Determination of catalytic stability in different buffers and substrates

The inactivation constant (k_{in}) and the half life ($t_{1/2}$) of recombinant β-galactosidase using *o*NPG or lactose as a substrate with different temperatures are shown in Table 4.7 and 4.8, respectively. The half life was calculated from ln of k_{in}. In different buffers with different Mg^{2+} concentrations, the result showed that the increase of temperature could induce the half life decrease. The half life rapidly decreased as the temperature was higher than 45°C in both different buffer systems (Table 4.7 and 4.8).

The increase of Mg^{2+} concentrations in a sodium phosphate buffer could extend the half life rather than the buffer without Mg^{2+} in both substrates. This means that no effect of the substrate type on the catalytic stability. However, the highest concentration of Mg^{2+} (10 mM) in the sodium phosphate buffer system decreased the half life of enzymes rather than the lower concentration of Mg^{2+} (1 mM). However, the half life of enzyme from *o*NPG or lactose hydrolysis in 1mM Mg^{2+} of sodium phosphate buffer was higher than that of the other buffers. This might be because of the presence of Mg^{2+} in the buffer could extend the catalytic stability (half life time) of the enzyme. Most of the half-life times of recombinant β-galactosidase in sodium phosphate buffer which contained 1 mM Mg^{2+} showed the higher value than that of the other buffers both in *o*NPG hydrolysis and lactose hydrolysis, especially in the temperature range 4–37°C. Nonetheless, the stability of recombinant β-galactosidase could rapidly lose activity beyond 45°C with or without Mg^{2+} both in *o*NPG hydrolysis and lactose hydrolysis. As a result of the lower half-life times with increased temperatures, it can be determined that the presence of Mg^{2+}

in the buffers did not impact the half-life times at high temperature up to 45°C. However, the presence of Mg^{2+} may increase the β-galactosidase stability of *L. bulgaricus* with the *alr*-based system. This result is consistent with other studies using β-galactosidases *LacLM* from *Lactobacillus* spp. and β-galactosidases *LacZ* from *E. coli* (Tenu et al., 1971; Edwards et al., 1990; Roth and Huber, 1996; Nguyen et al., 2006; Nguyen et al., 2007; Juers et al., 2009; Iqbal et al., 2010; Maischberger et al., 2010; Iqbal et al., 2011; Nguyen et al., 2012; He et al., 2016). Moreover, Nguyen and co-workers (2012) reported that the stability and activity of GH2 β-galactosidases could be affected by the ions such as Mg^{2+}, which is a common of enzyme characteristic. As the half-life time of recombinant β-galactosidase in sodium phosphate buffer containing 10 mM Mg^{2+} was lower values than that of sodium phosphate buffer containing 1 mM Mg^{2+} and milk buffer. This finding agrees with a work of Nguyen and co-workers (2012). Recombinant β-galactosidases from *L. bulgaricus* with an *erm*-based vector system decreased the enzyme activity of 31% in 10 mM bis-Tris buffer containing 10 mM Mg^{2+}. In addition, high concentrations of Mg^{2+} resulted in inactivation of β-galactosidases from an *E. coli* (E416Q and E416V strain) as the expression system (Roth and Huber, 1996). The presence of several metal-binding sites, locating near the active site of β-galactosidase enzyme has a second Mg^{2+} binding site, which is important to the catalysis (Adalberto et al 2010). Beta-galactosidase commonly requires Mg^{2+} for maximal activity because the Mg^{2+} is well known to be essential for the folding and stability of large RNA molecules. This binding of Mg^{2+} to the substrate could induce some conformational changes in the enzyme molecule (Adalberto et al 2010), resulting in the better enzyme activity (Edwards et al., 1990; Juers et al., 2009). Ions in buffers are believably took a direct

part in the catalytic mechanism of β-galactosidases and contribute to subunit interaction of β-galactosidases *LacZ* from *E. coli* (Nguyen et al., 2012). As the stabilization of β-galactosidases by presenting Mg^{2+}, similarly, the divalent ions such as Mn^{2+}, Mg^{2+}, and Ca^{2+} were important to catalytic activity and stability of β-galactosidases from *Kluyveromyces* (Huber et al, 1979; Harada et al., 1994; Page and Cera, 2006; Sutendra *et al*., 2007; Adalberto *et al*., 2010).

To mimic the real system of lactose hydrolysis in dairy manufacturing, the catalytic-stability of the enzyme was carried out in milk buffer with *o*NPG and lactose as a substrate. The half life significantly decreased when the incubation temperature increased. Nevertheless, the lactose hydrolysis in milk buffer at 65°C seemed to give the half life stability better than sodium phosphate buffer with 10 mM Mg^{2+} or without Mg^{2+}. These results might be because of the present of calcium ion (Ca^{2+}) in the milk buffer. Calcium is one of the important intrinsic components of milk and could induce the enzyme activity for the lactose hydrolysis reaction (Beard, 1992; Sendra et al., 2012). However, Pal and co-workers revealed that the β-galactosidases from many GRAS microorganisms were inhibited their activity by Ca^{2+} at approximately higher than 30 mM. Half life of enzyme in milk buffer with oNPG as a substrate at 4°C was the lowest when compared with that of the other buffers (Table 4.7). This might be because Ca^{2+}, dissociated from $CaCO_3$ which was the main composition in milk buffer, could attach and bind with the metal-binding site of enzyme. Moreover, Ca^{2+} can be released more when the temperature of *o*NPG hydrolysis increased (Ritchie, 2008).

Table 4.7 The catalytic stability of a recombinant β-galactosidase from *L. bulgaricus* in milk buffer for *o*NPG hydrolysis

Temperature (C°)	Sodium phosphate buffer, pH6.5		Sodium phosphate buffer+ 1mM Mg^{2+}, pH6.5		Sodium phosphate buffer + 10mM Mg^{2+}, pH6.5		Milk buffer, pH6.5	
	k_{in} (h^{-1})	$t_{1/2}$ (h)	k_{in} (h^{-1})	$t_{1/2}$ (h)	k_{in} (h^{-1})	$t_{1/2}$ (h)	k_{in} (h^{-1})	$t_{1/2}$ (h)
4	0.0013	533^{b}	0.0012	578^{c}	0.0013	533^{b}	0.0015	462^{a}
30	0.0048	144^{c}	0.0027	257^{d}	0.0090	77^{a}	0.0052	133^{b}
37	0.0050	139^{c}	0.0040	173^{d}	0.0059	117^{b}	0.0068	102^{a}
45	0.0955	7.3^{a}	0.0732	9.5^{b}	0.0967	7.2^{a}	0.428	16^{c}
50	0.256	2.7^{ab}	0.234	3.0^{b}	0.276	2.5^{a}	0.158	4.5^{c}
55	1.10	0.6^{a}	0.840	0.8^{a}	0.440	1.6^{b}	0.198	3.5^{c}

Note : Experiments were performed at least in duplicate, and the standard deviation was <5%. Values in each temperature marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

Table 4.8 The catalytic stability of a recombinant β-galactosidase from *L. bulgaricus* in milk buffer for lactose hydrolysis

Temperature (C°)	Sodium phosphate buffer, pH6.5		Sodium phosphate buffer + 1mM Mg^{2+}, pH6.5		Sodium phosphate buffer + 10mM Mg^{2+}, pH6.5		Milk buffer, pH6.5	
	k_{in} (h^{-1})	$t_{1/2}$ (h)	k_{in} (h^{-1})	$t_{1/2}$ (h)	k_{in} (h^{-1})	$t_{1/2}$ (h)	k_{in} (h^{-1})	$t_{1/2}$ (h)
4	0.0030	231^{a}	0.0023	301^{c}	0.0027	257^{b}	0.0024	289^{c}
30	0.0078	89^{a}	0.0038	182^{c}	0.0071	98^{b}	0.0040	173^{d}
37	0.0102	68^{c}	0.0136	51^{b}	0.0168	41^{a}	0.0113	61^{d}
45	0.196	3.5^{b}	0.132	5.2^{c}	0.188	3.7^{b}	0.211	3.3^{a}
50	0.579	1.2^{a}	0.202	3.4^{c}	0.215	3.2^{c}	0.263	2.6^{b}
55	0.809	0.9^{a}	0.596	1.2^{b}	0.198	3.5^{c}	0.311	2.2^{d}

Note : Experiments were performed at least in duplicate, and the standard deviation was <5%. Values in each temperature marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

4.5 Conclusions

L. plantarum TLG02 harboring pSIP609-*lacZ*and pSIP609-*lacZ-His* in an *alr* system yielded overexpression of recombinant β-galactosidase. The optimal temperature of the recombinant β-galactosidase was 55-60°C for lactose and 50°C for *o*NPG substrates. The temperature gave the highest affinity of attachment between lactose and recombinant enzyme was at 30°C. The presence of Mg^{2+} could increase the half-life times.

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CHAPTER V

APPLICATION OF RECOMBINANT BETA-GALACTOSIDASE IN MILK AND ICE CREAM

5.1 Abstract

The recombinant β-galactosidase from *Lactobacillus delbruekii* subsp. *bulgaricus* DSM 20081 expressed in *L. plantarum* TLG02 was applied for hydrolysis of lactose in the milk buffer and low fat milk. The crude enzyme of recombinant β-galactosidase at a concentration of 5-10 U/mL could hydrolyze lactose in milk buffer at least 50 and 90% within 1 hour at 4 and 65°C, respectively. The lactose hydrolysis and transgalactosylation of recombinant β-galactosidase in crude and purified enzymes was measured and compared with the commercial enzyme (Lactozym 2600L) with different enzyme concentrations (1, 5, and 10 U/mL) in low fat milk. All enzyme preparations could hydrolyze more than 85% of lactose within 18 hours at 4°C. Moreover, the optimal time for galacto-oligosaccharides formation (3.63 mg/mL) was at 3 hours. These were qualitatively confirmed by thin layer chromatography (TLC). Lactose-hydrolyzed milks were used as ingredients for a production of the ice cream which were evaluated for its qualities and sensory attributes. However, the sweetness and overall acceptance of regular-fat lactose-hydrolyzed milks hydrolyzed by crude enzyme had the higher acceptability than those of the other milks hydrolyzed by the other enzymes. The overrun of regular-fat

lactose-hydrolyzed ice cream was higher than that of low-fat lactose-hydrolyzed ice cream. The overall acceptance mean scores of low-fat lactose-hydrolyzed ice cream produced by difference enzymes were significantly different ($p<0.05$). However, crude and purified enzymes could give no significant mean scores of color, aroma, sandiness, and overall acceptance when these values were compared with the regular-fat lactose-hydrolyzed ice creams ($p>0.05$).

Keywords: recombinant β-galactosidase, milk buffer, transgalactosylation, thin layer chromatography, overall acceptance, overrun

5.2 Introduction

The most important reason for the use of β-galactosidase in the dairy industry is to allow lactose intolerant people to consume dairy products which are naturally high in lactose. Beta-galactosidase enzymes are commercially available from different sources with different enzyme formulations. Their major applications include the treatment of liquid milk and the use of the enzyme as a nutritional supplement (Dekker and Daamen, 2003; Mahoney, 2003). Another benefit of β-galactosidase is the transgalactosylation reaction, resulting in the formation of galacto-oligosaccharides (GalOS), which co-occurs during lactose hydrolysis (Nakayama and Arnachi, 1999; Nguyen et al., 2006, Splechtna et al., 2006; Nguyen et al., 2007; Splechtna et al., 2007; Nguyen et al., 2011).

Beta-galactosidases applied in liquid dairy products should be obtained from GRAS (generally recognized as safe) microorganisms. The enzyme should have a relatively high optimal pH (pH 6–7) to allow effective lactose hydrolysis at milk pH.

As many processors prefer to treat milk during overnight storage in low temperatures to avoid microbial growth, the enzyme needs to be able to be active at these temperatures (6-8°C) to avoid the introduction of additional processing steps. Moreover, the enzyme should be able to be inactivated by pasteurization and/or ultra-high temperature (UHT) treatment in order to prevent its activity in the final product. In addition, the enzyme should be sufficiently pure to prevent off-flavor formation upon extended storage of UHT milk (Rand, 1981; Pivarnik et al, 1995; Mahoney, 1997; Dekker and Daamen, 2003; Jelen and Tossavainen, 2003; Mahoney, 2003).

Lactose hydrolysis is used to prevent the crystallization of lactose, improve sweetness, increase the solubility of milk products, produce lactose-free food products, and improve scoopability and creaminess in ice cream, yogurt and frozen dessert products (Neelakantan et al., 1999). Many recombinant β-galactosidases from *Lactobacillus* spp., such as *L. sakei*, *L. acidophilus*, *L. plantarum*, *L. pentosus*, *L. bulgaricus*, were isolated/overexpressed, characterized, and applied for lactose hydrolysis and transgalactosylation (Vasiljevic and Jelen, 2003; Nguyen et al., 2007; Maischberger et al., 2010; Iqbal et al., 2010; Iqbal et al., 2011; Nguyen et al., 2012). There still has the limitation of the application of β-galactosidase from *Lactobacillus* spp. in milk hydrolysis by using milk lactose as a substrate. Also, by products known as oligosaccharides have been less studied when milk lactose has been used as a substrate (Schwab and Ganzle, 2011; Ganzle, 2012). Moreover, the application of β-galactosidase for lactose hydrolysis to get the oligosaccharides has been focused only in the use of the commercial enzyme derived from yeast and fungi, such as *Kluveromyces fragilis*, *Kluveromyces lactis*, *Aspegillus niger*, and *Aspegillus oryza* (Panesar et al., 2010; Oliveira et al., 2011). There are two sources of recombinant

β-galactosidase used for lactose-hydrolyzed milk, which are *L. delbrueckii* subsp. *bugaricus* ATCC11842 in *E. coli* expression system (Rhimi et al, 2009) and the cold-adapted *L. acidophilus* in *E. coli* expression system (Pan et al., 2010). Jolar and Karbassi (2011) have used the β-galactosidases in crude enzyme from *L. bulgaricus* to produce lactose-hydrolyzed milk for sensory evaluation to compare the lactose-hydrolyzed milk treated by commercial enzymes.

As the galacto-oligosaccharides (GalOS) obtain by transgalactosylation, which co-occurs during lactose hydrolysis, have prebiotic properties (Nakayama and Arnachi, 1999; Nguyen et al., 2006, Splechtna et al., 2006; Nguyen et al., 2007; Splechtna et al., 2007; Nguyen et al., 2011). GalOS can be produced from β-galactosidases derived from various *Lactobacillus* spp., such as *L. delbrueckii* subsp. *lactis*, *L. reuteri*, *L. acidophius*, *L. pentosus*, *L. sakei* as well as *L. plantarum* (Vasiljevic and Jelen, 2003; Spletchna et al., 2006; Nguyen et al., 2007; Iqbal et al., 2010; Maischberger et al., 2010; Iqbal et al., 2011; Oliveira et al., 2011). Given the higher yield rate of GalOS production by β-galactosidase from *Lactobacillus* spp. versus other sources, application of β-galactosidase from *Lactobacillus* spp. has gained attention as a prebiotic ingredient (Rhimi et al., 2009; Asraf and Gunasekaran, 2010; Iqbal et al., 2011). Moreover, production of GalOS by β-galactosidases from lactic acid bacteria can be augmented by increasing lactose concentration in milk (Smart, 1991; Ganzle, 2012).

For the production of lactose-free milk, there are three principle ways to produce this product (Dekker and Daamen, 2003). First, β-galactosidaseis added to the milk before heat treatment (pre-treatment) then the product is heated, packed, and sold at 6°C- 8°C to avoid microbial growth (Dekker and Daamen, 2003). This method is

not performed in the optimum temperature of the enzyme, the hydrolysis time is rather long (24-30 hours) depending upon the enzyme dosage (Dekker and Daamen, 2003). In addition, this method can be applied at high temperature (38-40°C) in good quality raw milk. Also, the milk should always be pasteurized or at least thermalized prior to hydrolysis for safety purposes. The second method involves the application of β-galactosidase after heat treatment of milk (post-treatment) (Dekker and Daamen, 2003). In this process, milk is packaged after a sterile β-galactosidase is added in-line immediately following UHT treatment of milk, allowing the lactose to be hydrolyzed in the final package at ambient temperature. Since active enzyme is present in the milk during storage, this method requires a high quality of β-galactosidase preparation (Dekker and Daamen, 2003). The last method is applying β-galactosidase into milk products by the consumer by adding a few drops of β-galactosidase prior to consumption of the milk. Consumers can buy small packages of neutral β-galactosidase, and the lactose will be hydrolyzed within 12–24 hours depending upon the dosage (Rand, 1981; Pivarnik et al, 1995; Mahoney, 1997; Dekker and Daamen, 2003; Jelen and Tossavainen, 2003; Mahoney, 2003).

In the review of Oliveira and co-workers (2011), the cold-adapted recombinant *L. acidophilus* β-galactosidases were produced and purified through the *E. coli* expression system. These recombinant enzymes were able to hydrolyze 73% of lactose in milk within 30 hours at 10°C (Pan et al., 2010). Another recombinant acid tolerant β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 was produced from the *E. coli* expression system. The lactose hydrolyses of both milk and whey using original recombinant enzymes were compared with the E491A mutated enzyme (substitution of glutamic acid at position 491 by alanine). The result showed that the

mutated enzyme had an 89% bioconversion at 42°C after 10 hours incubation with 1 mg/mL of β-galactosidase, as compared to 80% efficiency in the original version (Vasiljevic and Jelen, 2001; Rhimi et al., 2009). Although, the recombinant β-galactosidases expressed in *E. coli* systems is safe, few studies of recombinant β-galactosidases expressed in *Lactobacillus* spp. were performed. Jokar and Karbassi (2011) have studied the hydrolysis of sterile milk using the crude enzyme of β-galactosidases from *L. bulgaricus* CHR Hansen Lb-12 in comparison with the commercial enzyme (DSM Food specialist Maxilact 12000, France). The result showed that lactose-hydrolyzed milk by the crude enzyme did not exhibit any significant differences in sensory evaluation when compared with the ordinary UHT milk.

For ice cream applications, lactose reduction is mainly performed by neutral β-galactosidases (Illanes, 2003). The glucose and galactose from lactose hydrolysis produce a much softer ice cream than do disaccharides such as sucrose (Marshall et al., 2003). Lactose hydrolysis improves the scoopability of the ice cream due to the decreasing of the freezing point, leading to an increase in the amount of solutes in the solution (Matak, 1999). Lactose hydrolysis also has an effect on the sandiness, texture, and other sensory characteristics of ice cream (Mahoney, 1997; Jelen and Tossavainen, 2003; Mahoney, 2003). Only commercial grade β-galactosidases are used for lactose hydrolysis in ice cream production, most of which are derived from yeast (Stevenson et al., 1983; Matak, 1999; Abbasi and Saeedabadian, 2013). However, the purified and crude enzymes of recombinant β-galactosidases from *Lactobacillus* spp. have not been studied. The market trend of lactose-reduced products is driving demand for lactose-free products (Mahoney, 2003). The market

survey (Statista Inc.) of lactose-free dairy products in the U.S., the largest market for lactose-free food, has shown an increased demand for lactose-free food products of $701.5 million in 2012 to $807.7 million in 2015. The Thai dairy market had total revenues of $1.6 billion in 2012, representing a compound annual growth rate (CAGR) of 3.3% between 2008 and 2012 (Senadisai et al., 2015). Thus, the increased market demand for low-lactose milk and milk products such as ice cream should increase the overall demand for milk, thereby expanding the dairy market as a whole.

The objectives of this study were to study the efficacy of crude enzyme for lactose hydrolysis in the milk buffer and to compare the lactose hydrolysis and transgalactosylation of crude, purified and commercial enzymes in low fat milk for the potential use in the dairy industry. Lactose in low-fat and regular-fat pasteurized milk were hydrolyzed at 4°C using different types of β-galactosidases and were determined for sensory evaluation. Also, the qualitative presence of lactose was detected by thin layer chromatography (TLC). These lactose-hydrolyzed milks were used as ingredients in producing ice cream, which was evaluated for the sensory attributes too.

5.3 Materials and methods

5.3.1 Preparation of the crude enzymes of β-galactosidase from *L. plantarum* TLG02 harboring pSIP609-*lacZ*

L. plantarum TLG02 harboring pSIP609-*lacZ* was cultivated in 500 mL fermentations to obtain sufficient material for the crude enzyme. This strain was grown in MRS broth (Oxoid, Basingstoke, U.K.) in a one liter of Erlenmeyer flask with loosely closed screw-cap at 30°C in incubator (Memmert, BE 500, WTB Binder BD115). *L. plantarum* TLG02 harboring pSIP609-*lacZ* was grown overnight

in 50 mL MRS broth medium as a pre-culture and inoculated into 500 mL fresh pre-warmed MRS medium (30°C) until the density of cell concentration (OD_{600}) reached 0.1, which was measured by spectrophotometer (Ultrospec 2000, Pharmacia biotech, Cambridge, UK) at 600 nm absorbance. When the OD_{600nm} reached to ~0.3, 25 ηg/mL of the inducing peptide pheromone IP673 (supplied by Food Biotechnology Laboratory, Department of Food Sciences and Technology, BOKU University of Natural Resources and Life Sciences, Vienna, Austria) was added to the cultured medium. The cultures were incubated further for 20 hours until the cell concentration reached OD_{600} of 6. The induced cells were harvested and washed twice with 50 mM sodium phosphate buffer (pH 6.5). After centrifugation, the cell pellets were dissolved in 50 mM sodium phosphate buffer (pH 6.5) and were disrupted by the ratio of 1:3. Cells were disrupted via 5 minutes sonication (40% power output, with pulses set to 5 sec ON / 5 sec OFF, 2 times) on ice using a sonicator (Waken GE100 Ultrasonic processor, Japan). After sonication, cell debris was removed by the centrifuge machine (Thermoscientific, Sorvall legend XTR centrifuge, USA) at 9000 g (4°C) for 20 minutes to get the crude enzyme. The crude enzymes of β-galactosidase were stored at 4°C in refrigerator for 5 days.

5.3.2 Enzyme Assay

5.3.2.1 Assay with lactose as substrate

Lactose was used as the substrate in this assay. Twenty µL of enzyme solution was added to 480 µL of a 600 mM lactose solution in 50 mM sodium phosphate buffer (pH 6.5). The reaction mixture was incubated at 30°C for 10 minutes by 600 rpm speed agitation in dry bath incubator (HB1, Wealtec Corp., USA). After 10 minutes, the reaction was heat-inactivated at 99°C using dry bath incubator (HB1,

Wealtec Corp., USA) for 5 minutes. The sample had been cooled to room temperature and then, the release of D-glucose was assessed colorimetrically using the enzymatic assay of glucose oxidase (GOD/POD assay) from a wok of Kunst and co-workers (1988) by adding 60 µL of reaction mixture to 600 µL of a solution containing GOD (94 µg/mL; Sigma-Aldrich, CA, USA), POD (6.1 µg/mL; Sigma-Aldrich, CA, USA), 4-aminoantipyrine (157 µg/mL; Sigma-Aldrich, CA, USA), and phenol (1.95% v/v; Merck Ltd.) in 50 mM sodium phosphate buffer (pH 6.5).This assay mixture (660 µL) was incubated in a dark room at ambient temperature for 40 minutes, and the absorbance at 505 nm was measured using spectrophotometer (Ultrospec 2000, Pharmacia biotech, Cambridge, UK). One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. All measurements and experiments were performed at least in duplicate, and the experimental error was less than 5%. The glucose concentration (*C*) was defined as follows (Kunst et al., 1988):

$$C\,[\mathrm{g/l}] = \frac{\mathrm{OD}_{505}}{\mathrm{k}} \times (\text{sample dilution factor})$$

$$C\,[\mathrm{mM}] = \frac{C\,(\mathrm{g/l})}{Mr_{\mathrm{glucose}}} \times 1000$$

Where OD_{505} is the sample-measured absorption at 505 nm, k is the slope of the glucose standard curve, and Mr_{glucose} is the molecular mass of glucose (180.16 g/mol), after correcting for the blank.

The β-galactosidase activity was calculated from the following relationship (Nguyen *et al*., 2006):

$$\beta\text{-Galactosidase activity (U/mL)} = C\,[\text{mM}] \times \frac{1}{t} \times \frac{(V_{lactose} + V_{enzyme})}{V_{enzyme}} \times \text{Dil}$$

Where C [mM] is the glucose concentration (mM), t is the reaction time (10 minutes), $V_{lactose}$ is the volume of lactose (480 µL), V_{enzyme} is the volume of enzyme sample (20 µL), and Dil is the enzyme dilution factor, after correcting for the blank.

5.3.3 Lactose hydrolysis in the milk buffer

The effects of crude enzyme concentrations (1, 5, and 10 U/mL of reaction mixture), lactose concentrations (125 and 165 mM), and the reaction temperatures (4 and 65°C) on lactose hydrolysis in a milk buffer (Gutierrez et al., 2002) were determined. Milk buffer consists of KCL + $MgSO_4 \cdot 7H_2O$ + $NaH_2PO_4 \cdot H_2O$ + $CaSO_4 \cdot 2H_2O$ + $CaCl_2 \cdot 2H_2O$ + Citric acid at 1.10 + 0.71 + 1.87 + 1.00 + 0.99 + 2.00 g/L, respectively. The measured activities were compared with the blank standard reaction. The reaction of lactose hydrolysis affected by those effects was periodically observed at 0, 15, 30, 45 and 60 minutes after continuously agitation in a refrigerator. D-glucose as a product from lactose hydrolysis was measured by the GOD/POD assay. This assay provided the lactose content which could be calculated to be the percentage of lactose hydrolysis following (Mahoney, 1997):

$$\text{Percentage hydrolysis} = \frac{\text{Total monosaccharides}}{\text{Original lactose concentration}} \times 100$$

5.3.4 Lactose hydrolysis and transgalactosylation

The presence of lactose, glucose, galactose and galacto-oligosaccharides (GalOS) was detected in reduced fat milk using non-tagged purified

recombinant β-galactosidase from *L. bulgaricus* and commercial enzyme (5 U/mL of reaction mixture). The reactions were incubated at 4°C and continuously agitated at 300 rpm. Samples were collected periodically (at 0, 3, 6, 9, 12, 15 and 18 hours), and the composition of lactose, glucose, galactose and GalOS were analyzed by the high-performance liquid chromatography (HPLC; Phenomenex, Rezek RNM Carbohydrate column, 7.8 × 300 mm, CA, USA) following Osiriphun, (2010). A carbohydrate analysis column consisted of a refractive index detector (Waters model 410; Waters Corp., MA, USA), a pump (M510; Waters Corp., MA, USA), a column oven and a system for data analysis (Chromatopac CR-5A, Shimadzu Corp., Kyoto, Japan). The eluent used was pre-degassed distilled water at 80°C and fed at flow rate 0.4 ml/min. The degree of hydrolysis (DH) was calculated following the equation (Mahoney, 1997):

$$\text{Degree of hydrolysis} = \frac{\text{Total monosaccharides}}{\text{Original lactose concentration}} \times 100$$

5.3.5 Lactose-hydrolyzed milk for milk composition, thin layer chromatography, microbiological quality, and sensory evaluation

Low-fat (1.5% fat) and regular-fat pasteurized milk (full fat; 3.5% fat) samples were supplied by Dairy Home Co., Ltd (Nakorn Ratchasima, Thailand). The crude enzyme of recombinant β-galactosidase, the purified enzyme from chapter 4, and the commercial enzyme (Lactozym 2600L, β-galactosidase from *K. lactis*, Novozymes, Denmark) purchased from Sigma-Aldrich (CA, USA) were used for the preparation of the lactose-hydrolyzed milk samples. The crude, purified, and commercial enzymes were added into 200 mL of low-fat and regular-fat milk at

5 U/mL of reaction mixture. All milk lactose hydrolysis reactions were stirred at 200 rpm (Magnetic stirrer MSH300, Biosan, Riga, Latvia) with controlled temperatures at 4°C in a refrigerator. After 24 hours of hydrolysis reaction, the samples were collected and pasteurized at 80°C for 15 sec and then, cooled to 4°C in refrigerator for further analysis (thin layer chromatography detection, microbiological quality, lactose-hydrolyzed milk sensory evaluation, and detection of lactose-crystal).

5.3.6 Thin layer chromatography (TLC) detection of lactose hydrolysis

TLC was used for detecting the sugar derivatives from milk lactose hydrolysis. The sugar derivatives on chromatogram of TLC were dried and then sprayed with ninhydrin solution. Then, ninhydrin reacted with the amino acids and gave the colored, mainly brown and purple, compounds.

For sample preparation, 1,000 µL of either low-fat or regular-fat pasteurized milk samples were aliquoted into 1.5 mL Eppendorf microcentrifuge tubes. Lactose hydrolysis was performed using 5 U/mL concentrations for β-galactosidases (crude, purified, and commercial enzymes) in an orbital rotation shaker (Certomat TCC, B. Braun Biotech International, Germany) at 200 rpm, at 4°C. Lactose-hydrolyzed milk samples (100 µL) from the hydrolysis reaction were collected at 6, 9, 12, 18, and 24 hours for the TLC detection for which the lactose-hydrolyzed milk samples were heat-inactivated at 99°C for 5 minutes, then cooled to 4°C in refrigerator.

The sugar standards (glucose, galactose, and lactose) and lactose-hydrolyzed milk samples were analyzed by thin layer chromatography (TLC). TLC was carried out using high-performance TLC silica plates (HPTLC Lichrospher silica gel 60 F_{254}S, Merck). The sugar standards were prepared in 1g/L concentration, and

5 µL of these standards were applied to the TLC plate. The lactose-hydrolyzed milk samples were diluted 1:2 with dH_2O, and 2 µL were loaded on the TLC plate. The samples were applied to the marked start edge of the TLC plate (normally at 1.5 cm height from lower edge of the plate). The plate was then allowed to be air-dried for 10 min before its transferring to the TLC tank for the development.

The plate was run two times in solvent (n-butanol/n-propanol/ethanol/ water = 2:3:3:2) and dried in between. The plate was dipped quickly into the staining solution (ethanol/sulfonic acid = 19:1) and dried for 5 min at 130°C until black spots were visible. In addition to qualitative results, TLC provides a chromatogram measurement known as an R_f value. The R_f value is the "retardation factor" value expressed as a decimal fraction. The R_f value can be calculated following the equation (Spangenberg et al., 2011):

$$R_f = \frac{z_s}{z_f - z_0}$$

Where: z_s is the distance of the substance zone from the sample origin (mm), z_f is the solvent front migration distance (mm), and z_0 is the distance between immersion line and sample origin (mm).

5.3.7 Lactose-hydrolyzed milk composition

The determination of lactose-hydrolyzed milk composition was analyzed by Milko Sonic S/N: 5798 (supplied by Dairy Home Co., Ltd.).

5.3.8 Microbiological quality of lactose-hydrolyzed milk

Twenty-five mL of the lactose-hydrolyzed milk samples was added to 225 mL of sterile water and shaken to make a homogenous mixture (Andrews 1992). Serial dilutions were made to 10^{-2} and 10^{-3} for all samples and spread plate technique

was employed for determination of total plate count (3M™ Petrifilm™ Aerobic Count Plates), *E. coli*, Coliform (3M™ Petrifilm™ *E. coli*/Coliform Count Plates), and yeast and mold (3M™ Petrifilm™ Rapid Yeast and Mold Count Plates). The inoculation and interpretation of 3M™ Petrifilm™ was followed by Petrifilm guides (Microbiology Products, 3M Health Care Ltd.).

5.3.9 Lactose-hydrolyzed milk sensory evaluation

The lactose-hydrolyzed milk sample used for sensory evaluation was stored in a refrigerator approximately 24 hours prior to analysis. Each 600 mL sample of lactose-hydrolyzed milk was incubated in a temperature of less than 4-8°C, verified by digital thermometer (Shenzhen Shining Electric Technology Co., Ltd), prior to sensory evaluation.

The lactose-hydrolyzed milk samples from low-fat and regular-fat milk were separately served. Each set of servings consisted of a control sample, a lactose-hydrolyzed milk by crude enzyme-hydrolyzed milk, purified enzyme-hydrolyzed milk, and commercial enzyme-hydrolyzed milk. Approximately 20 mL of each lactose-hydrolyzed milk were poured into transparent plastic glasses Aro (makro, Nakhon Ratchasima, Thailand) labeled with random three-digit numbers and stored at room temperature for 5 minutes prior to the sensory session.

The sensory evaluations of low-fat and regular-fat lactose-hydrolyzed milk were analyzed by serving samples to 30 trained panelists (aged 20–45 years). Lactose-hydrolyzed milk samples were served simultaneously and in random order. Panelists were asked to taste the lactose-hydrolyzed milk samples in order to evaluate the attributes of milk by using 9-point hedonic test (Nelson and Trout (1964), which were color, aoma, sweetness, overall acceptance as shown in Appendix A (Figure

1A). A glass of water was also served to cleanse the palate between tasting each sample. Panelists could write additional comments on the bottom of the sensory evaluation form. The scores of each sample's attributes (color, aroma, sweetness, and overall acceptance) were averaged and used for comparing the lactose-hydrolyzed pasteurized milks derived from the different β-galactosidase types (Clarke, 2004; Wood, 2011; Abbasi and Saeedabadian, 2013).

5.3.10 Ice cream preparation

Five hundred mL of lactose-hydrolyzed milk was prepared from low-fat (1.5% fat) and regular-fat milk (3.5% fat) using the different enzyme types (crude and purified enzymes) at 5 U/mL reaction mixture. This ice cream was prepared following the method for lactose-hydrolyzed milk production in section 5.3.4. The commercial enzyme was not used to apply for this section since it gave the same results on enzyme ability as the purified enzyme did.

Lactose-hydrolyzed milks were used as a main ingredient in ice cream production. Low-fat and regular-fat milks were used as experimental controls. To prepare the ice cream, the milk (73.9 w/w %), glucose syrup (11.4 w/w%; 10-12 DE), and butter milk (9.5 w/w%) were mixed and heated to 60 °C, then a mixture of sugar (4.8 w/w%) and stabilizer (0.4 w/w%; fulfil 400, SKW Biosystems Co., LTD, French) were added until the mix had fully melted. Afterwards, the mix was pasteurized using a batch pasteurizer (15 sec at 80°C) and was then homogenized (1500/500 bar; APV Gaulin Homogenizer, Model: 1515MR-8TA, SPX FLOW, Inc., NC, USA). For purposes of aging, the mix was cooled to 4°C and then refrigerated at 4°C for at least 4 hours. After aging, the aeration process was performed by a Batch Ice Cream Freezer (Single Flavor Model104 – 40, Taylor, USA). The ice cream was drawn

at -5°C (approximately 15 minutes of whipping and freezing process). Then the ice creams were packed in 30 ml Aro plastic cups (Makro, Nakhon Ratchasima, Thailand) and stored (-30°C, 24 hours) for hardening in a freezer at -22°C (NT34T/404/43, Montecchio, Italy) prior to analysis (Abbasi and Saeedabadian, 2013; Marshall et al. 2003).

5.3.11 Characterization of ice cream qualities

5.3.11.1 Overrun of ice cream

The percentage of overrun was calculated following this equation (Marshall et al. 2003):

$$\text{Overrun (\%)} = \frac{\text{weight of mix} - \text{weight of equal volume of ice cream}}{\text{weight of equal volume of ice cream}} \times 100$$

5.3.11.2 Particle size analysis of ice cream

The particle size analysis of ice cream was used to measure fat globule size distribution in ice cream mix and ice cream. This measure was used either as an indicator of homogenizer performance or as a benchmark for fat destabilization (Goff and Hartel, 2013).

Two drops of melting ice cream samples (~20µL) were dropped in the laser scattering particle size distribution analyzer (HORIBA LA-960, HORIBA Scientific, Kyuto, Japan) operating transmittance (R) of 90.5%, transmittance (B) of 76.6%, circulation speed of 5 rpm, agitation speed of 10 rpm, and ultra-sonic of 2 minutes.

5.3.11.3 Microbiological quality of ice cream

The ice cream samples (25 g) was added to 225 mL of sterile water and shaken to make a homogenous mixture (Andrews 1992). Serial dilutions were made to 10^{-2} and 10^{-3} for all samples and spread plate technique was employed for determination of total plate count (3M™ Petrifilm™ Aerobic Count Plates), *E. coli*, Coliform (3M™ Petrifilm™ E. coli/Coliform Count Plates), and yeast and mold (3M™ Petrifilm™ Rapid Yeast and Mold Count Plates). The inoculation and interpretation of 3M™ Petrifilm™ was followed the Petrifilm guides (Microbiology Products, 3M Health Care Ltd.).

5.3.11.4 Sensory evaluation

The lactose-hydrolyzed ice cream samples from low-fat and regular-fat lactose-hydrolyzed milk were separately served. Each set of servings consisted of a control sample, crude enzyme-hydrolyzed milk, purified enzyme-hydrolyzed milk, and commercial enzyme-hydrolyzed milk. The ice cream samples stored in a freezer (-22°C) was then moved into a refrigerator (PTV19T/43, Montecchio, Italy) at 4°C for 30 minutes before sensory evaluation. Each sample of lactose-hydrolyzed ice cream was verified while at a temperature of less than -7 to -6°C by digital thermometer (Shenzhen Shining Electric Technology Co., Ltd). Ice cream samples consisted of approximately 30 mL of lactose-hydrolyzed ice cream in plastic cups and were labeled with random three-digit numbers prior to the sensory session.

The sensory evaluations of lactose-hydrolyzed ice creams were analyzed by serving samples to 30 trained panelists (aged 20–45 years). Lactose-hydrolyzed ice cream samples were served simultaneously and in random order.

Panelists were asked to taste the lactose-hydrolyzed milk samples in order of the sensory evaluation form placed on the tray. Each lactose-hydrolyzed ice cream sample was evaluated for 7 sensorial attributes: color, aroma, sweetness, firmness, meltability, sandiness, and overall acceptance as shown in Appendix A (Figure 2A) using a 9-point hedonic scale as mentioned in 5.3.9. A glass of tea was served to cleanse the palate between tasting each ice cream sample. Panelists could write additional comments on the bottom of the sensory evaluation form. The scores of each sample's attributes (Color, Aroma, Sweetness, Firmness, Meltability, Sandiness, and Overall acceptance) were averaged and used for comparing the lactose-hydrolyzed ice creams derived from the different β-galactosidase types (Clarke, 2004; Wood, 2011; Abbasi and Saeedabadian, 2013).

5.3.12 Statistical analyses

All experiments and measurements were performed at least in duplicate. Analysis of variance (ANOVA) was determined using SPSS program (SPSS version 16, Windows version). Duncan Multiple Range Testing (DMRT) was used to compare differences among the means at $P < 0.05$.

5.4 Results and discussion

5.4.1 Application of crude enzyme of recombinant β-galactosidase for lactose hydrolysis in the milk buffer

The effects of different crude enzyme concentrations (1, 5, and 10 U/mL), different lactose concentrations (125 for low-fat milk and 165 mM for regular-fat milk), and different temperatures (4 and 65°C) on lactose hydrolysis in milk buffer were determined. These temperatures were mimicked to the pre- and post-

treatment pasteurization, respectively for lactose hydrolysis in a general dairy manufacturing. However, the reaction time of lactose hydrolysis in this study was fixed at 1 hour because, from preliminary work, the crude enzyme was considered for the heat denaturation when the temperature was at 65°C and above.

For the effect of the concentration of crude enzyme on lactose hydrolysis, the result showed that the higher concentration of crude enzyme was used, the more percentage of lactose hydrolysis was found. The highest percentage of lactose hydrolysis (98.7%) in this study was found when using enzyme concentration at 10 U/mL and l25 mM of lactose was used as a substrate at 65°C. However, the lactose hydrolysis increased up to 99.8% when l65 mM of lactose was used as a substrate at the same temperature (Table 5.1). The percentage of lactose hydrolysis from using different concentrations of crude enzyme, thus, was significantly different as shown by ANOVA in Appendix B (Table 1B). Theoretically, the concentration of enzyme increases the percentage of lactose hydrolysis increases even though the substrate concentration is constantly maintained (Whitaker, 1994). This result agrees with Nagaraj and co-workers (2009) which reported that the increasing of β-galactosidase concentration from yogurt starter culture of *S. thermophilus* and *L. bulgaricus* could increase the percentage of lactose hydrolysis. Similarly, a work of Bosso and co-workers (2016) showed that the increase of β-galactosidases concentrations from *K. lactis* and *A. oryzae* gave a higher hydrolysis rate than that of using the lower concentrations. Also, a work of Sener and co-workers (2008) showed that the increase of β-galactosidase concentration as a commercial β-galactosidase from *K. marxianus lactis* provided more milk lactose hydrolysis rate compared to the use of a lower concentration.

Regarding the effect of lactose concentration on lactose hydrolysis, the result showed that the percentage of lactose hydrolysis with the lactose concentration about 125 mM and 165 mM as the substrate and with different crude enzyme concentrations was in the range 34.8–98.7% at 4°C and 35.4–99.8% at 65°C, respectively (Table 4.9). This might be because the lactose concentration used in this study (125 and 165 nM) was pretty close to each other, consequently the rate of lactose hydrolysis from using both concentrations was not significant difference as shown in ANOVA Table (Table 2C).

According to the effect of temperature on the lactose hydrolysis, the result showed that the lactose hydrolysis at 4°C was lower than that at 65°C. The percentage of lactose hydrolysis at 4 and 65°C with different lactose concentrations was in the range of 34.8–99.8% (Table 5.1). The percentage of lactose hydrolysis at 4 and 65°C with different lactose concentrations was significantly different as shown by ANOVA (Table 3C). Increase of the temperature could affect the reaction rate of lactose hydrolysis, resulting in more reaction rate as collision theory. This result agrees with Peterson and co-workers (1989) which showed that the high temperatures (40°C) augmented the lactose hydrolysis reaction when the immobilized β-galactosidase was used when compared to the low temperature (15°C). Similarly, a work of Bosso and co-workers (2016) revealed that higher temperature gave much higher percentage of lactose hydrolysis when the commercial β-galactosidase was used. Also, Rosolen and co-workers (2015) reported that the increase of temperature (55°C) could hydrolyze milk lactose by *K. lactis* β-galactosidase more than that at low temperature (10°C).

Thus, from the result of lactose hydrolysis by crude enzyme in this study, it shows that crude enzyme could be used to hydrolyze milk in both pre- (4°C) and post- (65°C) for a treatment of pasteurization. However, the lactose hydrolysis at 4°C is preferred as it still provides sufficient reaction time to complete lactose hydrolysis in milk.

Table 5.1 The effects of different crude enzyme concentrations, different lactose concentrations, and different temperatures on lactose hydrolysis in milk buffer

Temperature	Enzyme concentration	Lactose concentration	Percentage of lactose hydrolysis
4°C	1 U/mL	125 mM	34.8±0.1
		165 mM	35.4±0.6
	5 U/mL	125 mM	50.0±0.9
		165 mM	55.8±0.2
	10 U/mL	125 mM	63.0±1.4
		165 mM	75.1±0.1
65°C	1 U/mL	125 mM	34.4±0.7
		165 mM	38.3±0.1
	5 U/mL	125 mM	90.4±2.0
		165 mM	92.3±0.7
	10 U/mL	125 mM	98.7±1.2
		165 mM	99.8±0.3

Note: Experiments were performed in quadruplicate, and the standard deviation was <5%.

5.4.2 Determination of lactose hydrolysis and transgalactosylation

The different recombinant β-galactosidase forms (crude and purified enzymes) and commercial enzyme (Lactozym 2600L) were applied for lactose hydrolysis at 4°C. This temperature was the imitation of milk-pasteurized process condition that was a temperature of cooling tank before the pasteurization process. The β-galactosidase application was a pre-treatment of enzyme before the pasteurization process for lactose-hydrolyzed milk production. The hydrolysis of lactose of recombinant β-galactosidase with different concentrations (1, 5 and 10 U/ml) was studied in low fat milk containing 1.5% milk fat at 4°C. The lactose, glucose, galactose, and galacto-oligosaccharides (GalOS) content from milk lactose hydrolysis were measured by RP-HPLC. The percentage of lactose hydrolysis (Figure 5.1 and Table 5.2) and the GalOS content (Figure 5.2 and Table 5.3) of low-fat lactose-hydrolyzed milk was compared in different concentrations of crude, purified and commercial enzymes at 4°C.

The lactose content at 0 hour was 54.3 mg/mL that was defined as 100% of remaining lactose content. The glucose and galactose content at 0 hours were 6.96 and 4.33 mg/mL, respectively. Galacto-oligosaccharides (GalOS) content was not detected in low-fat milk at zero hour. Lactose hydrolysis of β-galactosidase from crude enzyme at 4°C for 18 hours (final hydrolysis reaction time) with 1, 5, and 10 U/mL of enzyme concentration showed the percentage of remaining lactose content to be 15.9, 13.2, and 11.2%, respectively. The use of commercial enzyme for milk lactose hydrolysis at 4°C for 18 hours with 1, 5, and 10 U/mL of enzyme concentration showed the percentage of remaining lactose content to be 6.2, 4.5, and 3.2%, respectively. The use of purified β-galactosidase for milk lactose hydrolysis at 4°C for 18 hours with 1, 5,

and 10 U/mL of enzyme concentration showed the percentage of remaining lactose content to be 10.3, 7.9, and 6.2%, respectively. β-galactosidase types ordered in terms of efficient lactose-hydrolyzation of milk from highest to lowest are commercial enzyme, purified enzyme, and crude enzyme. Nonetheless, all types of β-galactosidase with various concentrations hydrolyzed at least 80% of milk lactose within 6 hours and more than 85% by the final hydrolysis reaction time (18 hours). However, β-galactosidase concentration at 5 U/mL was chosen to continue for the lactose-hydrolyzed milk production because the lactose hydrolysis from using enzyme at using 5 and 10 U/mL (at 4°C for 18 hours) gave the same result. However, preliminarily, the use of enzyme concentration at 10 U/mL gave a little bit bitter and harsh taste rather than that at 5 U/mL. Thus, the enzyme concentration at 5 U/mL was chosen for a further study.

This result agrees with a work of Rhimi and co-workers (2009) that showed that mutated-type acidotolerant β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 expressed in *E. coli* system had more efficient lactose-hydrolysis than wild-type β-galactosidase. The mutated β-galactosidase hydrolyzed 89% of milk lactose at 42°C after 10 hours while the wild-type β-galactosidase hydrolyzed 80% of milk lactose under the same condition. Pan and co-workers (2010) found that the cold-adapted β-galactosidase *lacZ* from *L. acidophilus* hydrolyzed 73% of milk lactose within 30 hours at 10°C. Moreover, Jokar and Karbassi (2011) revealed that the crude enzyme of β-galactosidase from *L. bulgaricus* CHR Hansen Lb-12 and commercial enzyme (Maxilact, DSM, Netherlands) could produce lactose-hydrolyzed milk in a Ultra High Temperature (UHT) condition but the commercial enzyme gave the higher lactose hydrolysis than the crude one. Matioli and co-workers (2003) reported that the

commercial enzyme from *K. fragilis* (Lactozym 3000 LAU/L, Novozymes, Denmark) could hydrolyze 70 -80% of lactose from substrate solution within 2-3 hours at 40°C. However, the commercial enzyme was suggested to be not available after 6 hours because of the inactivation of milk protein and possible microbial contamination at 40°C.

The highest formation of GalOS from milk lactose hydrolysis reaction occurred in the range 1.5-3 hours. The crude and purified enzymes were more productive than the commercial enzyme for GalOS formation. There was no difference in GalOS formation (ranging from 2.83-3.63 mg/mL) among different enzyme concentrations (1, 5, and 10 U/mL). All types of enzyme with 1 U/ml of enzyme concentration showed the highest GalOS production.

The crude enzyme of β-galactosidase for lactose hydrolysis in this study is agreed with a GalOS production work of Vasiljevic and Jelen (2003). They reported that the crude enzyme of β-galactosidase from *L. delgaricus* DMF 3078, *L. delgaricus* ATCC 11842 and *Streptococcus thermophilus* 143 synthesized GalOS from a 5% (w/w) lactose concentration in skim milk salt buffer at 50°C within 120 minutes, the GalOS was a range of 3.59-4.79 mg/mL. In addition, Matinez-Villaluenga and co-workers (2008) reported that the GalOS production by β-galactosidase from the commercial enzyme (Lactozym 3000L HP G) had affected the reaction conditions, resulting in different formations of di- and tri-saccharides. Thus, the different compositions of GalOS were dependent upon the assayed conditions. However, the GalOS compounds from lactose hydrolysis using β-galactosidase from those studies were received from a very high concentration of other not-milk lactose sources (140-1000 mM) while the concentration of lactose in milk (~4.8% of milk) was about

133 mM (Vasiljevic and Jelen, 2003; Nguyen et al., 2007; Martinez-Villaluenga et al., 2008; Iqbal et al, 2010; Maischberger et al., 2010; Nguyen et al., 2011). Also, the condition of GalOS formation in those studies were different form our study condition, resulting in the difference of GalOS content (Vasiljevic and Jelent, 2003; Splechtna et al., 2007; Ganzle, 2012).

Thus, from this study, the purified enzyme had similar efficiency to the commercial enzyme. Even though, the percentage of lactose hydrolysis using crude and purified enzymes was slightly lower than that of using commercial one, crude and purified enzymes were found to be better for GalOS formation than commercial enzyme. However, lactose hydrolysis for lactose-hydrolyzed milk products and GalOS formation also depended on enzyme characterization, enzyme concentration, reaction time, temperature, substrates, and conditions of hydrolysis reaction.

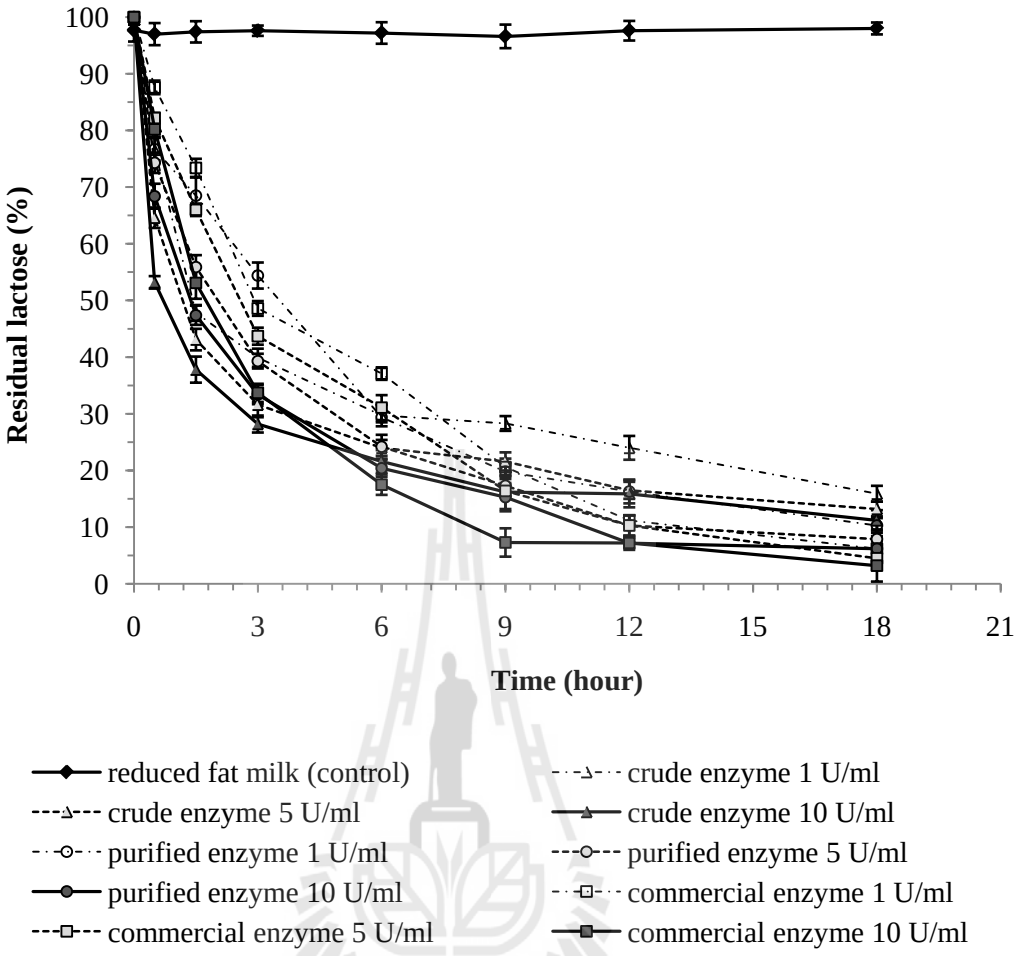

Figure 5.1 The percentage of lactose hydrolysis of low-fat lactose-hydrolyzed milk in different concentrations of crude, purified and commercial enzymes at 4°C.

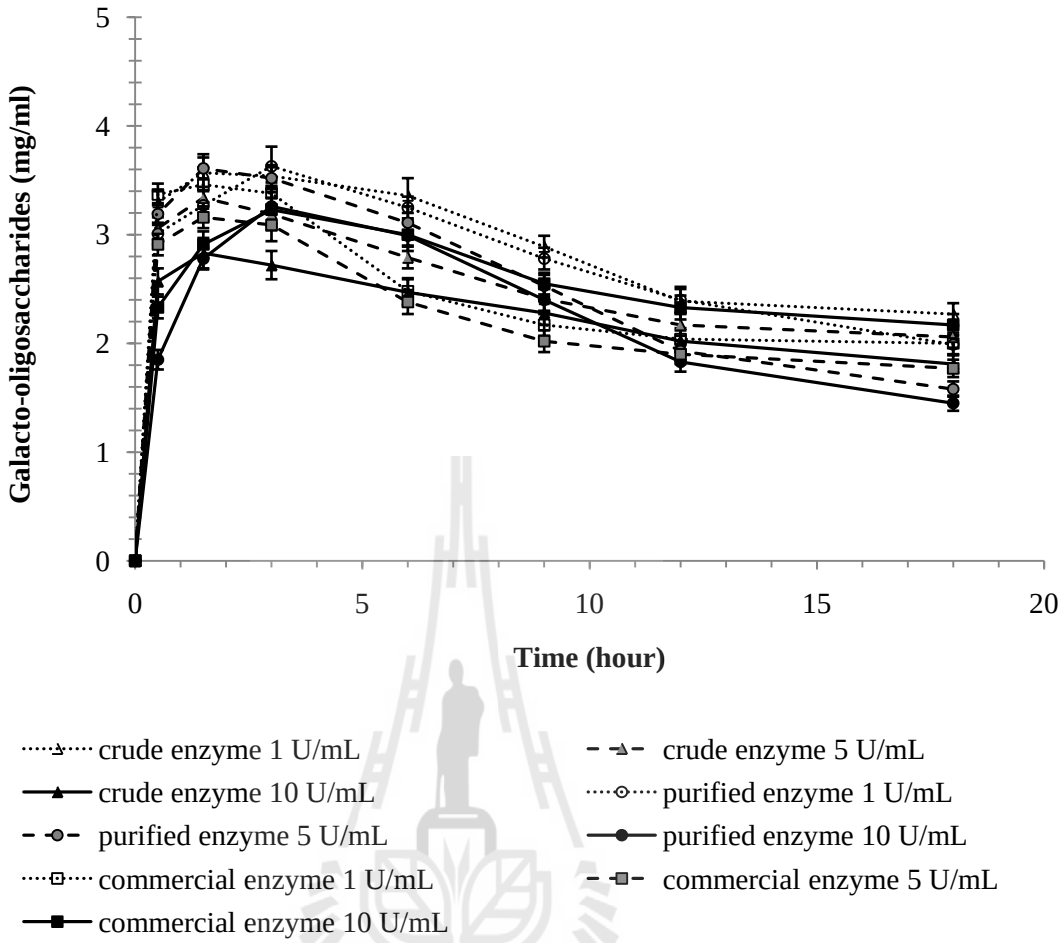

Figure 5.2 Galacto-oligosaccharides content (mg/ml) of low-fat lactose-hydrolyzed milk in different concentrations of crude, purified and commercial enzymes at 4°C.

Table 5.2 Percentage of remaining lactose content during milk lactose hydrolysis using different types of β-galactosidase in low-fat milk (1.5% fat)**.**

Time (h)	Concentration	0	0.5	1.5	3	6	9	12	18
Low-fat milk		100 ±2.0^{ns}	100±2.0^{ns}	100±1.9^{ns}	100±0.9^{ns}	100±1.9^{ns}	100±2.1^{ns}	100±1.7^{ns}	100±1.1^{ns}
crude enzyme	1 U/mL	100 ±2.0^{g}	76.6±2.3^{f}	47.8±1.4^{e}	39.9±1.6^{d}	29.7±0.2^{c}	28.3±1.3^{c}	24.0±0.2^{b}	15.9±0.1^{a}
commercial enzyme	1 U/mL	100 ±2.0^{h}	87.6±1.8^{g}	73.4±1.9^{f}	48.6±2.2^{e}	37.1±0.1^{d}	20.6±1.6^{c}	11.2±0.2^{b}	6.2±0.1^{a}
purified enzyme	1 U/mL	100 ±2.0^{h}	76.7±1.1^{g}	68.5±2.3^{f}	54.4±1.5^{e}	29.4±0.2^{d}	19.8±0.3^{c}	16.3±0.2^{b}	10.3±0.2^{a}
crude enzyme	5 U/mL	100 ±2.0^{h}	64.6±1.2^{g}	43.1±1.6^{f}	31.6±1.3^{e}	24.0±0.1^{d}	21.6±0.2^{c}	16.5±0.9^{b}	13.2±0.2^{a}
commercial enzyme	5 U/mL	100 ±2.0^{h}	82.2±0.90^{g}	66.0±1.1^{f}	43.7±1.5^{e}	31.1±0.2^{d}	16.4±0.1^{c}	10.3±0.2^{b}	4.5±0.2^{a}
purified enzyme	5 U/mL	100 ±2.0^{h}	74.3±1.5^{g}	55.9±2.8^{f}	39.3±1.6^{e}	24.2±0.2^{d}	17.3±0.3^{c}	10.3±0.9^{b}	7.9±0.3^{a}
crude enzyme	10 U/mL	100 ±2.0^{g}	53.2±2.0^{f}	37.8±3.2^{e}	28.2±1.3^{d}	21.6±0.9^{c}	16.2±0.1^{b}	15.9±0.2^{b}	11.2±0.1^{a}
commercial enzyme	10 U/mL	100 ±2.0^{g}	80.2±1.8^{f}	53.1±2.1^{e}	33.7±1.3^{d}	17.5±0.2^{c}	7.3±0.2^{b}	7.2±0.2^{b}	3.2±0.2^{a}
purified enzyme	10 U/mL	100 ±2.0^{g}	68.4±2.2^{f}	47.4±1.7^{e}	33.4±1.7^{d}	20.4±0.2^{c}	15.3±0.3^{b}	7.2±0.1^{a}	6.2±0.1^{a}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Different letters indicate means with significant differences (P<0.05).

Table 5.3 Galacto-oligosaccharides content (mg/mL) during lactose hydrolysis using different types of β-galactosidase in low-fat milk (1.5% fat).

Time(h)	concentration	0	0.5	1.5	3	6	9	12	18
crude enzyme	1 U/mL	0.00	3.30±0.1^{c}	3.57±0.2^{c}	3.54±0.1^{c}	3.36±0.2^{c}	2.89±0.1^{ab}	2.39±0.2^{a}	2.27±0.1^{a}
commercial enzyme	1 U/mL	0.00	3.37±0.1^{c}	3.46±0.2^{c}	3.38±0.1^{c}	2.48±0.2^{ab}	2.17±0.1^{a}	2.04±0.1^{a}	2.00±0.11^{a}
purified enzyme	1 U/mL	0.00	3.01±0.1^{b}	3.27±0.2^{c}	3.63±0.2^{c}	3.25±0.1^{c}	2.78±0.1^{ab}	2.40±0.2^{a}	1.99±0.2^{a}
crude enzyme	5 U/mL	0.00	3.06±0.1^{b}	3.34±0.1^{c}	3.19±0.2^{c}	2.79±0.1^{ab}	2.41±0.3^{ab}	2.17±0.2^{a}	2.06±0.2^{a}
commercial enzyme	5 U/mL	0.00	2.91±0.1^{b}	3.16±0.1^{c}	3.09±0.2^{c}	2.38±0.2^{ab}	2.02±0.1^{a}	1.90±0.2^{a}	1.77±0.1^{a}
purified enzyme	5 U/mL	0.00	3.19±0.1^{c}	3.61±0.1^{c}	3.52±0.1^{c}	3.11±0.2^{c}	2.53±0.1^{ab}	1.93±0.2^{a}	1.58±0.1^{a}
crude enzyme	10 U/mL	0.00	2.57±0.2^{bc}	2.83±0.2^{c}	2.72±0.2^{c}	2.47±0.3^{b}	2.28±0.2^{a}	2.02±0.1^{a}	1.81±0.2^{a}
commercial enzyme	10 U/mL	0.00	2.33±0.1^{a}	2.91±0.2^{ab}	3.23±0.2^{b}	3.00±0.1^{ab}	2.55±0.1^{a}	2.33±0.2^{a}	2.17±0.2^{a}
purified enzyme	10 U/mL	0.00	1.85±0.1^{a}	2.78±0.1^{c}	3.26±0.2^{c}	2.99±0.2^{bc}	2.40±0.1^{b}	1.83±0.2^{a}	1.45±0.3^{a}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Different letters indicate means with significant differences ($P<0.05$).

5.4.3 Thin layer chromatography detection of lactose hydrolysis

Thin layer chromatograpy (TLC) detection was used to qualitatively confirm the lactose hydrolysis at 4°C for 24 hours (Figures 5.3-5.6). The retardation factor of sugar derivatives from lactose hydrolysis was shown in Table 5.4 as well.

Both low-fat lactose-hydrolyzed milk and the regular-fat lactose-hydrolyzed milk using different enzyme types showed similar pattern of the presenting glucose and galactose bands on the TLC chromatogram. Also, there was no appearance of the band of lactose on the TLC plate after 18 hours of lactose hydrolysis. This confirmed that lactose in milk was completely hydrolyzed at that condition.

The retardation factor (R_f) of standard sugars and sugar derivatives from milk lactose hydrolysis was provided from TLC chromatogram. The R_f values of standard sugars were 0.71 of lactose, 0.82 of glucose, and 0.78 of galactose, respectively. The low-fat lactose-hydrolyzed milk and regular-fat lactose-hydrolyzed milk showed the similar R_f values of glucose and galactose, which were in a range of 0.80 – 0.83 and of 0.77 - 0.80, respectively, whereas the R_f value of lactose was 0.70 and 0.71 for low-fat milk and regular-fat milk, respectively.

This result agrees with the work of Bosch-Reig and co-workers (1992) which reported that the R_f values of sugars in aqueous solutions were 0.48 of lactose, 0.69 of glucose, and 0.65 of galactose. Smith and Dawson (1987) reported that the R_f values of different sugars in milk extract were 0.16 of lactose, 0.41 of glucose, 0.40 of galactose, and 0.16 of milk extract. Dafam and co-workers (2014) reported that the R_f values of sugars in *Anacardium occidentale* gum in solvent (butanol-ethanol-water) were 0.29 of lactose, 0.55 of glucose, and 0.48 of galactose, while Skalska-Kamińska

and co-workers (2009) found that the retardation factors (R_f) of *Malva arborae* hydrolysate were 0.43 of lactose, 0.67 of glucose, and 0.60 of galactose. The different R_f values obtained from the same type of sugar was the characteristic for each substance in these specific chemical reactions. The R_f values from these literatures were different from the result found in this study since the study conditions such as chamber saturation, constant composition of solvent mixtures, and constant temperature were different from the literatures (Sherma and Fried, 1996). However, these R_f values demonstrate similarities when arrayed in descending order: glucose, galactose, and lactose. The different R_f values of each sugar was the difference of raw material or experimental method. Even though there was a difference of R_f values in each a previous work, these values seemed to show the similarities when arrayed in descending order: glucose, galactose, and lactose that depended on raw material and experimental details which they used in each study.

Table 5.4 The retardation factor (R_f) of sugar derivatives from lactose hydrolysis at 24 hours.

	R_f		
	Standard lactose	**Standard glucose**	**Standard galactose**
	0.71 ± 0.001	0.82 ± 0.015	0.78 ± 0.015
Low-fat milk (control)	0.70 ± 0.003	-	-
Low-fat lactose-hydrolyzed milk with crude enzyme	-	0.82 ± 0.01	0.77 ±0.015
Low-fat lactose-hydrolyzed milk with purified enzyme	-	0.81 ± 0.015	0.78±0.02
Low-fat lactose-hydrolyzed milk with commercial enzyme	-	0.83 ± 0.03	0.79±0.01
Regular-fat milk (control)	0.71 ± 0.023	-	-
Regular-fat lactose-hydrolyzed milk with crude enzyme	-	0.81± 0.02	0.80 ±0.015
Regular-fat hydrolyzed milk with purified enzyme	-	0.82± 0.015	0.79 ±0.03
Regular-fat lactose-hydrolyzed milk with commercial enzyme	-	0.80± 0.01	0.78 ±0.03

Note: Experiments were performed in duplicate, and the standard deviation was <5%.

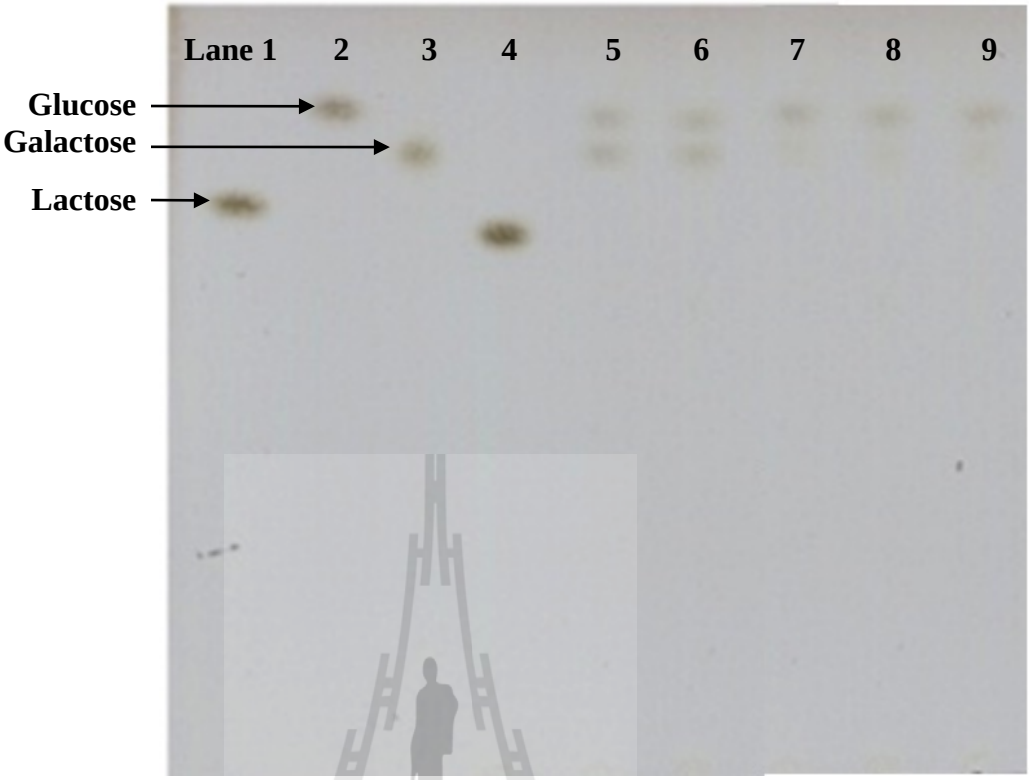

Figure 5.3 TLC Chromatogram of low-fat lactose hydrolyzed milks by crude enzyme (Lane 1, lactose standard; Lane 2, glucose standard; Lane 3, galactose standard; Lane 4, low-fat milk; Lane 5, lactose-hydrolyzed milk for 6 hour; Lane 6, lactose-hydrolyzed milk for 9 hour; Lane 7, lactose-hydrolyzed milk for 12 hour; Lane 8, lactose-hydrolyzed milk for 18 hour; Lane 9, lactose-hydrolyzed milk for 25 hour).

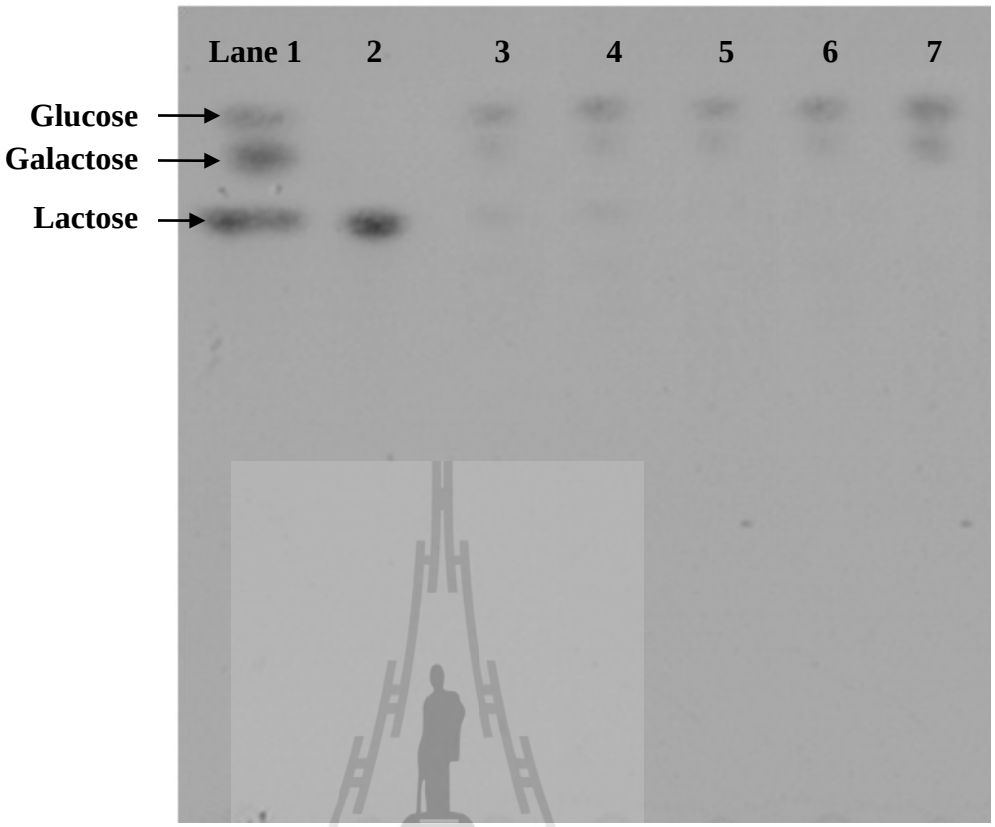

Figure 5.4 TLC Chromatogram of low-fat lactose hydrolyzed milks by purified enzyme (Lane 1, lactose, glucose, and galactose standard; Lane 2, low-fat milk; Lane 3, lactose-hydrolyzed milk for 6 hour; Lane 4, lactose-hydrolyzed milk for 9 hour; Lane 5, lactose-hydrolyzed milk for 12 hour; Lane 6, lactose-hydrolyzed milk for 18 hour; Lane 7, lactose-hydrolyzed milk for 24 hour).

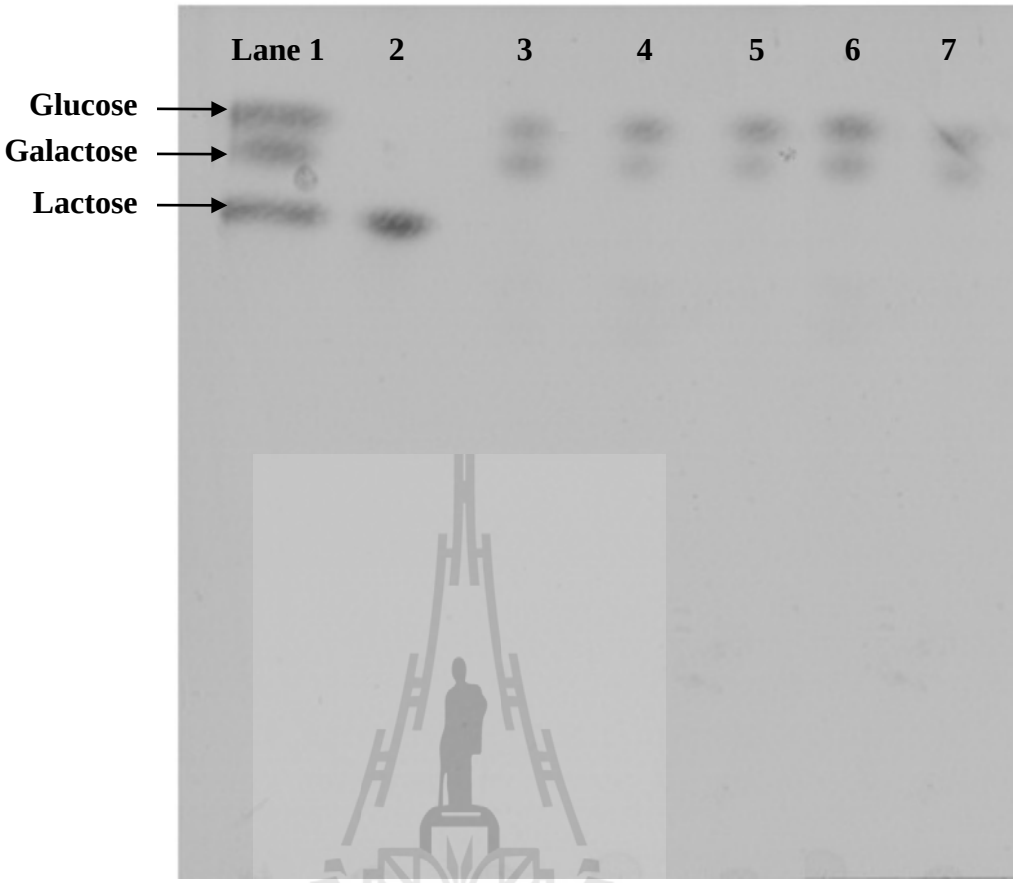

Figure 5.5 TLC Chromatogram of low-fat lactose hydrolyzed milks by commercial enzyme (Lane 1, lactose, glucose, and galactose standard; Lane 2, low-fat milk; Lane 3, lactose-hydrolyzed milk for 6 hour; Lane 4, lactose-hydrolyzed milk for 9 hour; Lane 5, lactose-hydrolyzed milk for 12 hour; Lane 6, lactose-hydrolyzed milk for 18 hour; Lane 7, lactose-hydrolyzed milk for 24 hour).

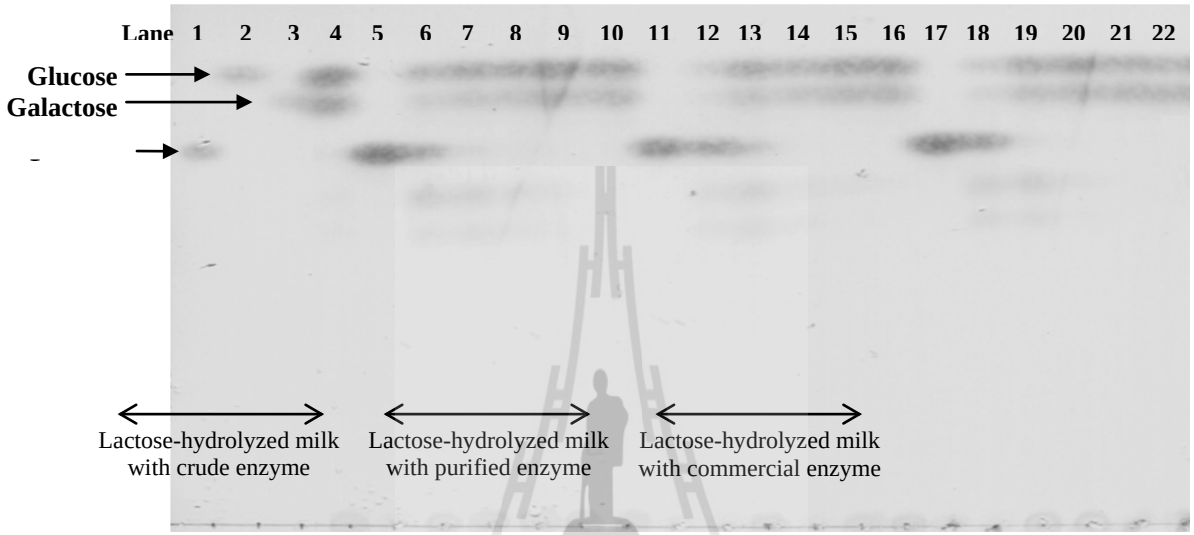

Figure 5.6 TLC Chromatogram of regular-fat lactose hydrolyzed milks (Lane 1, lactose standard; Lane 2, glucose standard; Lane 3, galactose standard; Lane 4, commercial lactose-hydrolyzed milk (Chokchai brand); Lane 5, regular-fat milk; Lane 6–10, lactose-hydrolyzed milk by crude enzyme at different time of lactose hydrolysis (6, 9, 12, 18, and 24 hours, respectively); Lane 11, regular-fat milk; Lane 12–16, lactose-hydrolyzed milk by purified enzyme at different time of lactose hydrolysis (6, 9, 12, 18, and 24 hours, respectively); Lane 17, regular-fat milk; Lane 18–22, lactose-hydrolyzed milk by commercial enzyme at different time of lactose hydrolysis (6, 9, 12, 18, and 24 hours, respectively).

5.4.4 Milk composition of lactose-hydrolyzed milk

The composition of lactose-hydrolyzed milk is shown in Table 5.5. This revealed that all parameters of milk composition obtained from this study were in the acceptable range for the consumption following Thai agricultural standard (TAS 6003-2010).

Table 5.5 Determination of lactose-hydrolyzed milk composition**.**

Parameter	Reference*	Raw material	Regular-fat lactose-hydrolyzed milk	Low-fat lactose-hydrolyzed milk
freezing point	≤-0.520°C	-0.514±0.003^{ns}	-0.522±0.006^{ns}	-0.519±0.005^{ns}
specific gravity (20°C)	>1.028	1.026±0.002^{a}	1.033±0.002^{b}	1.030±0.005^{ab}
Temperature	30-37°C	30.7±0.2^{b}	5.3±0.2^{a}	5.8±0.2^{a}
%Fat	>3.35%	5.04±0.04^{c}	3.85±0.05^{b}	1.48±0.03^{a}
%SNF	>8.25%	8.07±0.08^{a}	9.09±0.01^{c}	8.91±0.01^{b}
%Protein	>3.00%	2.85±0.06^{a}	3.24±0.1^{b}	3.11±0.09^{b}
Ph	6.6-6.9	6.7±0.1^{ns}	6.7±0.1^{ns}	6.7±0.1^{ns}

* Reference obtained from Thai agricultural standard (TAS 6003-2010).

Note: Experiments were performed in triplicate, and the standard deviation was <5%. Values in each attribute category marked with the same letter are not statistically different.

5.4.5 Microbiological quality and milk composition of lactose-hydrolyzed milk

The microbiological quality of milk samples was determined before the sensory evaluation to ensure the safety for consumer consumption (Table 5.6-5.8). The microbial results showed that the amount of microbial colonies was in the

acceptable level for the consumption following Thai agricultural standard (TAS 6003-2010) and Ministry of Public Health (No. 265) B.E. 2545 (2002).

Table 5.6 Microbiological quality of raw milk.

Sample	Dilution	Standard plate count (cfu/mL)	Coliform (cfu/mL)	*E.coli* (cfu/mL)	Yeast & Mold
Reference*	-	< 600,000	< 10,000	unidentified	unidentified
Raw milk**	$1x10^{-4}$	2.7×10^5	undetected	undetected	undetected

*Reference obtained from Thai agricultural standard (TAS 6003-2010).

**Data obtained from Dairy Home Co., Ltd.

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each attribute category marked with the same letter are not statistically different.

Table 5.6 Microbiological quality of low-fat pasteurized milk.

Sample	Sample dilution	Total plate count (cfu/mL)	Coliform	*E. coli*	Yeast & Mold
Reference pasteurize milk**	-	< 10,000 colony/mL	< 100 colony/mL	Not found in 0.1 mL	unidentified
Low-fat pasteurize milk*	$1x10^{-2}$ mL	4.0×10^2	undetected	undetected	undetected
Low-fat lactose-hydrolyzed pasteurize milk by crude enzyme	$1x10^{-2}$ mL	$6.9 \times 10^2 \pm 9$	undetected	undetected	undetected
Low-fat lactose-hydrolyzed pasteurize milk by purified enzyme	$1x10^{-2}$ mL	$5.6 \times 10^2 \pm 4$	undetected	undetected	undetected
Low-fat lactose-hydrolyzed pasteurize milk by commercial enzyme	$1x10^{-2}$ mL	$7.5 \times 10^2 \pm 8$	undetected	undetected	undetected

*Reference obtained from Notification of the Ministry of Public Health (No. 265) B.E. 2545 (2002)

**Data obtained from Dairy Home Co., Ltd.

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

Table 5.7 Microbiological quality of regular-fat pasteurized milk

Sample	Sample dilution	Total plate count (cfu/mL)	Coliform	*E. coli*	Yeast & Mold
Reference pasteurize milk**	-	< 10,000 colony/mL	< 100 colony/mL	Not found in 0.1 mL	unidentified
Regular-fat pasteurize milk*	$1x10^{-2}$	6.0×10^{2}	undetected	undetected	undetected
Regular-fat lactose-hydrolyzed pasteurize milk by crude enzyme	$1x10^{-2}$	$8.1 \times 10^{2} \pm 14$	undetected	undetected	undetected
Regular-fat lactose-hydrolyzed pasteurize milk by purified enzyme	$1x10^{-2}$	$7.8 \times 10^{2} \pm 11$	undetected	undetected	undetected
Regular-fat lactose-hydrolyzed pasteurize milk by commercial enzyme	$1x10^{-2}$	$7.4 \times 10^{2} \pm 12$	undetected	undetected	undetected

*Reference obtained from Notification of the Ministry of Public Health (No. 265) B.E. 2545 (2002)

**Data obtained from Dairy Home Co., Ltd.

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

5.4.6 Sensory evaluation of lactose-hydrolyzed milk

The mean sensory scores of the control low-fat milk and the milks hydrolyzed by crude, purified, and commercial enzymes are shown in Table 5.9. The sensory scores for color, aroma, sweetness and overall acceptance ranged from 8.05-7.96, 7.33-7.14, 7.11-6.03, and 7.05-6.68 respectively. No significant differences were observed between the control and lactose-hydrolyzed milk samples in color and aroma ($p>0.05$). However, the mean score of sweetness of samples prepared by crude enzyme was significantly different from that of the samples prepared by the rest of enzymes ($p<0.05$).

The overall acceptance scores of lactose-hydrolyzed milk samples prepared by the three different enzyme types were significantly different from the control's ($p<0.05$). The sweetness score of lactose-hydrolyzed milk by the crude enzyme was slightly less, "6 like slightly", whereas the other samples was "7 like moderately". This lower sweetness score may be the result of the relatively high volume of crude enzyme used, which was necessary to achieve the desired enzyme activity concentration of 5 U/mL of reaction mixture. The more volume of crude enzyme used in this study (10 U/mL), the more bitter flavor might occur. The overall acceptance score of the control, "7 like moderately", was slightly better than that of other three samples, which all scored at "6 like slightly". This might be because the add-flavors from hydrolysis such as glucose and galactose were predominant in lactose-hydrolyzed milk rather than in the control.

For the regular-fat milk, the mean sensory scores of the control and the milks hydrolyzed by crude, purified, or commercial enzymes are shown in Table 5.9.

The sensory scores for color, aroma, sweetness and overall acceptance ranged from 8.10-7.97, 7.30-7.17, 7.37-6.27, and 7.67-6.07 respectively.

The sweetness and overall acceptance scores of sample prepared by the crude enzyme were higher than those of the other β-galactosidase hydrolyzed samples. No significant differences were observed for color and aroma between the control and lactose-hydrolyzed milk samples ($p>0.05$). The overall acceptance score of the control was “7 like moderately”, whereas the regular-fat lactose-hydrolyzed milk samples by the three different enzyme types were “6 like slightly”. However, the overall acceptance score of lactose-hydrolyzed milk samples varied significantly ($P<0.05$) among the different types of enzymes used for lactose hydrolysis. This result agrees with a work of Jokar and Karbassi (2011) which showed that the UHT milk (control) was more acceptable than the lactose-hydrolyzed milks prepared from crude enzyme of *L. bulgaricus* CHR Hansen Lb-12 and the commercial enzyme.

Thus, there is a possibility that low-fat and regular-fat lactose-hydrolyzed milks prepared from recombinant β-galactosidase can be successfully marketed. Although the overall acceptance scores of low-fat and regular-fat lactose-hydrolyzed milks were lower than those of the control, they still scored positively.

Table 5.9 Sensory characteristics of low-fat lactose-hydrolyzed pasteurized milk.

Attributes	Low-fat milk (control)	Low-fat lactose-hydrolyzed milk by crude enzyme	Low-fat lactose-hydrolyzed milk by purified enzyme	Low-fat lactose-hydrolyzed milk by commercial enzyme
Color	8.05 ± 1.6^{ns}	7.96 ± 1.3^{ns}	7.98 ± 1.9^{ns}	8.01 ± 2.1^{ns}
Aroma	7.25 ± 1.3^{ns}	7.14 ± 1.7^{ns}	7.19 ± 2.3^{ns}	7.33 ± 1.6^{ns}
Sweetness	7.08 ± 1.4^{a}	6.03 ± 1.9^{b}	7.07 ± 1.7^{a}	7.11 ± 1.6^{a}
Overall acceptance	7.05 ± 1.7^{a}	6.04 ± 1.6^{b}	6.39 ± 1.3^{b}	6.68 ± 1.6^{b}

Note: Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

Table 5.10 Sensory characteristics of regular-fat lactose-hydrolyzed pasteurized milk.

Attributes	Regular-fat milk (control)	Regular-fat lactose-hydrolyzed milk by crude enzyme	Regular-fat lactose-hydrolyzed milk by purified enzyme	Regular-fat lactose-hydrolyzed milk by commercial enzyme
Color	8.10 ± 1.5^{ns}	7.97 ± 1.9^{ns}	8.03 ± 1.4^{ns}	8.00 ± 1.2^{ns}
Aroma	7.30 ± 1.7^{ns}	7.27 ± 1.2^{ns}	7.30 ± 1.5^{ns}	7.17 ± 2.0^{ns}
Sweetness	6.67 ± 1.1^{a}	6.27 ± 1.3^{a}	7.23 ± 1.5^{b}	7.37 ± 1.8^{b}
Overall acceptance	7.67 ± 1.9^{d}	6.07 ± 1.4^{a}	6.57 ± 1.2^{b}	6.80 ± 1.6^{c}

Note: Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

5.4.7 Characterization of ice cream qualities

5.4.7.1 Overrun of ice cream

The overrun results of lactose-hydrolyzed ice cream are shown in Table 5.11. The overrun of regular-fat lactose-hydrolyzed ice cream was higher than the overrun of low-fat lactose-hydrolyzed ice cream because of the higher fat content. The average percentage of lactose hydrolyzed in lactose-hydrolyzed milk samples for ice cream was about 90%, which affected the overrun. The different β-galactosidase types significantly decreased the overrun in each milk types. The overrun of ice cream from low-fat lactose-hydrolyzed milk could be ordered from highest to lowest as follows: low-fat milk (control sample), lactose-hydrolyzed milk with crude enzyme, and lactose-hydrolyzed milk with purified enzyme. The overrun of ice cream from regular-fat lactose-hydrolyzed milk could be ordered from highest to lowest as follows: regular-fat milk (control sample), lactose-hydrolyzed milk with crude enzyme, and lactose-hydrolyzed milk with purified enzyme. The results of overrun in this study aligned with the work of Abbasi and Saeedabadian (2013), which revealed that the ice cream hydrolyzed with lactase at 75% had the overrun of 107% while the ice cream without lactose hydrolysis had the overrun around 92%. Whereas, a work of Morr and Barrantes (1998) revealed that the presence of low-lactose whey powder in ice cream decreased overrun when compared to the control sample. However, a work of El-Neshawy and co-workers (1988) reported that the samples containing hydrolyzed lactose reconstituted milk (about 50% and 75% lactose hydrolysis) had higher overrun and better organoleptic properties than the control. The lactose-hydrolyzed ice cream exhibited less overrun but high in the hardness (Marshall et al. 2003; Clark, 2012). The hardness of ice cream was attributed to the

overrun (Muse & Hartel, 2004). The increase of glucose and lactose from lactose hydrolysis contributed to increase of total solids content of ice cream mix. This could increase the amount of unfrozen water in ice cream, resulting in the harder texture of ice cream than normal ice cream when it was frozen in the ice cream process (Hagiwara & Hartel, 1996; Tanaka et al., 1972; Goff et al., 1995; Wilbey et al., 1998).

The lactose-hydrolyzed milk for making ice cream could decrease the overrun and increased the hardness of ice cream. However, for ice cream manufacturing, the sensory evaluation had to be concerned for the final consideration of such enzyme application.

Table 5.11 The overrun of lactose-hydrolyzed ice cream

Milk type	Ice cream from milk control	Ice cream from lactose-hydrolyzed milk by crude enzyme	Ice cream from lactose-hydrolyzed milk by purified enzyme	Ice cream from lactose-hydrolyzed milk by commercial enzyme
Low-fat milk	39.8 ± 1^{d}	38.4 ± 1.2^{c}	37.6 ± 0.8^{b}	35.8 ± 1.3^{a}
Regular-fat milk	41.3 ± 1.5^{c}	40.5 ± 2.0^{b}	37.3 ± 1.2^{a}	39.6 ± 0.8^{ab}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each milk type category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

5.4.7.2 Particle size analysis of ice cream

The particle size analysis of lactose-hydrolyzed ice creams was measured by the laser scattering particle size distribution analyzer. The particle size obtained from low-fat lactose-hydrolyzed ice-cream samples ranged from

0.63–0.67 μm (Table 5.12). No significant differences were observed among the low-fat ice cream (control), ice-cream from low-fat lactose-hydrolyzed milk by crude enzyme, and ice-cream from low-fat lactose-hydrolyzed milk by purified enzyme ($p<0.05$). The particle size of regular-fat lactose-hydrolyzed ice-cream samples was in the rage of 1.21–1.23 μm. The particle size of regular-fat ice cream (control) was different from that of ice-cream from regular-fat lactose-hydrolyzed milk by crude enzyme, and ice-cream from regular-fat lactose-hydrolyzed milk by purified enzyme.

The particle size of ice cream from the low-fat lactose-hydrolyzed milk was smaller than that of ice cream from the regular-fat lactose-hydrolyzed milk which might be due to the fat content in milk. The mean diameter of the fat globules of low-fat lactose-hydrolyzed ice-cream mix in this study was slightly less than 1 μm while the fat globule size of regular-fat milk was around 2–3 μm (Goff and Hartel, 2013).

The larger particle size of regular-fat lactose-hydrolyzed ice-cream could contribute a pronounced flocculation of ice cream mixes than low-fat lactose-hydrolyzed ice-cream.

Table 5.12 The particle size analysis of lactose-hydrolyzed ice cream.

Milk type	Particle size (μm)			
	Ice cream from milk control	**Ice cream from lactose-hydrolyzed milk by crude enzyme**	**Ice cream from lactose-hydrolyzed milk by purified enzyme**	**Ice cream from lactose-hydrolyzed milk by commercial enzyme**
Low-fat milk	0.67 ± 0.036^{ns}	0.66 ± 0.024^{ns}	0.63 ± 0.015^{ns}	0.64 ± 0.021^{ns}
Regular-fat milk	1.33 ± 0.013^{c}	1.27 ± 0.038^{bc}	1.24 ± 0.054^{ab}	1.21 ± 0.029^{ab}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each milk type category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

5.4.7.3 Microbiological quality of lactose-hydrolyzed ice cream

The microbiological quality of lactose-hydrolyzed ice cream were evaluated in order to confirm the hygienically production and a safe for consumption as shown in Table 5.13.

Table 5.13 Microbiological quality of lactose-hydrolyzed ice cream.

Milk type	β-galacotsidase type	Dilution	TPC	Coliform	*E.coli*	Yeast & Mold
low-fat milk	without β-galacotsidase	$1x10^{-3}$	$7.5 \times 10^2 \pm 14$	Undetected	undetected	undetected
	crude enzyme	$1x10^{-3}$	$7.0 \times 10^2 \pm 21$	Undetected	undetected	undetected
	purified enzyme	$1x10^{-3}$	$6.3 \times 10^2 \pm 28$	Undetected	undetected	undetected
regular-fat milk	without β-galacotsidase	$1x10^{-3}$	$9.4 \times 10^2 \pm 11$	Undetected	undetected	undetected
	crude enzyme	$1x10^{-3}$	$1.1 \times 10^3 \pm 21$	Undetected	undetected	undetected
	purified enzyme	$1x10^{-3}$	$9.8 \times 10^2 \pm 18$	Undetected	undetected	undetected

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

5.4.7.4 Ice Cream Sensory Evaluation

The mean sensory scores of the low-fat ice cream (control), the ice cream from low-fat lactose-hydrolyzed milk by crude enzyme and the ice cream from low-fat lactose-hydrolyzed milk by purified-enzyme are shown in Table 5.14. The sensory scores for color, aroma, sweetness, firmness, meltability, sandiness, and overall acceptance varied from 5.69-6.44, 4.31-5.69, 6.14-6.44, 5.19-5.88, 5.31-5.69, 4.81-5.38, and 5.07-6.29, respectively. No significant differences of meltability were observed among the control, the ice cream from low-fat lactose-hydrolyzed milk by crude enzyme, and the ice cream from low-fat lactose-hydrolyzed milk by purified enzyme ($p<0.05$). However, the overall acceptance showed significant differences among them but the use of lactose-hydrolyzed milks for ice cream did not provide sufficient change in sweetness to warrant a statistical difference between the control and lactose-hydrolyzed pasteurized milk samples. Similar results were observed in the study of Sutton and co-workers (1995) in which the lactose reduction did not produce a perceived increase in sweetness among custard samples that were treated with lactase. Also, Lindamood and co-workers (1989) reported that the lactose and/or sucrose hydrolysis in the range of 0-78% used for ice cream making were not considered too sweet for ice cream, as untrained panels were unable to discern a difference.

The mean sensory scores of the regular-fat ice cream (control) and the ice creams hydrolyzed by crude and purified enzymes are shown in Table 5.15. The sensory scores for color, aroma, sweetness, firmness, meltability, sandiness, and overall acceptance varied from 6.89-7.19, 5.19-5.50, 5.50-6.38, 5.56-6.06, 5.56-6.44, 5.38-5.75, and 6.43-6.60, respectively. No significant differences were observed

among the control, and ice cream samples with lactose hydrolysis for color, aroma, sandiness, and overall acceptability ($p<0.05$). The firmness of the control was significantly higher than that of the other ice cream samples made from lactose-hydrolyzed milks by crude or purified enzymes. This was possibly due to the higher levels of glucose and galactose in the lactose-hydrolyzed milks existed more than that found in the control. Increasing the amounts of monosaccharides in the ice cream mix could increase viscosity but decrease free water content, resulting in a decreased firmness. Consequently, the texture of the lactose-hydrolyzed ice cream was softer than that of the control. However, the higher sugar levels (polysaccharides) caused a smoother texture by lowering the freezing point, resulting in decreasing the amount of frozen material, decreasing free water content, and increasing viscosity (Abdullah et al., 2003; Marshell and Arbuckle, 1996). This result agrees with a work of Lindamood and co-workers (1989) which reported that firmness values of non-hydrolyzed ice cream samples were higher than that of lactose-hydrolyzed ice cream samples while Matak (1999) showed that the firmness of the ice cream control was lower than that of 83% lactose-hydrolyzed ice cream. Moreover, Guy (1980) revealed that firmness values of ice cream decreased significantly while increasing the percentage of lactose-hydrolyzed sweet whey in the formulation.

Thus, the production of ice cream from regular-fat lactose-hydrolyzed milk is possible. This means that the application of recombinant β-galactosidase from this study can used in both forms of crude and purified enzymes. However, it seemed to be that the purified enzyme was suitable for low-fat lactose-hydrolyzed ice cream rather than the crude one.

Table 5.14 Sensory characteristics of low-fat lactose-hydrolyzed ice cream.

Attributes	Ice cream from low-fat milk	Ice cream from low-fat lactose-hydrolyzed milk by crude enzyme	Ice cream from low-fat lactose-hydrolyzed milk by purified enzyme
Color	6.44 ± 1.5^{b}	5.69 ± 1.5^{a}	6.31 ± 1.6^{b}
Aroma	5.69 ± 1.8^{b}	4.31 ± 2.0^{a}	5.38 ± 1.9^{b}
Sweetness	6.44 ± 1.6^{ns}	6.14 ± 2.4^{ns}	6.38 ± 2.0^{ns}
Firmness	5.63 ± 1.9^{b}	5.19 ± 1.8^{a}	5.88 ± 2.0^{b}
Meltability	5.69 ± 1.8^{ns}	5.31 ± 1.8^{ns}	5.44 ± 1.9^{ns}
Sandiness	4.81 ± 2.4^{a}	5.38 ± 1.8^{b}	5.25 ± 1.9^{b}
Overall acceptance	5.93 ± 1.4^{b}	5.07 ± 1.3^{c}	6.29 ± 2.0^{a}

Note: Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

Table 5.15 Sensory characteristics of regular-fat lactose-hydrolyzed ice cream.

Attributes	Ice cream from regular-fat milk	Ice cream from regular-fat lactose-hydrolyzed milk by crude enzyme	Ice cream from regular-fat lactose-hydrolyzed milk by purified enzyme
Color	7.19 ± 1.0^{ns}	6.89 ± 1.2^{ns}	7.09 ± 1.1^{ns}
Aroma	5.19 ± 1.9^{ns}	5.25 ± 1.3^{ns}	5.50 ± 2.1^{ns}
Sweetness	5.81 ± 1.2^{a}	5.50 ± 1.1^{a}	6.38 ± 1.5^{b}
Firmness	6.06 ± 2.3^{b}	5.94 ± 1.2^{b}	5.56 ± 1.5^{a}
Meltability	5.56 ± 2.0^{a}	5.88 ± 1.3^{a}	6.44 ± 1.5^{b}
Sandiness	5.75 ± 1.8^{ns}	5.38 ± 1.6^{ns}	5.50 ± 1.4^{ns}
Overall acceptance	6.60 ± 1.5^{ns}	6.43 ± 0.7^{ns}	6.60 ± 1.4^{ns}

Note: Values in each attribute category marked with the same letter are not statistically different. Values marked with different letters are statistically different ($P<0.05$).

5.5 Conclusions

The recombinant β-galactosidase can be used in either the crude or purified forms to produce lactose-hydrolyzed milk. In addition, crude and purified enzymes were more productive than the commercial enzyme for the GalOS formation. The hydrolyzed milk can be used to produce the low-lactose ice cream. The possibility of ice cream production from lactose-hydrolyzed milks was well-confirmed by their physical, microbiological, and sensory qualities.

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CHAPTER VI

Summary

Beta-galactosidase is an important enzyme in the dairy industry. This study developed a recombinant β-galactosidase (*lacZ*) from *L. delbrueckii* subsp. *bulgaricus* DSM 20081, expressed in *L. plantarum* TLG2 through a pSIP vector system for used as a food grade expression system. This is an essential step towards food-grade production of β-galactosidase in *L. plantarum*. The expression plasmids (pSIP609-*lacZ* and pSIP609–*lacZ*-His) were successfully constructed by replacing the *erm* gene with the *alr* gene from *L. plantarum*, resulting in efficient overproduction of β-galactosidases. The purified β-galactosidase from *L. plantarum* TLG02 harboring pSIP609-*lacZ*-histag was determined for the biochemical characteristics. The optimal temperature of this β-galactosidase was 55-60°C and 50°C, using lactose and *o*NPG as a substrate. The kinetic parameters of β-galactosidase were determined in milk buffer at different temperatures (4–65°C). The temperature at 30 and 50°C were the suitable temperature for giving the Michaelis-Menten constant (K_m) and the catalytic efficiency (k_{cat}/K_M) values, respectively. The presence of Mg^{2+} in the buffer could extend the catalytic stability (half-life time) of the enzyme but its stability could rapidly lose activity beyond 45°C with or without Mg^{2+}. All types of β-galactosidase with various concentrations (1-5 U/mL) hydrolyzed more than 85% of milk lactose within 18 hours at 4°C. All enzyme preparations (1–10 U/mL) could produce the highest GalOS content at 3 hours. Crude and purified enzymes provided more GalOS

content than the commercial one. The overall acceptance of such lactose-hydrolyzed milk was scored in “like slightly”. For the production of lactose-hydrolyzed milk, crude and purified enzymes could be used and substituted to the commercial enzyme. The overall acceptance of lactose-hydrolyzed ice cream was scored in “like slightly”. However, the purified enzyme seemed to be suitable for the production of lactose-hydrolyzed ice cream.

Thus, in this study, the production and application of the recombinant β-galactosidase has a very high potential for using in dairy industry for producing the low-lactose product.

APPENDICES

APPENDIX A

Sensory evaluation form of lactose-hydrolyzed milk

Name:……. Date:…….Panelist no. ……

Instructions: You are presented with four samples of lactose-hydrolyzed milk which labeled with three-digit numbers code in order to taste four sensorial attributes (Color, Aroma, Sweetness, and Overall acceptance). Taste the sample and give the score which best describes your feeling. Rinse your mouth with tea between samples. Pease write down any comments.

Score value assigned: 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely.

Attributes	**Code**			
Color				
Aroma				
Sweetness				
Overall acceptance				

Comment:……

……

……

Figure 1A: Sensory evaluation form of lactose-hydrolyzed milk.

Sensory evaluation form of lactose-hydrolyzed ice cream

Name:……Date:…….Panelist no. ……

Instructions: You are presented with four samples of lactose-hydrolyzed ice cream which labeled with three-digit numbers code in order to taste seven sensorial attributes (Color, Aroma, Sweetness, Firmness, Meltability, Sandiness, and Overall acceptance). Taste the sample and give the score which best describes your feeling. Rinse your mouth with tea between samples. Pease write down any comments.

Score value assigned: 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely.

Attributes	**Code**			
Color				
Aroma				
Sweetness				
Firmness				
Meltability				
Sandiness				
Overall acceptance				

Comment:……

……

……

Figure 2A: Sensory evaluation form of lactose-hydrolyzed ice cream.

APPENDIX B

Table 1B ANOVA Table (The effect of crude enzyme concentrations on lactose hydrolysis).

Tests of Between-Subjects Effects

Dependent Variable: result

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13539.449[a]	4	3384.862	39.487	.000
Intercept	98259.205	1	98259.205	1.146E3	.000
Temp	3260.836	1	3260.836	38.041	.000
Enzyme	10171.298	2	5085.649	59.329	.000
Lac	107.315	1	107.315	1.252	.277
Error	1628.679	19	85.720		
Total	113427.332	24			
Corrected Total	15168.127	23			

a. R Squared = .893 (Adjusted R Squared = .870)

Table 2B ANOVA Table (The effect of lactose concentrations on lactose hydrolysis).

Tests of Between-Subjects Effects

Dependent Variable: result

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	107.315[a]	1	107.315	.157	.696
Intercept	98259.205	1	98259.205	143.532	.000
Lac	107.315	1	107.315	.157	.696
Error	15060.812	22	684.582		
Total	113427.332	24			
Corrected Total	15168.127	23			

a. R Squared = .007 (Adjusted R Squared = -.038)

Table 3B ANOVA Table (The effect of temperatures on lactose hydrolysis).

Tests of Between-Subjects Effects

Dependent Variable: result

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3260.836[a]	1	3260.836	6.025	.022
Intercept	98259.205	1	98259.205	181.544	.000
Temp	3260.836	1	3260.836	6.025	.022
Error	11907.291	22	541.241		
Total	113427.332	24			
Corrected Total	15168.127	23			

a. R Squared = .215 (Adjusted R Squared = .179)

APPENDIX C

Table 1C Lactose content (mg/mL) during lactose hydrolysis using different types of β-galactosidase in low-fat milk (1.5% fat).

Time (h)	Concentration	0	0.5	1.5	3	6	9	12	18
crude enzyme	1 U/mL	54.3±0.8^{g}	41.6±1^{f}	25.9±0.8^{e}	21.7±0.9^{d}	16.2±0.6^{cd}	15.3±0.7^{c}	13.1±1^{b}	8.61±0.2^{a}
commercial enzyme	1 U/mL	54.3±0.8^{h}	47.6±0.7^{g}	39.8±0.9^{f}	26.4±0.7^{e}	20.2±0.6^{d}	11.2±0.5^{c}	6.11±0.1^{b}	3.38±0.1^{a}
purified enzyme	1 U/mL	54.3±0.8^{g}	41.6±1^{f}	37.2±1^{e}	29.5±1^{d}	16.0±0.5^{c}	10.7±0.5^{bc}	8.85±0.4^{b}	5.59±0.1^{a}
crude enzyme	5 U/mL	54.3±0.8^{g}	35.1±1^{f}	23.4±1^{e}	17.2±0.6^{d}	13.0±0.6^{cd}	11.7±0.6^{c}	8.98±0.3^{b}	7.16±0.2^{a}
commercial enzyme	5 U/mL	54.3±0.8^{h}	44.7±0.5^{g}	35.9±0.6^{f}	23.7±0.8^{e}	16.9±1^{d}	8.90±0.2^{c}	5.60±0.^{2b}	2.46±0.1^{a}
purified enzyme	5 U/mL	54.3±0.8^{g}	40.4±0.9^{f}	30.4±1^{e}	21.3±0.7^{d}	13.1±0.5^{c}	9.41±0.3^{b}	5.59±0.2^{a}	4.27±0.1^{a}
crude enzyme	10 U/mL	54.3±0.8^{g}	28.9±0.6^{f}	20.6±0.9^{e}	15.3±0.8^{d}	11.7±0.4^{c}	8.78±0.2^{b}	8.61±0.2^{b}	6.11±0.1^{a}
commercial enzyme	10 U/mL	54.3±0.8^{g}	43.6±0.8^{f}	28.8±0.7^{e}	18.3±0.9^{d}	9.5±0.1^{c}	3.94±0.1^{b}	3.93±0.1^{b}	1.73±0.04^{a}
purified enzyme	10 U/mL	54.3±0.8^{f}	37.1±1^{e}	25.8±0.9^{d}	18.1±0.9^{c}	11.1±0.2^{b}	8.32±0.2^{a}	3.93±0.1^{a}	3.38±0.1^{a}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Different letters indicate means with significant differences (P<0.05).

Table 2C Glucose content (mg/mL) during lactose hydrolysis using different types of β-galactosidase in low-fat milk (1.5% fat).

Time(h)	Concentration	0	0.5	1.5	3	6	9	12	18
crude enzyme	1 U/mL	6.96±0.1^{a}	7.59±0.3^{ab}	10.9±0.2^{b}	15.3±0.5^{c}	18.5±0.3^{d}	19.2±0.4^{e}	19.5±0.4^{f}	19.5±0.5^{g}
commercial enzyme	1 U/mL	6.96±0.1^{a}	8.42±0.4^{b}	10.35±0.7^{c}	15.2±0.5^{d}	18.5±0.3^{e}	19.8±0.5^{ef}	20.1±0.3^{f}	20.6±0.3^{f}
purified enzyme	1 U/mL	6.96±0.1^{a}	7.61±0.3ab	11.5 ±0.7b	16.7±0.3^{c}	18.5±0.6^{cd}	19.2±0.3^{d}	19.7±0.5^{d}	20.6±0.4^{d}
crude enzyme	5 U/mL	6.96±0.1^{a}	8.61±0.4^{b}	12.4 ±0.4^{c}	16.9±0.2^{e}	18.9±0.5^{f}	19.2 ±0.5^{f}	19.5±0.3^{f}	20.2 ±0.6^{f}
commercial enzyme	5 U/mL	6.96±0.1^{a}	8.52±0.3^{b}	12.9 ±0.5^{c}	16.9±0.9^{d}	19.4±0.7^{e}	20.2±0.3^{f}	20.7±0.3^{f}	20.8 ±0.5^{f}
purified enzyme	5 U/mL	6.96±0.1^{a}	9.32±0.4^{b}	13.0 ±0.3^{c}	16.9±0.2^{d}	18.7 ±0.3^{e}	19.7±0.7^{ef}	20.1±0.8^{f}	20.5 ±0.4^{f}
crude enzyme	10 U/mL	6.96±0.1^{a}	8.67±0.5^{b}	14.5±0.6^{c}	18.1±0.3^{d}	19.0±0.3^{de}	19.7±0.4^{e}	19.8±0.4^{e}	20.6 ±0.3^{e}
commercial enzyme	10 U/mL	6.96±0.1^{a}	10.12±0.6^{b}	15.0 ±0.3^{c}	17.9±0.4^{d}	19.8±0.7^{e}	20.8±0.4^{ef}	21.0±0.3^{f}	21.1±0.4^{f}
purified enzyme	10 U/mL	6.96±0.1^{a}	10.74±0.3^{b}	14.4 ±0.3^{c}	17.9±0.7^{d}	19.2 ±0.6^{e}	19.8±0.8^{f}	20.3±0.3^{f}	20.6±0.5^{f}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Different letters indicate means with significant differences (P<0.05).

Table 3C Galactose content (mg/mL) during lactose hydrolysis using different types of β-galactosidase in low-fat milk (1.5% fat).

Time(h)	Concentration	0	0.5	1.5	3	6	9	12	18
crude enzyme	1 U/mL	4.33±0.3^{a}	4.88±0.4^{b}	11.7±0.7^{c}	16.1±0.7^{d}	19.0±0.5^{e}	21.1±0.7^{ef}	21.5±1^{ef}	22.9±0.4^{f}
commercial enzyme	1 U/mL	4.33±0.3^{a}	6.38±0.3^{b}	10.7±0.4^{c}	16.8±0.7^{d}	22.1±0.6^{e}	23.3±0.7^{ef}	24.4±0.5^{f}	25.0±0.8^{f}
purified enzyme	1 U/mL	4.33±0.3^{a}	10.8±0.3^{b}	16.9±0.5^{c}	19.0±0.5^{d}	21.4±0.5^{e}	22.9±0.8^{f}	23.3±0.3^{f}	23.5±0.3^{f}
crude enzyme	5 U/mL	4.33±0.3^{a}	7.56±0.8^{b}	14.3±0.8^{c}	18.3±0.8^{d}	20.0±0.3^{e}	21.6±0.4^{f}	22.4±0.7^{f}	24.0±0.6^{g}
commercial enzyme	5 U/mL	4.33±0.3^{a}	9.12±0.5^{b}	16.1±0.7^{c}	22.1±0.7^{d}	24.1±0.6^{e}	25.5±0.8^{f}	26.9±0.7^{g}	27.6±0.6^{g}
purified enzyme	5 U/mL	4.33±0.3^{a}	11.6±0.5^{b}	19.0±0.3^{c}	22.3±0.7^{d}	24.0±0.9^{e}	24.0±0.6^{e}	25.5±0.4^{f}	26.5±0.2^{f}
crude enzyme	10 U/mL	4.33±0.3^{a}	8.93±0.5^{b}	16.8±0.5^{c}	18.8±0.7^{d}	21.5±0.9^{e}	22.3±0.6^{ef}	23.1±0.4^{f}	24.1±0.8^{f}
commercial enzyme	10 U/mL	4.33±0.3^{a}	10.3±0.6^{b}	19.6±0.4^{c}	23.3±0.6^{d}	26.0±0.7^{e}	27.6±0.6^{f}	28.8±0.7^{f}	28.5±0.5^{f}
purified enzyme	10 U/mL	4.33±0.3^{a}	17.4±0.3^{b}	20.0±0.9^{c}	24.4±0.4^{d}	24.9±0.3^{d}	26.9±0.5^{e}	27.6±0.7^{e}	29.5±0.5^{f}

Note: Experiments were performed in duplicate, and the standard deviation was <5%. Different letters indicate means with significant differences ($P<0.05$).

BIOGRAPHY

Numphon Thaiwong was born on June9th, 1976 in Suphanburi, Thailand. She attended Satri Wat Mahapruettaram High School (1992-1994), and in 2000 she received the Bachelor degree of Agro-Indrustry (Product Development) from Kasetsart University, Bangkok, Thailand. From 2000-2001 she worked as production supervisor at the Union Frozen Products Co., Ltd., Samutsakhon. She later worked as R&D supervisor at Thai Fishsauce Factory (Squid brand) Co., Ltd., Bangkok (2002-2003) and at Thai Spirit Industry Co., Ltd., Chachoengsao (2007-2008). In 2008 she received the degree of Master of Science (Food Sanitation) from King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. In 2008-2010 she worked as quality system and R&D consultant at IQS Management Co., Ltd., Bangkok. In 2010-2015 she received a scholarship for the degree of Doctor of Philosophy in Food Technology at Suranaree University of Technology (SUT). During her graduate study she presented her research at the 5th KKU International Engineering Conference (KhonKaen, Thailand, 28–29 March, 2014) under the title of "Production of Recombinant β-galactosidase in *Lactobacillus plantarum*, using a pSIP-Based Food-Grade Expression System". She then published this research project in Advanced Materials Research Journal. In the last year of her PhD she received a scholarship from the Erasmus Mundus project TECHNO to participate in the PhD exchange program for 6 months at BOKU University of Natural Resources and Life Sciences, Vienna, Austria, (1 October, 2014 – 31 March, 2015).