SYNTHESIS OF ALKYL GLYCOSIDES BY TRANSGLYCOSYLATION REACTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE

BY

MR. RITTICHAI CHAROENSAPYANAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF THE DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY FACULTY OF MEDICINE THAMMASAT UNIVERSITY ACADEMIC YEAR 2016

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ENTITLED

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was approved as partial fulfillment of the requirements for
the degree of Doctor of Philosophy in Biochemistry and Molecular Biology

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Dissertation Title	SYNTHESIS OF ALKYL GLYCOSIDES BY TRANSGLYCOSYLATION REACTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE
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ABSTRACT

Alkyl glycosides are non-ionic surfactants which have been applied in a variety of household, industrial, biotechnological and pharmaceutical levels. In this study, alkyl glycosides of propyl glycosides ($ProG_n$), pentyl glycosides ($PenG_n$) and isopentyl glycosides ($IsoPenG_n$) were enzymatically synthesized by recombinant cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* A11 using β-cyclodextrin as donor and 1-propanol, 1-pentanol and isopentanol as acceptors. When the reaction products were analyzed by thin layer chromatography (TLC), it was found that each alcohol acceptor gave at least three alkyl glycoside products. These products were assumed to be alkyl glycoside derivatives having one to three glucose units attached to the alkyl groups of each alcohol. After the reactions were optimized and prepared in larger scale, the 37, 48 and 42% (w/w) yields of $ProG_n$, $PenG_n$ and $IsoPenG_n$ were obtained, respectively. Two products of each glycoside derivative were then isolated by preparative TLC and their structures were identified by the combination of amylolytic enzymes treatment, mass spectrometry and NMR analyses to be propyl-α-D-glucopyranoside ($ProG_1$), propyl-α-D-maltopyranoside ($ProG_2$), pentyl-α-D-glucopyranoside ($PenG_1$), pentyl-α-D-maltopyranoside ($PenG_2$), isopentyl-α-D-glucopyranoside ($IsoPenG_1$) and isopentyl-α-D-maltopyranoside ($IsoPenG_2$). The emulsifying properties of the

synthesized products were determined by measuring their ability to form and stabilize oil-in-water emulsion using n-hexadecane as substrate. The results showed that $PenG_2$ was the best emulsifying agent as compared to the other synthesized products. The antibacterial activity of the synthesized products was also tested by disc diffusion technique, minimal inhibitory concentration and minimal bactericidal concentration against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The results revealed that the structure and length of alkyl chain and the number of glucose had an effect on the antibacterial activity of alkyl glycosides. $PenG_2$ which contained linear primary pentyl chain and disaccharide exhibited the highest antibacterial activity and was the most effective agent for inhibiting *E. coli* growth. Furthermore, the addition of $PenG_2$ to the insoluble *Corynebacterium glutamicum* amylomaltase protein during cell lysis resulted in the increasing of both solubility and activity of amylomaltase. From these biological properties, it can be concluded that these synthesized alkyl glycosides could be applied for the use as emulsifying, antibacterial and insoluble protein solubilizing agents.

Keywords: Antibacterial activity, Cyclodextrin glycosyltransferase, Emulsifying properties, Insoluble protein solubilization, Isopentyl glycosides, Pentyl glycosides, Propyl glycosides

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Mr. Rittichai Charoensapyanan

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percentage
/	Per
<	Less than
=	Equivalent
>	More than
±	Plus-minus
×	Multiplication
Δ	Delta
°C	Degree celsius
µg	Microgram
µL	Microlitre
µmol	Micromole
A	Absorbance
CDs	Cyclodextrins
CFU	Colony forming unit
CGTase	Cyclodextrin glycosyltransferase
cm	Centimetre
CMC	Critical micelle concentration
E.C.	Enzyme commission number
et al.	Et alii, and colleagues
g	Gram
$IsoPenG_1$	Isopentyl-α-D-glucopyranoside
$IsoPenG_2$	Isopentyl-α-D-maltopyranoside
$IsoPenG_3$	Isopentyl-α-D-maltotriopyranoside
K_d	Dissociation constant
kDa	Kilodalton
M	Molar
m/z	Mass-to-charge ratio

mA	Milliamp
MBC	Minimal bactericidal concentration
mg	Milligram
MG_1	Methyl-α-D-glucopyranoside
MHz	Megahertz
MIC	Minimal inhibitory concentration
mL	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
MS	Mass spectrometry
nm	Nanametre
NMR	Nuclear magnetic resonance
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
$PenG_1$	Pentyl-α-D-glucopyranoside
$PenG_2$	Pentyl-α-D-maltopyranoside
$PenG_3$	Pentyl-α-D-maltotriopyranoside
$ProG_1$	Propyl-α-D-glucopyranoside
$ProG_2$	Propyl-α-D-maltopyranoside
$ProG_3$	Propyl-α-D-maltotriopyranoside
R_f	Relative mobility
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
U	Unit(s)
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
α-CD	α-Cyclodextrin
β-CD	β-Cyclodextrin
γ-CD	γ-Cyclodextrin

CHAPTER 1
INTRODUCTION

1.1 Background and rationale

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) is a member of the α-amylase family of glucosyl hydrolases, which can catalyze four reactions: cyclization, coupling, disproportionation and hydrolysis reactions. These different catalytic reactions of the enzyme lead to several researches in the synthesis of novel glycosides. Recently, CGTase has been used to transfer α-1,4-linked oligosaccharides from glycosyl donor molecules to interesting organic compounds. The obtained glycosides from this transfer showed the new physicochemical and biological properties. Ones of the organic compounds such as alcohols were also used as glycosyl acceptor molecules to produce alkyl glycosides.

Alkyl glycosides are environmentally friendly surfactants which composed of an alkyl hydrophobic and sugar hydrophilic parts. They exhibit the good water solubility, antibacterial, protein and lipid solubilization, and mild detergent properties. Therefore, the demand of these surfactants in many industries is rapidly increased. As reported, alkyl glycosides can be synthesized by both chemical and enzymatic routes. However, the advance in enzyme technology shows more advantageous than chemical method. The enzymatic syntheses can produce alkyl glycosides by incubating raw materials such as alcohols and carbohydrates with the enzyme. Several types of enzymes from many organisms such as mannosidases, galactosidases and, as indicated in most reports on glucosidases have been studied to achieve alkyl glycoside production. For CGTases, they have been used much less for alkyl glycoside synthesis than other amylolytic enzymes. The use of CGTase for alkyl glycoside synthesis is of great interest because this enzyme can synthesize alkyl glycoside products having one or more monosaccharide units.

CGTase has previously reported for alkyl glycoside syntheses using cyclodextrins (CDs) and linear short chain alcohols i.e. methanol, ethanol and 1-butanol as substrates. However, the new developments in the use of CGTase for the synthesis of alkyl glycosides with longer hydrophobic and hydrophilic chains are still

more attention for several research groups. Due to the fact that the surfactant properties as well as the applications of alkyl glycosides are depend on their structure and length of alkyl and carbohydrate groups. Thus, in this study, more attention has been paid to the enzymatic synthesis of medium chain alkyl glycosides (linear and iso alcohols) having one or more glucose units. The enzymatic reactions are catalyzed by recombinant CGTase from *Bacillus circulans* A11 using β-CD as a glucosyl donor. In addition, the synthesized alkyl glycosides are investigated their properties such as emulsifying, antibacterial and insoluble protein solubilizing properties.

1.2 Objectives of this study

1.2.1 To purify and characterize the recombinant CGTase from *Bacillus circulans* A11.

1.2.2 To establish an optimal process for the synthesis of alkyl glycosides.

1.2.3 To identify the structure of the synthesized alkyl glycosides.

1.2.4 To investigate the properties of the synthesized alkyl glycosides such as emulsifying, antibacterial and insoluble protein solubilizing properties.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) is an extracellular starch degrading enzyme and belongs to glycosyl hydrolase family 13 or α-amylase family. This large family contains most important starch processing enzymes such as hydrolases, transglycosidases, debranching enzymes and isomerases (MacGregor, Janeček, & Svensson, 2001). These enzymes use a similar α-retaining bond cleavage mechanism to process their substrates because they have a similar catalytic site residues. However, the major difference is the inclination for hydrolysis or transfer reactions and specificity for α-1,4 or α-1,6 glycosidic bonds (McCarter & Withers, 1994).

CGTase can catalyze α-1,4-linked glucan in four main ways: cyclization, coupling, disproportionation and hydrolysis reactions. On their first reactions, CGTase converts starch to non-reducing cyclic α-1,4-maltooligosaccharides product (cyclodextrins, CDs) through an intra-molecular transglycosylation reaction called cyclization. CGTase also presents the inter-molecular transglycosylation reactions which are composed of the coupling and disproportionation reactions. The coupling reaction is the reversible reaction of cyclization which CDs are opened and the obtained α-1,4-linked linear glucan is transferred to an acceptor molecule. The cyclization and coupling reactions are shown below:

$$(\alpha\text{-}1,4\text{-glucan})_m \leftrightarrow \text{cyclic } (\alpha\text{-}1,4\text{-glucan})_x + (\alpha\text{-}1,4\text{-glucan})_{m-x}$$

For disproportionation, CGTase catalyzes this reaction by transferring the glycosyl groups from α-1,4-glucan donor molecule to another oligosaccharide acceptor as shown in the following equation:

$$(\alpha\text{-}1,4\text{-glucan})_m + (\alpha\text{-}1,4\text{-glucan})_n \rightarrow (\alpha\text{-}1,4\text{-glucan})_{m-x} + (\alpha\text{-}1,4\text{-glucan})_{n+x}$$

In addition to intra- and inter- molecular transglycosylation reactions, CGTase shows a lesser extent of hydrolysis reaction on α-1,4-glucan (van der Veen, van Alebeek, Uitdehaag, Dijkstra, & Dijkhuizen, 2000). All four reactions are summarized in Figure 2.1.

2.2 The occurrence of CGTase

Bacteria are the only known source of CGTase. The main function of CGTase in bacteria is the production of external CDs (Figure 2.2). These CDs are transported into the cell and metabolized them to release glucose, while other organisms are not accessible and able to metabolize (Thombre & Kanekar, 2016). Since the first discovery of capable CGTase producer secreted by *Bacillus macerans* (Takano et al., 1986), CGTases have been successively isolated and biochemically characterized from a variety of microorganisms, especially in *Bacillus* species, for example, *Bacillus firmus* (Gordon, Hyde, & Moore, 1977), *Bacillus cereus* (Jamuna, Saswathi, Sheela, & Ramakrishna, 1993) and *Bacillus circulans* (Vassileva, Atanasova, Ivanova, Dhulster, & Tonkova, 2007). In addition, CGTases are also discovered from *Thermoanaerobacterium thermosulfurigenes* (Wind et al., 1995) and *Klebsiella pneumoniae* (Gawande & Patkar, 2001). CGTases are classified into three subgroups: α-CGTase, β-CGTase and γ-CGTase according to their main types of CDs produced, and enzymes from different sources exhibit different properties as shown in Table 2.1.

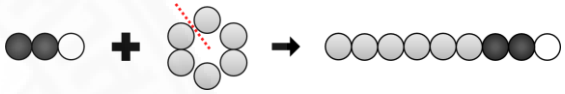
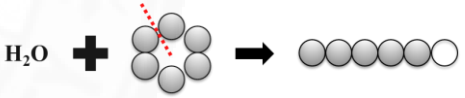
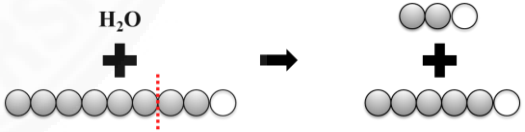
Reaction	Action (equation)	Action (picture)
(A) Cyclization	$(\text{linear glucan})_m$ ↓ $(\text{cyclic glucan})_x + (\text{linear glucan})_{m-x}$	
(B) Coupling	$(\text{cyclic glucan})_x + (\text{linear glucan})_m$ ↓ $(\text{linear glucan})_{m+x}$	
(C) Disproportionation	$(\text{linear glucan})_m + (\text{linear glucan})_n$ ↓ $(\text{linear glucan})_{m-x} + (\text{linear glucan})_{n+x}$	
(D) Hydrolysis	H_2O + cyclic glucan ↓ linear glucan	
	$H_2O + (\text{linear glucan})_m$ ↓ $(\text{linear glucan})_x + (\text{linear glucan})_{m-x}$	

Figure 2.1 Schematic representation of the CGTase catalyzed reactions (A) Cyclization (B) Coupling (C) Disproportionation and (D) Hydrolysis. Glucose residues (○ and ●) and Glucose residues with reducing end (○) (modified from van der Veen et al., 2000)

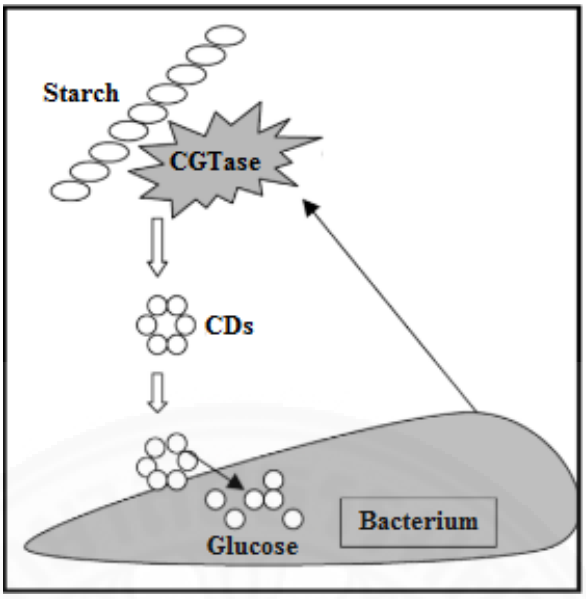


Figure 2.2 Schematic representation of the function of CGTase (copy from Thombre & Kanekar, 2016)

Table 2.1 Properties of some bacterial CGTases (modified from Tonkova, 1998)

Producer	Optimum pH	Optimum temp. (°C)	Mass (Da)	Main CD produced	Ref.
Bacillus firmus	7.5-8.5	65	75,000	γ-CD	1
Bacillus macerans IAM 1243	No data	No data	74,000	α-CD	2
Bacillus sp. AL-6	7.0-10.0	60	74,000	γ-CD	3
Bacillus cereus NCIMB	5.0	40	No data	α-CD	4
Thermoanaerobacterium sp. EM1	4.0-4.5	80-95	68,000	β-CD	5
Bacillus stearothermophilus ET1	6.0	80	67,000	β-CD	6
Paenibacillus sp. A11	6.0-7.0	40-60	72,000	β-CD	7
Brevibacillus brevis CD162	8.0	55	75,000	β-CD	8
Thermococcus sp. B1001	7.0	85	83,000	α-CD	9
Paenibacillus sp. RB01	6.0	55	65,000	β-CD	10
Klebsiella pneumoniae AS-22	5.5-9.0	35-50	75,000	α-CD	11
Thermococcus kodakaraensis	5.5-6.0	80	77,000	β-CD	12
Bacillus sp. G1	6.0	60	75000	β-CD	13
Bacillus circulans ATCC 21783	6.0-8.6	65	66,000	β-CD	14
Bacillus lehensis	7.0	60	74,000	β-CD	15

[1](Yim, Sato, Park, & Park, 1997); [2](Takano et al., 1986); [3](Fujita et al., 1990); [4](Jamuna et al., 1993); [5](Wind et al., 1995); [6](Chung et al., 1998); [7](Kaskangam, 1998); [8](Kim, Sohn, & Oh, 1998); [9](Tachibana et al., 1999); [10](Tesana, 2001); [11](Gawande & Patkar, 2001); [12](Rashid et al., 2002); [13](Sian et al., 2005); [14](Vassileva et al., 2007); [15](Elbaz, Sobhi, & ElMekawy, 2015)

2.3 The structure of CGTase

CGTase shows the apparent feature of its primary structure, and enzymes from different sources have 47%-99% similarity in the amino acid sequence (van der Veen et al., 2000). In comparison of the amino acid sequence of CGTases, there are 31 amino acid residues located in the four conserved amino acid sequence regions of α-amylase family (Machovič & Janeček, 2007). These residues are related to the catalytic activity and substrate binding site of CGTase. The arrangement of conserved regions of α-amylase family is shown in Table 2.2. The differences in primary structure of CGTases are closely related to the product-specificity.

The secondary and tertiary structures of CGTases are analyzed by X-ray crystallographic technique. A typical CGTase consists of five structural domains (labeled A to E domains) as shown in Figure 2.3. Domain A is the main catalytic structural domain which found in common with other α-amylase family members. The structure of domain A is an eight parallel β-sheet in closed state outsourcing eight α-helix, which is also called $(\alpha/\beta)_8$ barrel or TIM barrel structure. The three important catalytic residues such as Asp229, Glu257 and Asp328 are located at the C-terminal ends of the β-strands of this domain. Domain B is an extended loop region which embedded between the third β-strand and the third α-helix of domain A. Domain B contributes to substrate binding by providing several amino acid side chains alongside a long groove on the surface of the enzyme that interacts with the substrate. Domains C to E have a β-sheet structure. Domain C has a similar antiparallel β-sandwich structure of the C-terminal with that of amylase structure. This domain is known to be a substrate binding site and stability function. Domain E is a raw starch binding domain and has two maltose binding sites (MBS). MBS1 has a large capacity of binding amylose and consists of important Trp616 and Trp662 residues, while Tyr633 is relatively important in MBS2 that is responsible for guiding the substrate to the active site center (Lawson et al., 1994; Penninga et al., 1996). For domain D, which is almost exclusively found in CGTase, its function is still unclear. However, Rimphanitchayakit and co-workers (2005) investigated the function of domain D by assembling all structural domains of CGTase from different strains and reported that domain D had effect on the enzyme reaction and could help in the space

Table 2.2 Conserved regions of enzymes in α-amylase family (modified from Takaha & Smith, 1999; Machovič & Janeček, 2007).

Enzyme	**Species**	**Region 1**[a,b]	**Region 2**[a,b]	**Region 3**[a,b]	**Region 4**[a.b]
CGTase	*Bacillus circulans* 251	133 IIDFAPN**H**	226 IRM**D**AVKH	253 FTFG**E**WFL	322 TFIDNH**D**
	Bacillus sp. 1011	133 IIDFAPN**H**	226 IRV**D**AVKH	253 FTFG**E**WFL	322 TFIDNH**D**
	Bacillus stearothermophilus	129 IIDFAPN**H**	222 IRM**D**AVKH	249 FTFG**E**WFL	318 TFIDNH**D**
	Thermoanaerobacterium sp.	134 IIDFAPN**H**	227 IRL**D**AVKH	254 FTFG**E**WFL	323 TFIDNH**D**
	Klebsiella pneumoniae	128 VLDYAPN**H**	120 IRI**D**AIKH	253 FFFG**E**WFG	327 VFMDNH**D**
Debranching enzyme	Human muscle	196 ITDVVYN**H**	506 VRL**D**NCHS	534 YVVA**E**LFT	604 FMDITH**D**
	Caenorhabditis elegans	358 VQDVVWN**H**	672 LRI**D**NAHG	700 YVFA**E**LFT	770 FLDQSH**D**
	Saccharomyces cerevisiae	222 LTDIVFN**H**	532 FRI**D**NCHS	560 YVVA**E**LFS	664 FMDCTH**D**
α-Amylase	*Aspergillus oryzae*	115 MVDVVAN**H**	202 LRI**D**TVKH	226 YCIG**E**VLD	291 TFVENH**D**

[a] Numbers indicate the amino acid positions in each polypeptide.

[b] Bold fonts indicate the proposed catalytic residues.

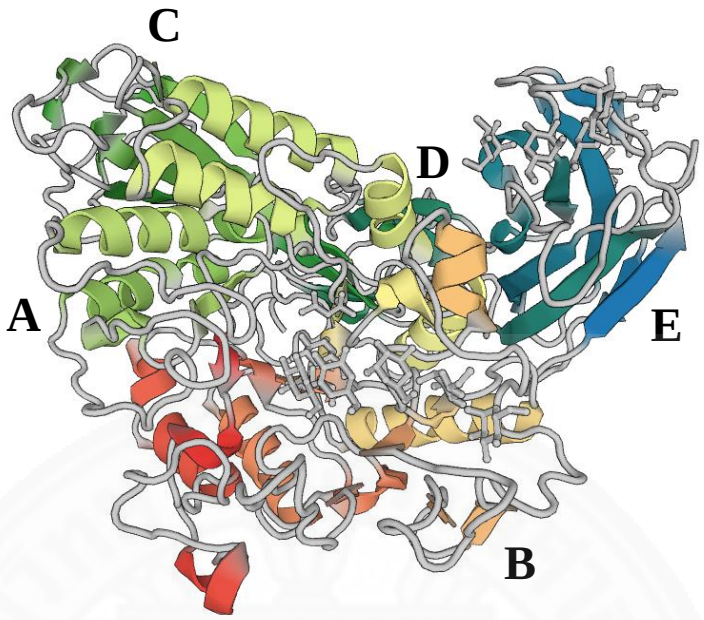


Figure 2.3 Stereo-view of the structure of *B. circulans* strain 251 β-CGTase. Domains A to E are labeled. (copy from Uitdehaag & Dijkstra, 2000)

orientation of domains A, B and E. In addition to these five domains, tandem subsite architecture is found in the substrate binding groove at the active site center of CGTase with at least nine subsites (Davies, Wilson, & Henrissat, 1997). Each monomer-binding site is given a number from -7 to +2, starting from the bond that is to be cleaved. The subsites toward the non-reducing end are donor sites, while those toward reducing end are acceptor sites. These donor and acceptor sites are given as positive and negative numbers, respectively (Figure 2.4).

In the structural comparison to other enzymes in α-amylase family, there are two different structural features between CGTase and α-amylases. One major difference observed is the presence of a Tyr195 in the catalytic site in CGTase as shown in Figure 2.5, while α-amylases have a smaller side chain amino acid. The mutating Tyr195 to glycine or leucine resulted in significant reduction of coupling and cyclization activities. Many studies suggested that Tyr195 and other aromatic residues of CGTase acts as cyclization axis during the formation of CDs (Penninga et al., 1995; Uitdehaag, van der Veen, Dijkhuizen, Elber, & Dijkstra, 2001). Another difference is an extended loop in amylases that block the binding site of substrate at -3 and -4 sites. This loop found to be a factor in interruption of cyclization and disproportionation activites (Leemhuis, Kragh, Dijkstra, & Dijkhuizen, 2003). Moreover, there are many factors which may have an effect on the ratio of hydrolysis and transglycosylation activities in α-amylases. For example, the A230V mutation was found to reduce the cyclization and disproportionation activities, while the catalytic rate of hydrolysis was improved. This mutation in the subsite +1 inhibited the binding substrate at the acceptor sites (Leemhuis, Rozeboom, Wilbrink, et al., 2003). It has been reported that an induced-fit mechanism may happen after binding of substrate (not water) at the acceptor site, which activates the catalysis action of CGTase (Leemhuis, Uitdehaag, Rozeboom, Dijkstra, & Dijkhuizen, 2002). The S77P mutation changes conformation of tyrosine in the same strand due to a loss of hydrogen bond, which in turn affects the hydrogen bond to an arginine. This arginine directly interacts with catalytic acid/base and the distortion requires substrate binding to restore the active conformation of the catalytic acid/base, resulting in the increasing of the cyclization ratio (Figure 2.6) (Kelly et al., 2008).

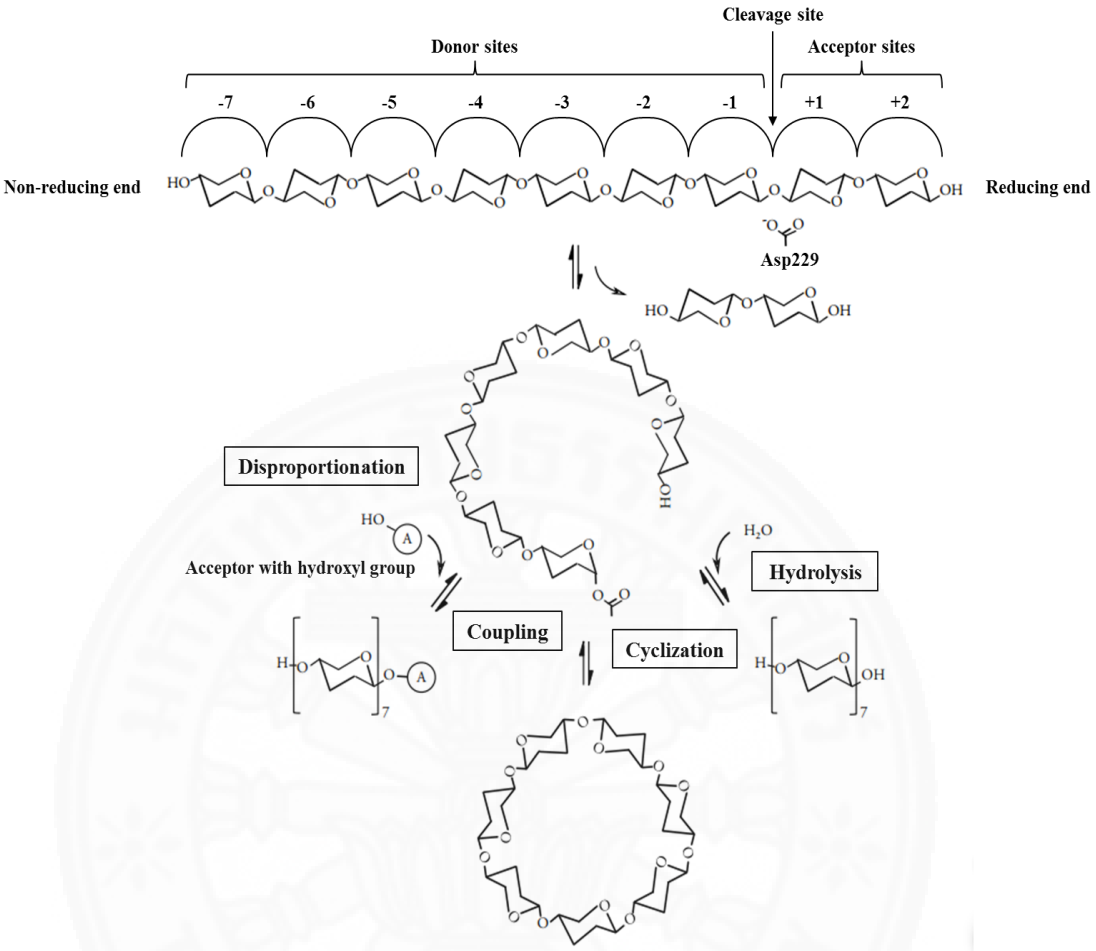


Figure 2.4 Schematic representation of the active site and the catalytic reactions of CGTase (modified from Uitdehaag, van der Veen, Dijkhuizen, & Dijkstra, 2002)

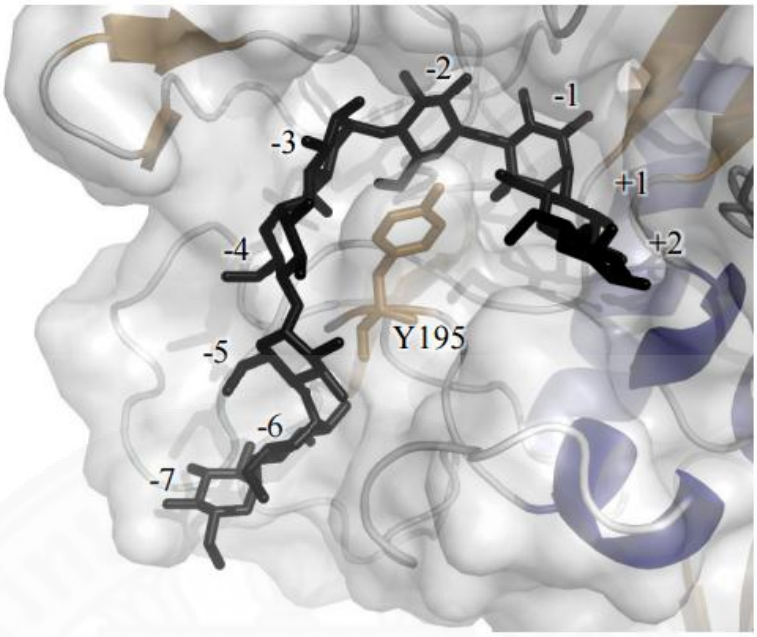


Figure 2.5 Maltononaose substrate binding at the active site center of *B. circulans* A251 CGTase with Tyr195 (copy from Uitdehaag et al., 2001)

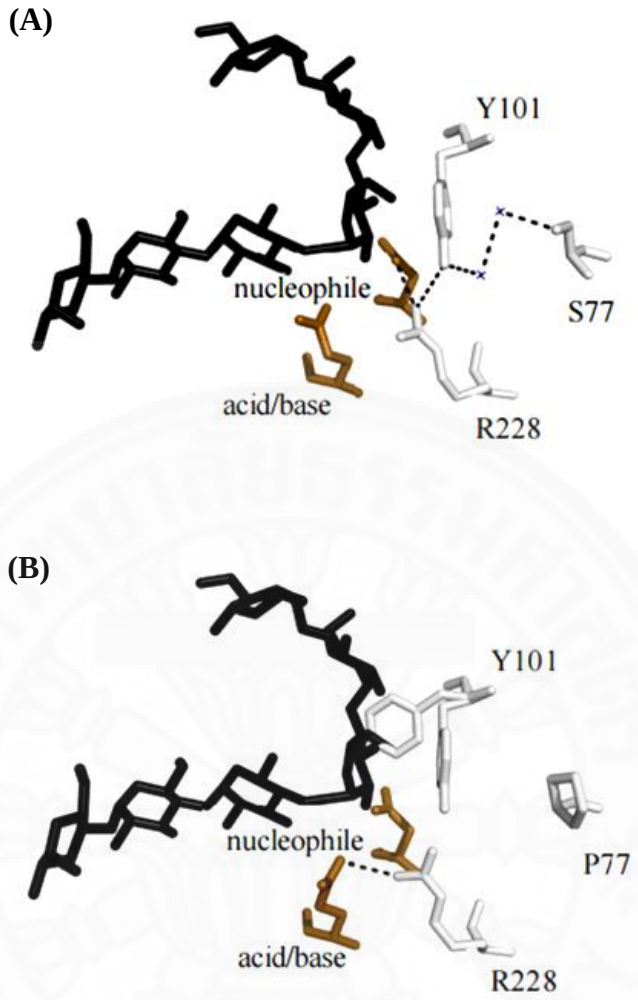


Figure 2.6 Hydrogen bonding network of the (A) wild type and (B) S77P mutated *Thermoanaerobacterium thermosulfurigenes* CGTases (copy from Kelly et al., 2008)

2.4 The catalytic mechanism of CGTase

The catalytic mechanism of enzymes in the α-amylase family is known to act by two amino acid residues *via* a general acid/base catalysis mechanism. An acid/base catalyst (usually glutamic acid in CGTase) and nucleophile (usually aspartic acid in CGTase) residues participate in a single- or double- displacement reactions, resulting in inversion or retention of configuration at the anomeric carbon atom of the hydrolyzed glycoside, respectively (Sinnott, 1990). For inversion mechanism, the catalytic acid residue of the enzyme (Glu257) donates a proton to the anomeric carbon, while water molecule which is removed a proton by catalytic base residue (Asp229) attacks on the anomeric center of the sugar (Figure 2.7(A)).

In case of CGTase, enzyme proceeds with retention of the substrate's anomeric α-configuration *via* a double displacement reaction involving a covalent enzyme-intermediate complex as shown in Figure 2.7(B). With retention mechanism, a general acid/base catalyst works first as an acid and then as base in two steps: glycosylation and deglycosylation. The first step is the departure of the leaving saccharide group. When the substrate binds to the active site center, many important amino acid residues play a role in the interactions between CGTase and substrate as shown in Figure 2.8. The general acid (Glu257) subsequently donates a proton to the glycosyl oxygen atom of the glucose residue at the subsite +1, while the nucleophile (Asp229) forms an enzyme confiscated covalent intermediated by attack the C1 of non-reducing end glucose residue at subsite -1. During reaction, the substrate goes through an oxocarbenium ion-like transition state. Then, the protonated glucose molecule at subsite +1 leaves the active site (break the substrate strand). In the second step, to activate acceptor molecule, the deprotonated Glu257 acts as a general base to accept a hydrogen ion from acceptor molecule at subsite +1. The oxygen of the activated acceptor molecule carries out a nucleophilic attack on the covalent glycosyl-enzyme intermediate (covalent bond between the non-reducing end glucose at subsite -1 and the aspartate residue) *via* another oxocarbenium ion-like transition state, resulting in net retention of configuration at the anomeric center. Thus, the final α-1,4-glycosidic product bond is formed. In addition to Glu257 and Asp229, the third important residue (Asp328) is believed to function by maintaining the proper

protonation state of Glu257 and stabilizes the transition state of the reaction by interaction with the OH-2 and OH-3 groups of the substrate. It has been reported that the hydrogen bond at the OH-2 group could become a strong low-barrier hydrogen bond that stabilizes the transition state of the reaction (Matsuura, Kusunoki, Harada, & Kakudo, 1984; Strokopytov et al., 1995; Brzozowski & Davies, 1997). Based on this mechanism, the acceptor molecule which binds at the acceptor site can be water (hydrolysis), the non-reducing end of the glucose of donor (cyclization) or other acceptors with a hydroxyl group (disproportionation and coupling) as shown in Figure 2.4 .

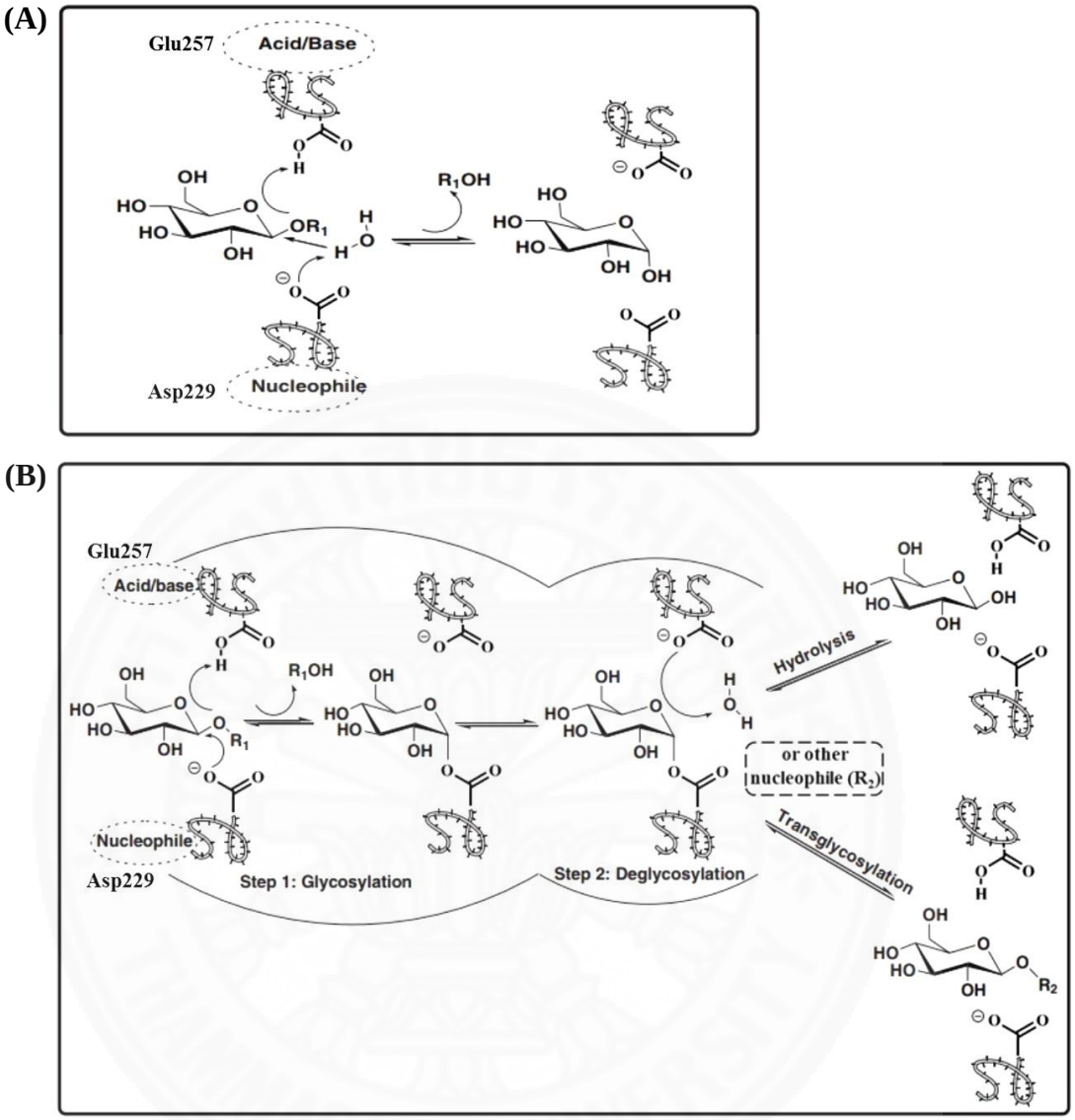


Figure 2.7 The cleavage modes of enzymes in α-amylase family. (A) Inversion and (B) Retention mechanisms (modified from Rather & Mishra, 2013)

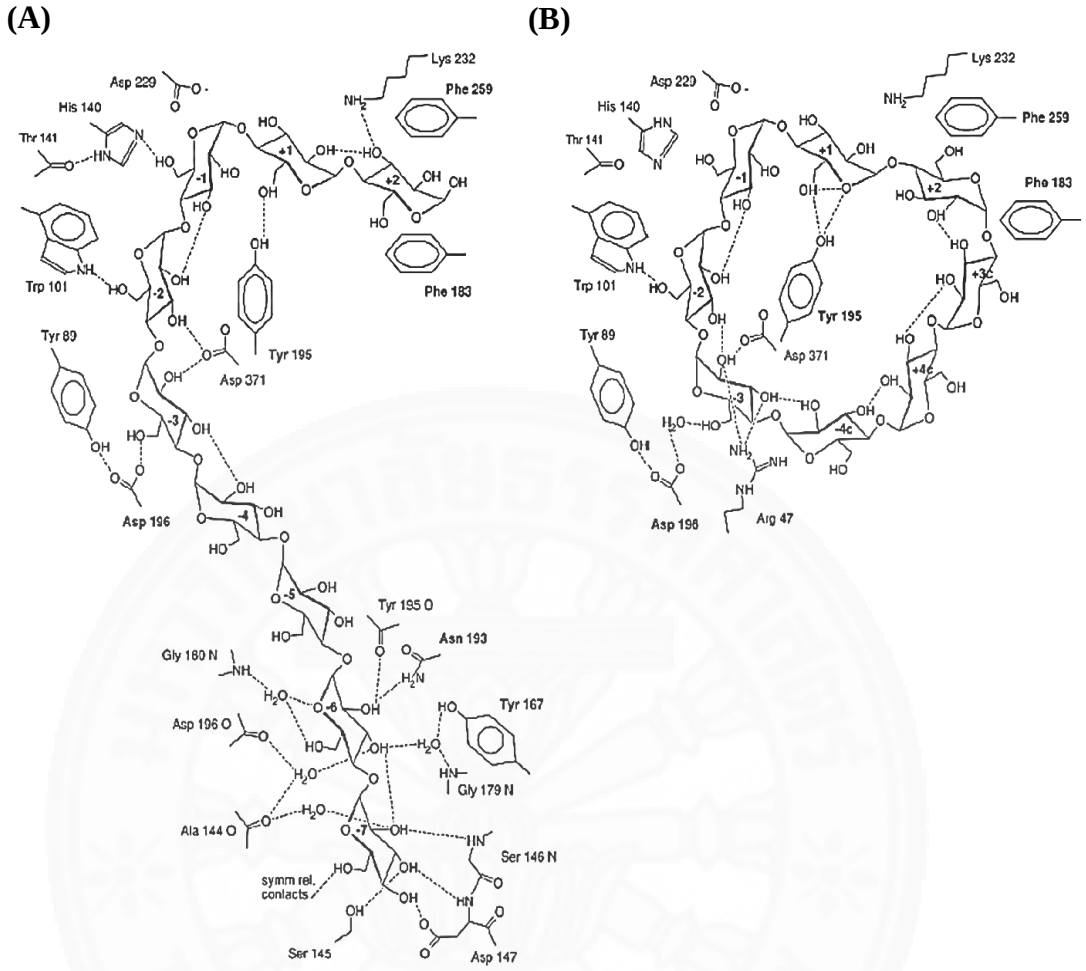


Figure 2.8 Schematic representation of the interactions between CGTase and (A) maltononaose or (B) γ-CD (copy from Uitdehaag, Kalk, van der Veen, Dijkhuizen, & Dijkstra, 1999)

2.5 Application of transglycosylation reaction of CGTase

From the catalytic machinery of CGTase in the transglycosylation reaction (transferring from donor to acceptor), CGTase has been utilized for many industrial applications of saccharide and glycoside products:

2.5.1 Synthesis of cyclodextrins (CDs)

CGTase is generally used to synthesize CDs from starch *via* the cyclization reaction, which is the basis of their industrial application. CDs exhibit helical structure and hydrophobic channel, therefore, they show the ability to form an inclusion complex with a variety of hydrophobic compounds by molecular complexation. This can lead to the change of physiological and chemical properties of guest molecules. Recently, CDs are applicable to be used in food science, biotechnology, analytical chemistry, agriculture and pharmaceutical. For example, CDs are used as drug containers, stabilizer agents and artificial chaperones for protein refolding (Rajewski & Stella, 1996; Machida et al., 2000; Tomono et al., 2002; Challa, Ahuja, Ali, & Khar, 2005; Loftsson, Jarho, Másson, & Järvinen, 2005).

2.5.2 Synthesis of novel glycosides

The recent developments also concentrate on the use of the CGTase catalyzed coupling and disproportionation reactions for the synthesis of novel gluco-conjugates by using alternative acceptor substrates. There are many groups of substances which are able to be used as a nucleophile acceptor for CGTase to produce the useful glycosides. Examples are as follows:

(1) Aga and co-workers (1991) reported on the use of CGTase from *B. stearothermophilus* in the production of 2-O-α-D-glucopyranosyl L-ascorbic acid (ascorbyl glycosides). The result was a greater stability under oxidative conditions through the coupling reaction using α-CD and ascorbic acid as a glycosyl donor and acceptor, respectively.

(2) Kitahata (2000) produced the coupling sugar, called maltooligosyl sucrose, from the mixture of starch hydrolyzate donor and sucrose acceptor using disproportionation reaction of several *Bacillus* sp. CGTases. The coupling sugar has half sweetness of sucrose, prevents starch ingredients from

retrogradation and retains moisture of foods. Furthermore, this product was studied its cariogenicity and proved to be an antcariogenic sweetener by Japanese National Insitute of Health.

(3) Aramsangtienchai and co-workers (2011) reported the efficient synthesis of epicatechin glycosides *via* coupling reaction catalyzed by CGTase from *Paenibacillus* sp. using β-CD and epicatechin as glycosyl donor and acceptor, respectively. The obtained epicatechin glycosides showed the high water solubility and stability against UV irradiation properties. The advantage of the enhanced properties makes the epicatechin glycosides more beneficial as food ingredient than their parent compounds.

(4) Mathew and co-coworkers (2012) synthesized piceid glycosides using piceid as an acceptor and maltodextrin as glycosyl donor through the disproportionation reaction catalyzed by CGTase from *B. macerans*. Piceid is the main component of *Polygonum cuspidatum* root and widely used as cardiac drugs. The adsorbability in the digestive tracts of the picied conjugated glucose was significantly higher than that of piceid.

2.5.3 Synthesis of alkyl glycosides

In addition to saccharides and different natural compounds, alcohols can also be used as acceptor for transglycosylation reaction of CGTase to produce alkyl glycoside. Alkyl glycosides are amphipathic non-ionic surfactants which become dominant components in the products of food, detergent, cosmetic, pharmaceutical and protein research as a result of their emulsifying, antibacterial and protein solubilizing properties (Matsumura, Imai, Yoshikawa, Kawada, & Uchibori, 1990; Sarney & Vulfson, 1995; Jones, 1999). In the initial use of CGTase for the synthesis of alkyl glycosides, it has only been used to elongate the length of sugar group of commercially available alkyl glycosides *via* its coupling reaction. For example, Yoon and Robyt (2006) used methyl- and phenyl- glucoside in α- and β-configurations as acceptors and α-CD as donor to yield the longer maltodextrin glycosides by the activity of CGTase from *B. macerans*. The dodecyl-β-D-maltooctaoside was synthesized from dodecyl-β-D-maltoside as acceptor and α-CD as donor with the action of CGTase from *B. macerans* (Svensson, Ulvenlund, & Adlercreutz, 2009). However, in the year 2011, Chotipanang and co-workers (2011)

discovered a new characteristic of CGTase from *Paenibacillus* sp. RB01 in the synthesis of methyl glycosides with one-to-several glucose residues in alcohol-buffer system. The reaction was performed using 1-methanol acceptor and β-CD donor.

2.6 Cyclodextrins (CDs)

2.6.1 Structure and characteristic of CDs

CDs are the oligomers of anhydroglucose units that join to form a ring structure with α-1,4-glycosidic bonds. CDs produced from CGTase consisted of 6 to 8 units of glucose namely α-CD, β-CD and γ-CD, respectively. Figure 2.9 demonstrates the structure of CDs. Each of the glucose unit is in the rigid $^4C_1$ chair conformation. CDs are the ring of conical cylinder structure which is frequently characterized as a doughnut or wreath-shaped truncated cone (Szejtli, 1998; Cserháti & Forgács, 2003). The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges with the secondary hydroxyl groups (C-2 and C-3) on the wider edge of the ring and the primary hydroxyl groups (C-6) on the other edge. The side of located secondary hydroxyl groups has the diameter of the cavity larger than the side with the primary hydroxyls because free rotation of the latter reduces the effective diameter of the cavity. The non-bonding electron pairs of the glycosidic oxygen bridges (the apolar C-3 and C-5 hydrogens and ether-like oxygens) are at the inside of the cavity. These CDs have a hydrophilic outer surface which can dissolve in water, and a hydrophobic cavity which provides a hydrophobic guest molecule. Furthermore, the C-2-OH group of one glucose unit can form a hydrogen bond with C-3-OH group of the near glucose unit. These H-bonds involve in the formation of a secondary belt of CDs. In the β-CD molecule, a complete secondary belt is formed. As a result, the β-CD has a rather rigid structure and lowest water solubility of all CDs, while the H-bond belt is incomplete in the α-CD because one glucose unit is in a distorted position, only four H-bonds can be established fully instead of six possible bonds. Finally, γ-CD is a non-coplanar and more flexible structure. Therefore, it is the most soluble of all CDs. The main properties of CDs are summarized in Table 2.3.

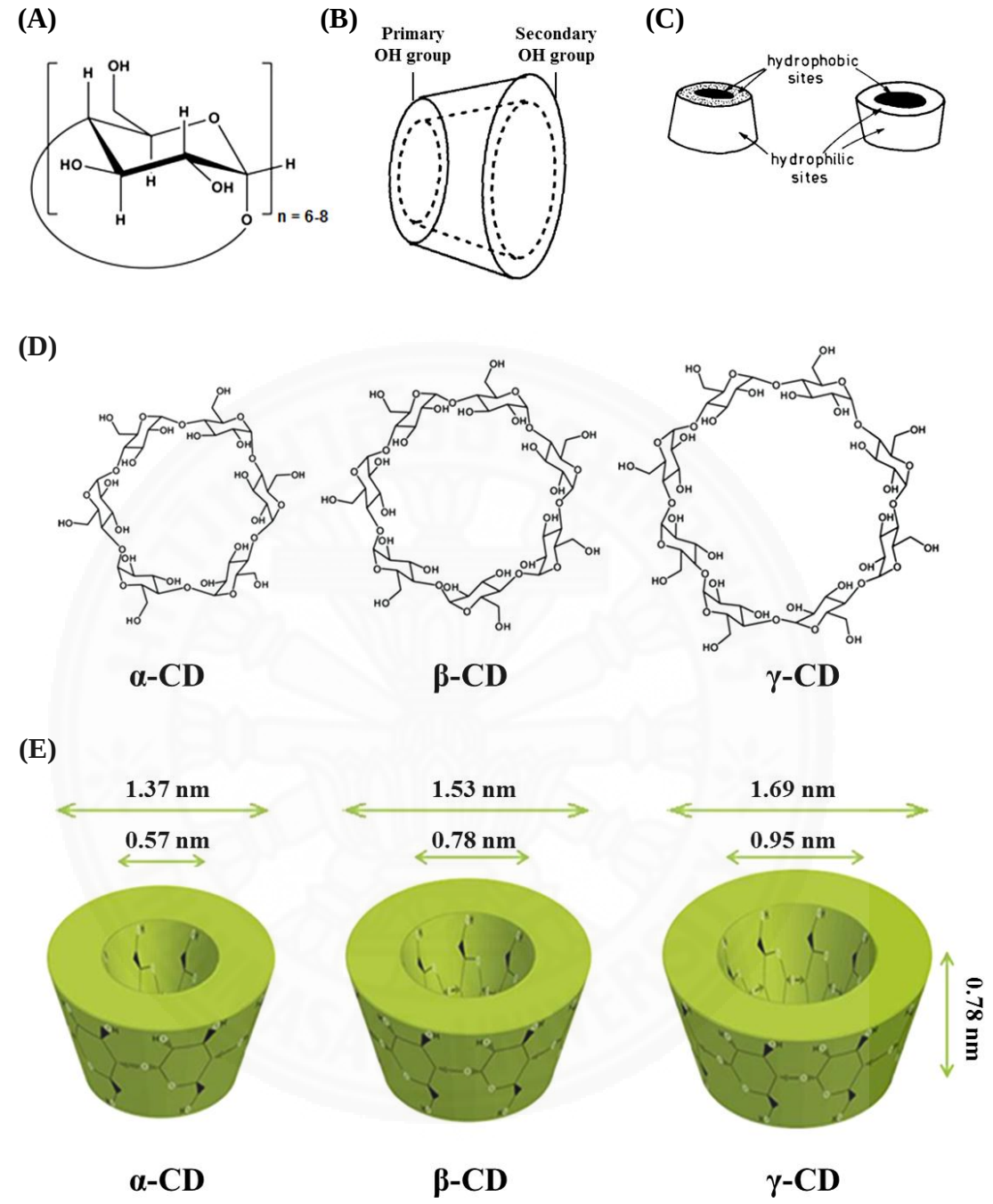


Figure 2.9 Structure of α-CD, β-CD and γ-CD. (A) Glycosyl unit, (B) Primary and secondary hydroxyl groups, (C) Hydrophobic and hydrophilic sites, (D) Chemical structures and (E) 3D-structures (modified from Szejtli, 1998; Cserháti & Forgács, 2003)

Table 2.3 Properties of α-CD, β-CD and γ-CD (modified from Szejtli, 1998)

Property	**α-CD**	**β-CD**	**γ-CD**
Number of glucose	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 mL) at 25 °C	14.50	1.85	23.20
pKa	12.33	12.20	12.08
Inner diameter (nm)	0.57	0.78	0.95
Outer diameter (nm)	1.37	1.53	1.69
Cavity volume (nm^3)	0.174	0.262	0.472

The outstanding characteristic of CDs is their capability to form complex with a wide variety of guest molecules. The inclusion of a guest in CD cavity consists basically of a substitution of the included water molecules by the less polar guest. In an aqueous solution, the apolar CD cavity is occupied by water (polar-apolar interaction) that is unfavorable. Thus, the guest molecules can be rapidly substituted when they are less hydrophilic or less polar than water, and there is a positive correlation between stability of CD complexes and hydrophilic character of molecules or certain parts of the guests (Davis & Brewster, 2004). The guest:CD complex in 1:1 ratio is the simplest and most frequent case, which is the essence of molecular encapsulation. However, the ratio of 1:2, 2:1, 2:2 or even more complicated associations and higher-order equilibrium also exist as shown in Figure 2.10. The result of the complexation between CDs and guests includes alteration of the solubility of guest compound, prevention of light, heat and oxidation exposure, masking unwanted physiological effects and reduction of volatility. From these beneficial effects of complexes, CDs should be used in many kinds of application such as food, cosmetic, analytical chemistry, biotechnology and pharmaceutical.

2.6.2 Application of CDs

2.6.2.1 Food industry

The application of CDs-assisted molecular encapsulation in foods offers many advantages. CDs are widely used as food additives for stabilization of flavors, elimination of undesired tastes or other undesired compounds and to protect browning reactions. For example, CDs are used in the preparation of controlled release powdered flavors, candy and chewing gum to retain flavor and smell for longer duration (Mabuchi & Ngoa, 2001). β-CD is used to remove the cholesterol from egg, butter and milk in the production of low cholesterol dairy products (Szejtli, 2004). Recently, CDs are also used in the food packaging materials with anti-microbiological contamination properties. Qian and co-workers (2008) produced the fiber-β-CD-cinnamaldehyde-based film as an active food packing material for food products. β-CD was grafted onto cellulose fibers using polyacrylic acid and the resultant fiber-β-CD complex was successfully used to encapsulate the antimicrobial cinnamaldehyde. This antimicrobial film can extend a long shelf-life of bread.

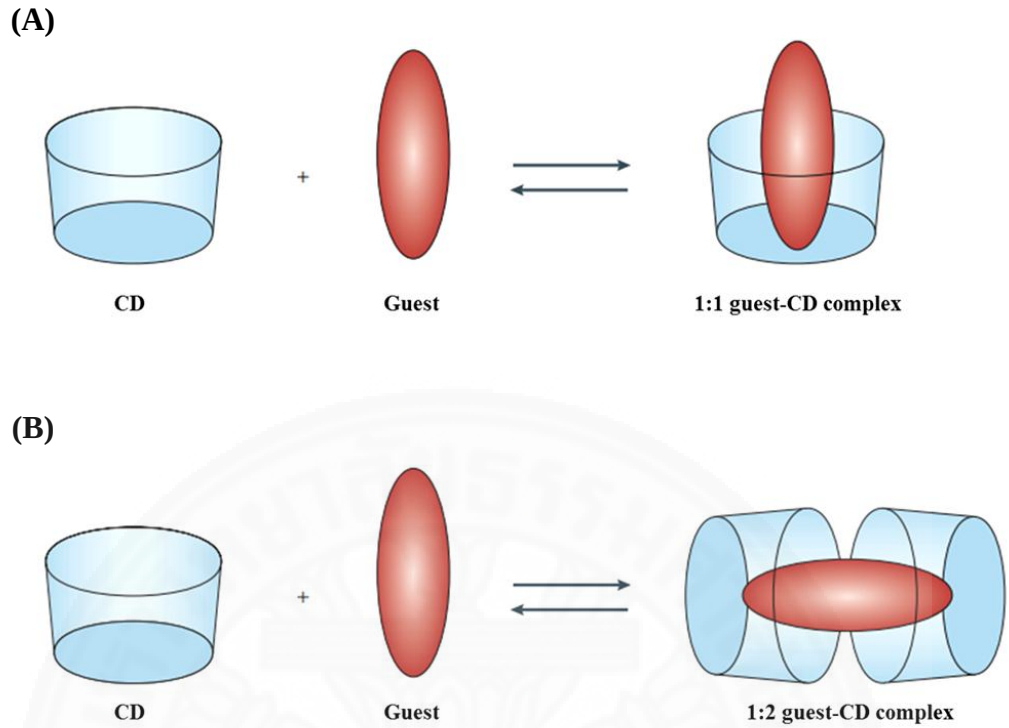


Figure 2.10 Schematic representation of the guest-CD complexes. (A) 1:1 guest-CD complex and (B) 1:2 guest-CD complex (modified from Davis & Brewster, 2004)

2.6.2.2 Cosmetic and personal care products industries

The number of applications dealing with CDs in cosmetic and personal care products is enormous. The important advantage for the use of CDs in cosmetic manufacturing is suppressing the volatilization of perfumes by stabilizing and controlling the release of the fragrance from the inclusion complexes. The interaction of CDs with guests produces a higher van der Waals force to form inclusion compounds (Martin Del Valle, 2004). Thus, these compounds are released slowly for prolonging the fragrance. For example, the use of CD-included fragrances in skin preparations stabilizes the fragrance against loss caused by evaporation and oxidation over a long period (Hedges, 1998). Moreover, CDs can also generate an improvement of handling of liquid or oily substances as powders. The oily tocopherol and octyl methoxycinnamate, which use as an antioxidant and oil-soluble sun screen, respectively, are combined with CDs. As a CD complex is not soluble in water or oil. It can be used in formulations as a powder or suspended in lotions and creams. The formation of complexes with CD serves to enhance the stability of tocopherol and controls the release of octyl methoxycinnamate, resulting in a long-lasting protection of the skin (Szejtli & Bolla, 1980; Pitha, 1981). Some of the other applications in personal care products are the use of dry CDs powder for masking odors in diapers, fabric softeners and paper towels. These powders are also used in hair care shampoo for reducing the odor and volatility of mercaptan (Szejti, 1998; Buschmann & Schollmeyer, 2002).

2.6.2.3 Agricultural and chemical industries

CDs can form complexes with a wide variety of agricultural chemicals including herbicides, insecticides, fungicides, repellents and growth regulators. The CD-pesticide complexes increase stability and acceleration in degradation of pesticides (Khan et al., 2011). CDs can also be used to delay germination of seed. The grain is treated with CDs, making the amylases in the grain are inhibited. So, the amylases cannot function to degrade the starch supplies of the seed, leading to delay in the germination of seed. Initially the plant grows more slowly, but later on this is largely compensated by an improved plant growth yielding a 20–45% larger harvest (Szejti, 1998). Furthermore, CDs are applied in conservation of wood products. Early, the water-insoluble fungicides have to be impregnated into

the woods by dissolving these fungicides in organic solvent, now it is possible to use simply aqueous CDs solution for this purpose (Szejti, 2004). In chemical industry, CDs have been used to discriminate between positional isomers, functional groups, homologous and enantiomers by high performance liquid chromatography (HPLC) or gas chromatography (GC). The stationary phases of these columns contain immobilized CDs or their derivatives. Other applications can be found in the analysis of spectroscopic of nuclear magnetic resonance (NMR). CDs can act as chiral shift agents and selective agents altering spectra in circular dichroism analysis. Recently, CDs also have application in separation through liquid composite membranes, microdialysis, solid- or liquid- phase extraction and molecularly imprinted polymers (Li & Purdy, 1992; Mosinger, Tománková, Němcová, & Zýka, 2001).

2.6.2.4 Biotechnological and pharmaceutical industries

The interesting application of CDs in biotechnological field is the use of CDs as artificial chaperone to refold the inactive inclusion bodies into its biologically active form. In the refolding process by artificial chaperone, the inclusion bodies must first be denatured by detergents. Then, CDs selectively interacts with a denatured protein by hydrophobic interaction to prevent irreversible aggregation and releases the protein in its refolded form with the aid of ATP and another co-chaperone (Sasaki & Akiyoshi, 2010). Several studies reported that many proteins such as carbonic anhydrase B, lysozyme, antibodies and other recombinant proteins were successfully refolded by various CDs and detergents (Rozema & Gellman, 1996; Machida et al., 2000; Hamada, Arakawa, & Shiraki, 2009). As a consequence, artificial chaperone-assisted refolding kit using a mixture of CDs as one of the active components is presently on to the market. For pharmaceutical industry, the main benefits of CDs in this sector are drug solubility, stability and bioavailability. The majority of pharmaceutical active agents do not have sufficient solubility in water. Thus, the normal formulation system for these insoluble drugs is a combination with organic solvents, extreme pH and high temperature conditions, which often cause irritation or other adverse reactions. CDs are not irritant and can increase the water solubility of the various poorly water-soluble drug, biomedical peptide and proteins including hormones, aspartame, tumor necrosis factor, interleukin-2 and albumin (Uekama, Hirayama, & Irie, 1998). In some cases, the addition of CDs in

drugs results in improved bioavailability, increasing the pharmacological effect allowing a reduction in the dose of the drug administration. For example, CDs have been successfully applied for the increase of the drug permeability by direct interaction with mucosal membranes. This is mainly explained by the fact that the ability of CDs to remove cholesterol can increase fluidity of membrane and cause cell lysis (Matsuda & Arima, 1999). CDs are capable to assist in drug stabilization and irritation. These advantages improve the drug contact time at the biological adsorption surface such as skin, mucosa and eye cornea. The property of CDs in the formation of inclusion complexes is also used to improve the durability of drugs against hydrolysis, oxidation, heat, light, humidity, temperature and metal salts. Inclusion complexes can facilitate the handling of volatile drugs. This can lead to a different way of drug administration. Furthermore, the inclusion complex between irritating drugs and CDs can protect the gastric mucosa for the oral route, reduce skin damage for the dermal route and mask the bitter or irritant taste and bad smell (Zhao, Temsamani, & Agrawal, 1995; Hedges, 1998; Szejtli, 1998; Irie & Uekama, 1999). In the safety of CDs usage, it is well recognized that only 2-4% of CDs are absorbed in the small intestines, and that the remaining section is degraded and taken up as glucose. This can explain the low toxicity observed on the oral administration of CDs (Szente & Szejtli, 2004).

2.7 Alkyl glycosides

Alkyl glycosides are composed of the hydrophilic sugar headgroup that connects to the alkyl hydrophobic tail of different chain length of saturated or unsaturated nature *via* a glycosidic bond. The sugar group can be glucose, galactose, maltose and xylose, while the alkyl tail is usually a primary alcohol and the glycosidic bond can form in α- or β- linkages. The general structure of alkyl glycosides is shown in Figure 2.11. Naturally, alkyl glycosides are biosynthesized by different microorganisms in form of glycolipids which have many environmental properties (Saharan, Sahu, & Sharma, 2011). For example, glycolipids of cellobiose lipid, rhamnolipid and sophorolipid exhibit antimicrobial, antiviral, bioemulsifying and bioremediation properties (Figure 2.12).

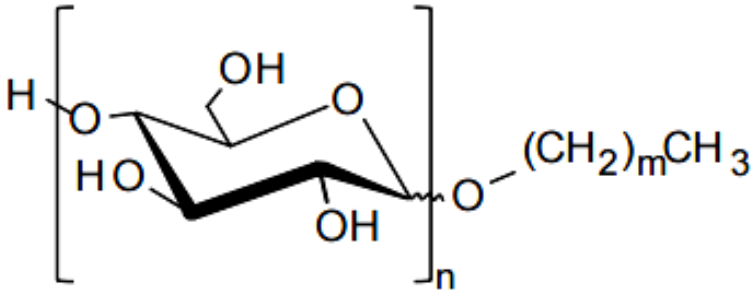


Figure 2.11 General structure of alkyl glycosides, when (n) is the number of glucose residues and (m) is the length of the alkyl chains.

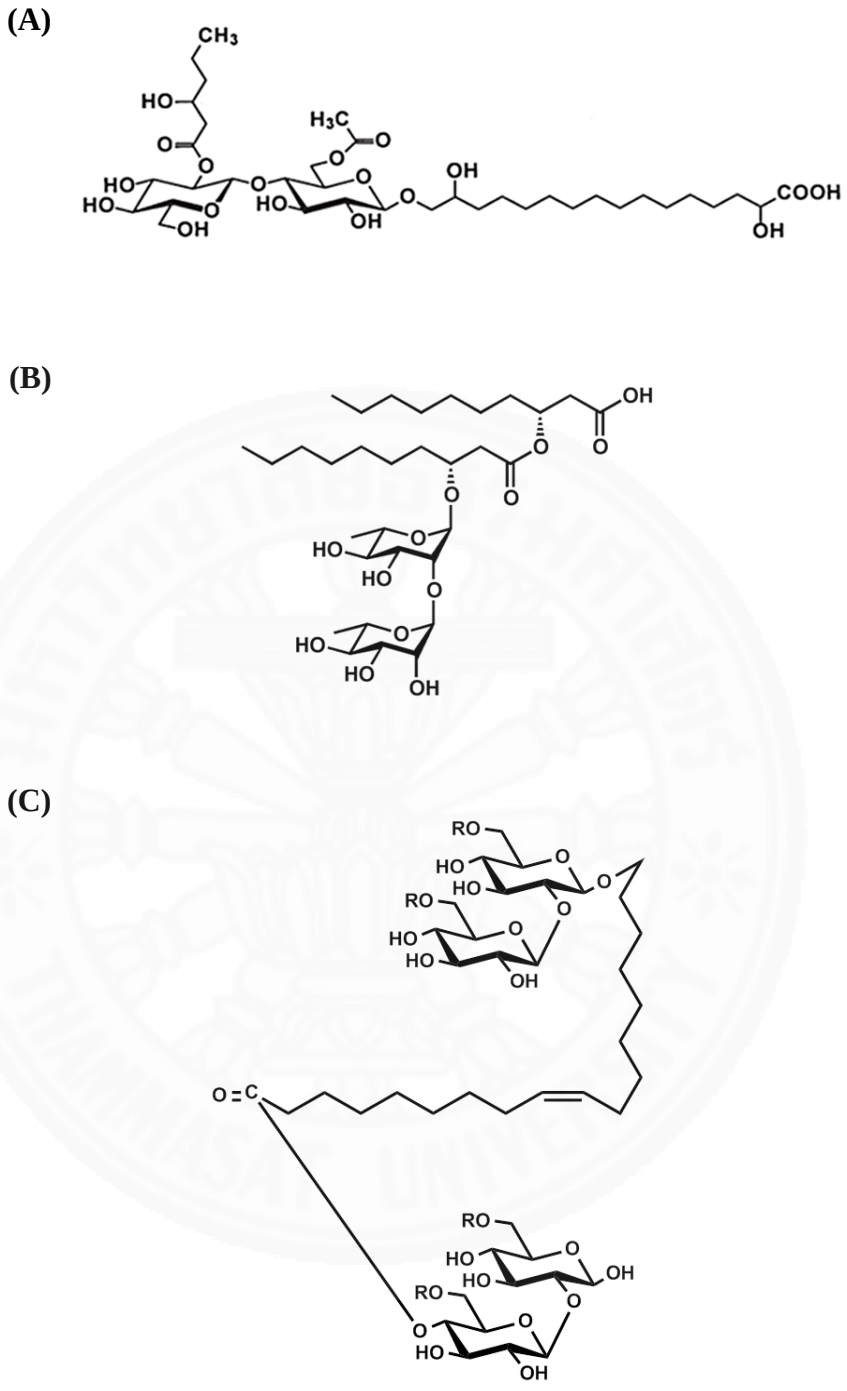


Figure 2.12 Alkyl glycosides in natural glycolipid form from living organisms. (A) Cellobiose lipid from *Pseudozyma fusiformata* (copy from Kulakovskaya et al., 2009), (B) Rhamnolipid from *Pseudomonas aeruginosa* (copy from Lang & Wullbrandt, 1999) and (C) Sophorolipid from *Candida* sp. NRRLY-27208 (copy from Price, Ray, Vermillion, Dunlap, & Kurtzman, 2012)

Because alkyl glycosides are amphipathic molecules which contain both hydrophobic and hydrophilic parts, they show the properties of surfactants. Surfactants are compounds that lower the surface tension between a liquid and a solid or that between two liquids. Surfactants can be classified into four types which are anionic, cationic, non-ionic and zwitterionic agents dependent on their net charge. For alkyl glycosides, the hydrophilic carbohydrate derived moiety could ionize but no charge presented on their molecules, thus they are classified as non-ionic surfactant. The advantage of alkyl glycosides is that they produce from naturally occurring and renewable raw materials (lipids and carbohydrates), so they are rapidly biodegradable and non-toxic as compared to other surfactants. However, the use of alkyl glycosides is not popular in the industrial sector because they have more expensive than other surfactants. Therefore, the development in the synthesis of alkyl glycosides has been extensively studied to reduce the production cost.

2.8 Production of alkyl glycosides

2.8.1 Chemical synthesis of alkyl glycosides

At present, the main commercial production of alkyl glycosides is performed by chemical method. Alkyl glycosides are produced from glucose and primary alcohol or mixture of primary alcohols containing 8-18 carbon atoms *via* one of the two common reactions (Igarashi, 1977). All reactions require extreme conditions of temperature, pH and pressure and use the multiple steps of protection, deprotection and activation (Figure 2.13). The first reaction is the direct reaction or one-step reaction. In this reaction, glucose source reacts directly with an excess of the alcohol or alcohol mixture in the presence of a strong acid catalyst. The reaction is performed under the range of temperature of 95-115 $^{\circ}$C and relatively high vacuum. The water formed during the acetalization reaction is removed as a distillate. Another reaction belongs to the transacetalization reaction or two-step reaction. The two-step reaction uses a shorter alcohol in the first reaction, followed by a transferase reaction with a larger alcohol in the second step. A carbohydrate is first coupled to short chain alcohol (usually butanol) producing butyl glycosides which

then act as substrate in the transacetalization with longer alcohol. This reaction is performed under the temperature of 90 to 140 °C and the low vacuum conditions.

2.8.1.1 Koenigs-Knorr reaction

The Koenigs-Knorr reaction is the earliest known glycosylation method. It is named after Wilhelm Koenigs and Edward Knorr. This reaction is applicable to the preparation in both of aryl and alkyl glycosides, and is widely used for the synthesis of glycosides having complex groups attached to the anomeric carbon atom, particularly oligosaccharides. Generally, the procedure involves the treatment of acylated glycosyl halide with an alcohol in the presence of a heavy metal salt or an organic base as the acid acceptor such as mercuric bromide, mercuric oxide, mercuric cyanide and silver trifluoromethanesulfonate. For example, Wolfrom and co-workers (1963) reported the treatment of galactopyranosyl bromide with methanol and silver carbonate. The result yields the corresponding methyl-β-D-galactopyranoside as shown in Figure 2.14.

2.8.1.2 Fischer reaction

The Fischer reaction, named after Emil Fischer, is the simplest and most effective method for the preparation of alkyl glycosides by combining the free sugar with an alcohol under acidic condition. The production can be done in either a one-step (direct synthesis) or a two-step process (transacetalization). The two-step process is mainly used when polymeric carbohydrates are used as substrates. However, the reaction is carried out in the presence of an acid catalyst in alcohols under reflux, resulting in a mixture of α- and β- anomers of the pyranoside and furanoside forms. The proportion of these isomers importantly depends on the reaction conditions and the stability of the four isomers (Figure 2.15). The Fischer method has been successfully applied for the synthesis of α- and β- benzyl fucopyranoside by treatment with benzyl alcohol under saturation with HCl at 0 °C. L-fucose was converted into products in the ratio of 5:1 as shown in Figure 2.16 (Shealy, O'Dell, & Arnett, 1987).

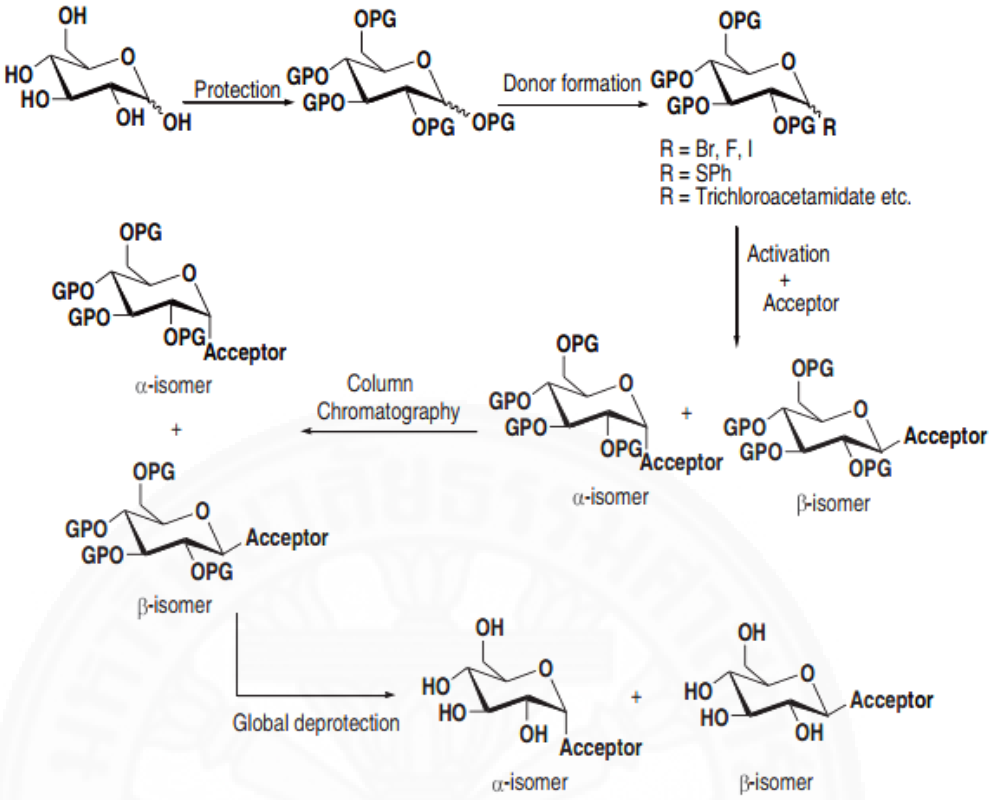


Figure 2.13 Overview of chemical synthesis of alkyl glycosides. The reaction starts by protection of all hydroxyl groups using acetic anhydride, sodium acetate, aldehydes or benzoyl halides at 140 °C. Activation step involves exchange of anomeric group by bromine (Br), fluorine (F), iodine (I), thiophenol (SPh) or tricholoroacetamide. The final step is deprotection of final product by sodium methoxide in methanol. (copy from Rather & Mishra, 2013)

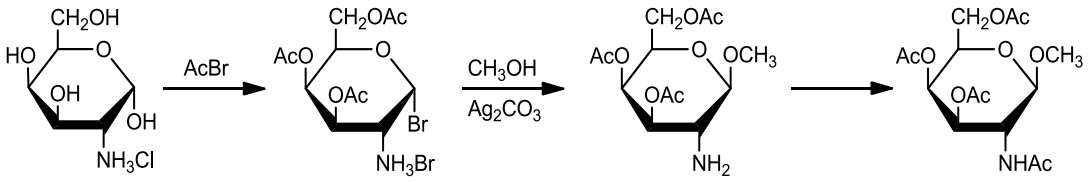


Figure 2.14 Chemical synthesis of methyl-β-D-galactopyranoside using the Koenigs-Knorr reaction (copy from Wolfrom, Cramp, & Horton, 1963)

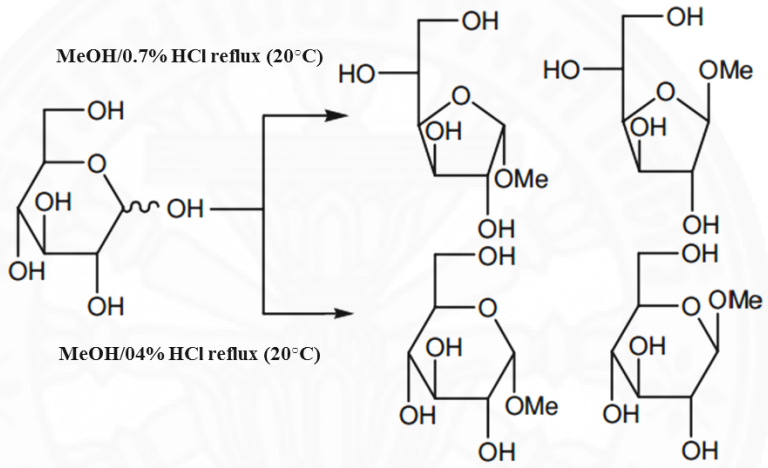


Figure 2.15 Mixture of glycoside isomers from the Fischer reaction (copy from Brito-Arias, 2007)

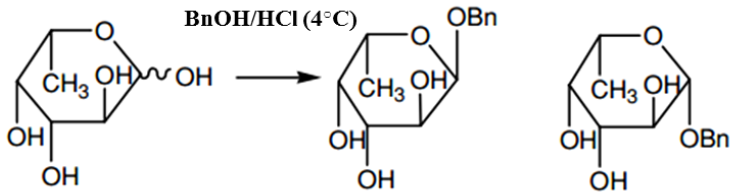


Figure 2.16 Chemical synthesis of α- and β- benzyl fucopyranoside using the Fischer reaction (copy from Shealy et al., 1987)

2.8.2 Enzymatic synthesis of alkyl glycosides

From the literature, it was apparent that the synthesis of alkyl glycosides by chemical method had the limitation in the regulation of ratio and types of final product. Furthermore, the process was performed under extreme conditions *via* several steps which resulted in the toxic and waste generation, expensive cost and high energy consumption. Therefore, the enzymatic method has been developed to anomerically synthesize pure alkyl glycosides by a simple one-step reaction under non-toxic and mild conditions. Within this enzymatic route, the synthesis can perform at near neutral pH, ambient temperature, atmospheric pressure and highly give the specific product (Crout & Vic, 1998; van Rantwijk, van Oosterom, & Sheldon, 1999). The enzymatic synthesis of alkyl glycosides can be achieved through a two main types of enzymes from many organisms such as glycosidases (E.C. 3.2) and glycosyltransferase (E.C. 2.4).

2.8.2.1 Glycosidases (E.C. 3.2)

Glycosidases are hydrolytic enzymes which mainly catalyze the cleavage of a glycosidic bond. However, glycosidase can also be used for synthesis of alkyl glycosides by two classes of glycosidases: one is inverting and another is retaining glycosidases (van Rantwijk et al., 1999; Rather & Mishra, 2013). In inverting glycosidases, alkyl glycosides are synthesized *via* thermodynamically controlled reverse hydrolysis reaction by reacting a monosaccharide with an alcohol when the equilibrium of the reaction is shifted towards synthesis. The equilibrium can be shifted by reducing water activity, increasing the concentration of substrates and addition of co-solvents (Ljunger, Adlercreutz, & Mattiasson, 1994; Panintrarux, Adachi, Araki, Kimura, & Matsuno, 1995; Ducret, Carrière, Trani, & Lortie, 2002) as shown below:

$$\text{Glycosyl-OH + ROH} \leftrightarrow \text{Glycosyl-OR + } H_2O \qquad K = \frac{[GlcOR][H_2O]}{[GlcOH][ROH]}$$

Unlike inverting glycosidases, the transglycosylation reaction is used in retaining mechanism for the synthesis of alkyl glycosides. Transglycosylation reaction, which is similarly found in glycosyltransferase enzymes, uses the activated glycoside as donor and alcohol as nucleophilic acceptor to generate

a new glycosidic bond. Because this reaction is a kinetically controlled reaction, the potentiality of alkyl glycosides production depends more on the properties and structural conformation involved in the hydrolysis and transglycosylation reactions of enzyme than thermodynamic equilibrium. Most studies have reported the enzymatic synthesis of alkyl glycosides *via* both reverse hydrolysis and transglycosylation reactions of glycosidases as shown in Table 2.4.

2.8.2.2 Glycosyltransferases (E.C. 2.4)

The glycosyltransferases mainly catalyze the transfer of saccharide moieties from an activated donor molecules to specific acceptor molecules. The resulting product of glycosyl transfer can be a carbohydrate, glycoside, oligosaccharide and polysaccharide. The high selectivity transfer of glycosyl donor to acceptor molecule and the high yield obtained are interesting benefits of these enzymes (Nilsson, 1988). Although glycosyltransferases have been widely used for the synthesis of oligosaccharides by transfer reactions between various kinds of donors and acceptors, very few studies regarding the synthesis of alkyl glycosides by glycosyltransferases have been reported. For example, levansucrase from *Rahnella aquatilis* (Kim, Kim, Lee, Song, & Rhee, 2000), dextransucrase from *Leuconostoc mesenteroides* (Kim et al., 2009) and CGTase from *Paenibacillus* sp. RB01 (Chotipanang, Bhunthumnavin, & Prousoontorn, 2011) were used to synthesize alkyl glycosides from saccharides and short chain alcohols.

Table 2.4 Examples of the synthesis of alkyl glycosides by glycosidases (modified from Rather & Mishra, 2013)

Enzyme	Source	Donor	Acceptor	Product	Ref.
β-xylosidase	*Aspergillus niger*	Xylobiose	Methanol	Methyl-β-D-xylopyranoside	1
			Ethanol	Ethyl-β-D-xylopyranoside	
			Propanol	Propyl-β-D-xylopyranoside	
			Butanol	Butyl-β-D-xylopyranoside	
			Pentanol	Pentyl-β-D-xylopyranoside	
			Hexanol	Hexyl-β-D-xylopyranoside	
			Heptanol	Heptyl-β-D-xylopyranoside	
			Octanol	Octyl-β-D-xylopyranoside	
			2-Propanol	2-Propyl-β-D-xylopyranoside	
			2-Hexanol	2-Hexyl-β-D-xylopyranoside	
			Cyclohexanol	Cyclohexyl-β-D-xylopyranoside	
			Benzanol	Benzyl-β-D-xylopyranoside	
β-glucosidase	Almond	Xylose	Methanol	Methyl-β-D-xylopyranoside	2
			Ethanol	Ethyl-β-D-xylopyranoside	
			Propanol	Propyl-β-D-xylopyranoside	
			Butanol	Butyl-β-D-xylopyranoside	
β-glucosidase	*Candida molischiana*	Cellobiose	Decanol	Decyl-β-D-glucopyranoside	3
β-glucosidase	Almond	Glucose	Hexanol	Hexyl-β-D-glucopyranoside	4
β-glucosidase	*Fusarium oxysporum*	Cellobiose	Methanol	Methyl-β-D-glucopyranoside	5

[1](Shinoyama, Kamiyama, & Yasui, 1988); [2](Drouet, Zhang, & Legoy, 1994); [3](Gueguen, Chemardin, Pommares, Arnaud, & Galzy, 1995); [4](Vic, Thomas, & Crout, 1997); [5](Makropoulou et al., 1998)

Table 2.4 (cont.) Examples of the synthesis of alkyl glycosides by glycosidases (modified from Rather & Mishra, 2013)

Enzyme	Source	Donor	Acceptor	Product	Ref.
β-glucosidase	Almond	Glucose	Methanol	Methyl-β-D-glucopyranoside	6
		Cellobiose	Methanol	Methyl-β-D-glucopyranoside	
β-glucosidase	*Aspergillus niger*	Cellobiose	Methanol	Methyl-β-D-glucopyranoside	7
			Ethanol	Ethyl-β-D-glucopyranoside	
β-glucosidase	Almond	Nitrophenyl glucoside	Methanol	Methyl-β-D-glucopyranoside	8
			Ethanol	Ethyl-β-D-glucopyranoside	
			Propanol	Propyl-β-D-glucopyranoside	
			Butanol	Butyl-β-D-glucopyranoside	
			Pentanol	Pentyl-β-D-glucopyranoside	
			Hexanol	Hexyl-β-D-glucopyranoside	
β-glucosidase	Almond	Glucose	Butanol	Butyl-β-D-glucopyranoside	9
β-glucosidase	Thai rosewood	Nitrophenyl glucoside	Methanol	Methyl-β-D-glucopyranoside	10
			Ethanol	Ethyl-β-D-glucopyranoside	
			Propanol	Propyl-β-D-glucopyranoside	
			Butanol	Butyl-β-D-glucopyranoside	
			Hexanol	Hexyl-β-D-glucopyranoside	
			Octanol	Octyl-β-D-glucopyranoside	
			Isobutanol	Isobutyl-β-D-glucopyranoside	
			2-Butanol	2-Butyl-β-D-glucopyranoside	

[6](Millqvist-Fureby, Gill, & Vulfson, 1998); [7](Yan, Lin, & Lin, 1998); [8](Yi, Sarney, Khan, & Vulfson, 1998); [9](Ismail, Soultani, & Ghoul, 1999); [10](Lirdprapamongkol & Svasti, 2000)

Table 2.4 (cont.) Examples of the synthesis of alkyl glycosides by glycosidases (modified from Rather & Mishra, 2013)

Enzyme	Source	Donor	Acceptor	Product	Ref.
β-glucosidase	Almond	Glucose	Octanol	Octyl-β-D-glucopyranoside	11
β-galactosidase	*Penicillium canescencs*	Lactose	Heptanol	Heptyl-β-D-galactopyranoside	11
			Octanol	Octyl-β-D-galactopyranoside	
			Nonanol	Nonyl-β-D-galactopyranoside	
β-glucosidase	*Sclerotinia sclerotiorum*	Cellobiose	Butanol	Butyl-β-D-glucopyranoside	12
			Pentanol	Pentyl-β-D-glucopyranoside	
			Hexanol	Hexyl-β-D-glucopyranoside	
			Heptanol	Heptyl-β-D-glucopyranoside	
			Octanol	Octyl-β-D-glucopyranoside	
β-glucosidase	*Thermotoga neapolitana*	Nitrophenyl glucoside	Hexanol	Hexyl-β-D-glucopyranoside	13
β-glucosidase	Almond meal	Glucose	Methanol	Methyl-β-D-glucopyranoside	14
			Ethanol	Ethyl-β-D-glucopyranoside	
			Propanol	Propyl-β-D-glucopyranoside	
			Butanol	Butyl-β-D-glucopyranoside	
			Pentanol	Pentyl-β-D-glucopyranoside	
			Hexanol	Hexyl-β-D-glucopyranoside	
			Heptanol	Heptyl-β-D-glucopyranoside	
			Octanol	Octyl-β-D-glucopyranoside	
			Nonanol	Nonyl-β-D-glucopyranoside	
			Decanol	Decyl-β-D-glucopyranoside	

[11](Kouptsova, Klyachko, & Levashov, 2001); [12](Smaali, Maugard, Limam, Legoy, & Marzouki, 2007); [13](Turner, Svensson, Adlercreutz, & Karlsson, 2007); [14](Wang et al., 2012)

2.9 General properties of alkyl glycosides

Alkyl glycosides are a class of non-ionic surfactants which exhibit the general properties such as critical micelle concentration and emulsifying properties.

2.9.1 Critical micelle concentration (CMC)

The important characteristic of alkyl glycosides is the critical micelle concentration (CMC). CMC is the concentration at which monomers of alkyl glycosides aggregate into micelle (Figure 2.17(A)). Micelle is spherical, cylindrical or bilayer structure formed through the entropically driven process of minimizing the exposure of the hydrophobic part to a hydrophilic solvent. The formation of micelle in water resulted from a delicate balance of intermolecular forces, including hydrophobic, steric, electrostatic, hydrogen bonding, and van der Waals interactions of the surfactant monomers. The various shapes of micelle are occurred due to the different of the length of hydrophilic and hydrophobic parts and also the concentration of surfactants (Figure 2.17(B)). At CMC, the physicochemical properties of the surfactant solution change such as turbidity, osmotic pressure and surface tension. CMC of alkyl glycoside depends on many factors such as temperature, pressure and sometimes strongly on the presence and concentration of other surfactant compounds. Moreover, some alkyl glycosides only form above the critical micelle temperature which is the minimum temperature at which alkyl glycosides form micelle. Many methods have been used to measure the CMC such as surface tension measurements, fluorescence, light scattering and molal volumes (Balzer & Lüders, 2000). As is typical for all surfactants, the CMC of alkyl glycosides significantly decreases with the length of the hydrocarbon as shown in Table 2.5.

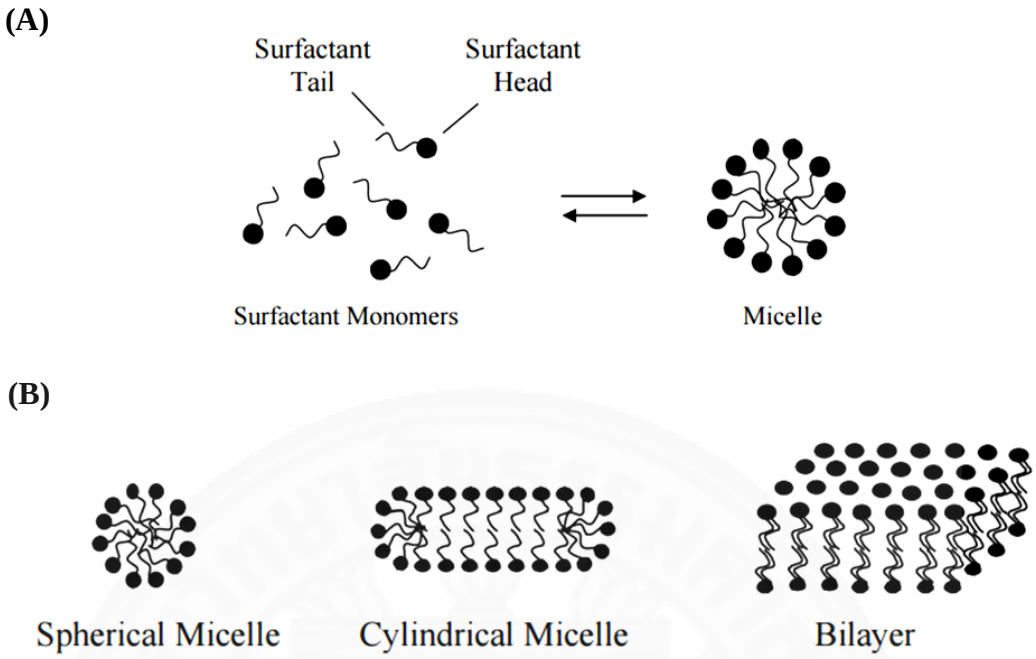


Figure 2.17 Schematic representation of (A) the formation of micelle and (B) three common shapes of micelle in aqueous solution (copy from Rangel-Yagui, Pessoa, & Blankschtein, 2004)

Table 2.5 CMC of alkyl glycoside monomers (modified from Balzer & Lüders, 2000)

Alkyl glycoside	**CMC (mM)**	**Temperature (°C)**
Octyl-α-D-glucoside	12	25
Octyl-β-D-glucoside	17	25
Decyl-α-D-glucoside	0.35	25
Decyl-β-D-glucoside	2.2	25
3-Octyl glucoside-3-ethyl	8.5	25
4-Heptyl glucoside-4-propyl	12	25
5-Hexyl glucoside-5-butyl	15	25
Dodecyl-α-D-glucoside	0.072	60
Dodecyl-β-D-glucoside	0.19	25
Tetradecyl-α-D-glucoside	0.022	20
Tetradecyl-β-D-glucoside	0.015	20

2.9.2 Emulsifying properties

The emulsifying properties of alkyl glycosides are derived from the original hydrophilic group of sugars and the original lipophilic group of alkyl chains. When alkyl glycosides are added to a mixture of two fluids (typically oil and water), they are absorbed into one or more layers at the interface between water and oil and position themselves so that their hydrophilic portions are oriented toward water and their lipophilic portions are oriented toward oil droplet (Payet & Terentjev, 2008). This event results in the formation of emulsion and reduction of surface and interfacial tension. Emulsion is a class of disperse system consisting of two immiscible liquids (Binks, 1998; Tadros, 2005). In the simplest case, there is either an oil-in-water (o/w) or water-in-oil (w/o) emulsion. However for the technical applications, multiphase emulsions are also quite common; for example, water-in-oil emulsion can be dispersed in water to obtain a water-oil-water (w/o/w) emulsion. Generally, emulsions are thermodynamically unstable and eventually separate into oil and water phases (Pearce & Kinsella, 1978). Thus, the emulsifying properties of alkyl glycosides can determine in terms of emulsification activity and stability. Emulsification activity is the ability to form an emulsion, while the emulsification stability measures the rate of separation of emulsion into oil and water phases (Cheng, Jian, & Wang, 2008; Tadros, 2013).

2.10 Application of alkyl glycosides

Based on the structure and general properties of alkyl glycosides, they are applied in a variety of household, industrial, biotechnological and pharmaceutical levels. However, varying in the types and lengths of the alkyl chain and sugar group as well as the configuration of the linkages have a direct effect on the usages and properties of alkyl glycosides (von Rybinski, 1996).

2.10.1 Detergent, personal care cleaner and cosmetic products

Alkyl glycosides have been used as the components in detergent, personal care cleaner and cosmetic products. The alkyl chain of alkyl glycosides can interact with dirty compound such as oil, while the hydrophilic part faces outward and attracts water molecules. When many alkyl glycoside molecules attach to an oil droplet, they surround it and make micelles. Alkyl glycosides were certified from Food and Drug Administration (FDA) that they can be used as food detergents in the cleaning of food products and food processing equipments (Steele, 2008). For liquid laundry detergents, alkyl glycosides show the increasing of storage stability of the enzymes used such as cellulases, lipases and proteases (von Rybinski & Hill, 1998). Alkyl glycosides also have high washing performance over a broad pH range. This makes them suitable in highly alkaline dishwashers and in slightly acidic cleaners that are mild for human skin. The efficacy of alkyl glycosides as mild cleaners is clearly shown in the following arm-flex wash test (Rosen, 1978). This mildness of alkyl glycosides can reduce the tensile strength of hair in shampoo (von Rybinski & Hill, 1998). Products containing alkyl glycosides are also reported to be used on baby skin and applied to the eye area, and mucous membranes may be exposed to these products. Furthermore, alkyl glycosides provide a foam stabilization effect and improve foam volume when used as co-surfactants with anionic surfactants. These properties are expected by the consumer and important for purchasing decisions (Shinoda, Yamanaka, & Kinoshita, 1959). In cosmetic products, alkyl glycosides have been mainly used as emulsifiers and emulsion-stabilizing agents to make a homogeneous mixture of components in skin cream.

2.10.2 Biotechnological and biochemical researches

Alkyl glycosides are shown to be useful in membrane protein functional and structural studies. They are used as solubilizers and stabilizing agents for many proteins. This application of alkyl glycosides is based on the fact that alkyl glycosides can interact with hydrophobic and hydrophilic parts of proteins and form water-soluble micelles in solution, while proteins can also be reconstituted into detergent-lipid mixed micelles. This may be the closest representative bilayer-mimetic system. However, alkyl glycosides are specific in biotechnological and biochemical uses because they have variable sugar and alkyl group structures and form different linkages to other molecules. For example, short chain alkyl glucosides and maltosides have been successfully used in activating the crystallization of membrane proteins (Allen, 1994; Istvan, Hasemann, Kurumbail, Uyeda, & Deisenhofer, 1995; Pebay-Peyroula, Garavito, Rosenbusch, Zulauf, & Timmins, 1995; Rosenow, Brune, & Allen, 2003). Most alkyl glycosides with greater than C8 alkyl in length showed the ability to solubilize and preserve the structure and function of various oligomeric states of G-protein coupled receptor and rhodopsin (Jastrzebska et al., 2006). Dodecyl maltosides have been used to cystallize the membrane protein cytochrome c oxidase from *Rhodobacter sphaeroides* (Qin, Hiser, Mulichak, Garavito, & Ferguson-Miller, 2006). In addition to general proteins, alkyl glycosides are also applied to solubilize insoluble proteins. The insoluble proteins are proteins that are insoluble in water because their hydrophobic amino acids are located on the outer surface of the proteins. Insoluble proteins are usually found in the recombinant bacteria that contained the recombinant gene from different species. They may also be formed due to misfolding of soluble proteins. These proteins then form aggregates and lead to the loss of biological activity. Generally, the insoluble proteins have the native-like secondary structure. Thus, the common method for solubilization of insoluble proteins is the use of mild solubilizing agents such as alkyl glycosides. As reported, octyl glucosides have been successfully applied for the recovery of cytochrome P450 from *Cunninghamella elegans* (Leibly et al., 2012).

2.10.3 Pharmaceutical and medical researches

The interesting application of alkyl glycosides in medical field is the use of alkyl glycosides as drug carriers, surfactant compositions in drugs and antimicrobial agents. Drug carriers are used in sundry drug delivery systems such as controlled-release technology to prolong *in vivo* drug actions and increase the effectiveness of drug delivery to the target sites of pharmacological actions. Kiwada and co-workers (1985) reported that the alkyl glycosides from alcohols (myristyl, cetyl and steryl) and sugars (glucose, galactose and mannose) formed lamellar vesicles like phosphatidylcholine vesicles (liposomes), and can be applied as drug carriers that serve as mechanism to improve the delivery and effectiveness of drugs. These alkyl glycoside vesicles showed the outstanding stability for 48 hours in plasma. Alkyl glycosides are also used as non-irritating and non-toxic compositions in therapeutic agents. The limitation of the development and use of therapeutic agents is the ability to deliver them to the site of action. Therefore, the enhancing surfactant will stabilize the therapeutic agents. Alkyl glycosides increase the passage of adsorption of the therapeutic agents through skin, mucosal surfaces and various membrane barriers without damaging the structural integrity and biological function of the membranes and also increase bioavailability of the agents (Maggio, 2012). Another valuable application of alkyl glycosides is their antimicrobial activity. This activity of alkyl glycosides comes from the interaction of alkyl chains with cell membranes of bacteria, which causes the cell break. Both gram-positive and gram-negative bacteria are affected, but the susceptibility of the other organisms to alkyl glycosides varies significantly. Several studies in the use of alkyl glycosides as antimicrobial agents have been already reported (Matsumura et al., 1990; Matin, Bhuiyan, & Azad, 2013).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Equipments

Autopipette: Pipetman, Gilson, France
Biosafety carbinet: Model Power wave XS, BioTek, U.S.A.
Centrifugal evaporator: Model Concentrator 5301, Eppendorf, Germany
Centrifuge filter units: Amicon® Ultra, Merck Millipore, Ireland
DEAE-Toyopearl 650M: TOSOH Corporation, TOSOH, Japan
Dialysis tubing: Model D-9527, Sigma, U.S.A.
Fraction collector: Model Frac-920, GE Healthcare, Sweden
High pressure steam sterilizer: Model ES-315, TOMY, Japan
Incubator: Model UNB 500, Memmert, Germany
Magnetic stirrer: Model 500P-2, PMC, U.S.A.
McFarland densitometer: Model DEN-1, Grant-bio, England
Microcentrifuge: Model Legend Micro 17, Thermo scientific, UK
Microplate spectrophotometer: Model Power Wave™ XS, BioTek, U.S.A.
Mini-Gel electrophoresis unit: Mini protein, Bio-Rad, U.S.A.
Nanodrop spectrophotometer: Model Nanodrop 2000, Thermo scientific, U.S.A.
Orbital incubator shaker: Model 240, Thermo scientific, U.S.A.
Peristaltic pump: Model Pump P-1, GE Healthcare, Sweden
pH meter: Model PHS-3BW, BANTE instrument, China
Power supply: Model Power PAC™ Basic, Bio-Rad, U.S.A.
Quick spin: Model C1301B-230, Labnet, Korea
Refrigerated centrifuge: Allegra® X-15R, BECKMAN COULTER, U.S.A.
Scanning densitometer: Model GS-800 Calibrated Densitometer, Bio-Rad, U.S.A.
Sonicator: Model VCX130, SONICS, U.S.A.
Spectrophotometer: Model UV-1601, Shinmadzu, Japan
Vortex mixer: Genie 2 G-560E, Scientific Industries™, U.S.A.
Water bath: Model WNB Basic, Memmert, Germany

3.2 Chemicals

Acetic acid: BDH Prolabo chemicals, UK

Agar: Merck, Germany

Alcohols:

- 1-Butanol: BDH Prolabo chemicals, UK
- 1-Ethanol: Carlo ERBA Reagents, France
- 1-Pentanol: BDH Prolabo chemicals, UK
- 1-Propanol: Carlo ERBA Reagents, France
- 1-Methanol: Merck, Germany
- 3-Methyl-1-butanol: Carlo ERBA Reagents, France

Ammonium persulfate: Sigma, U.S.A.

Antibiotics:

- Ampicillin: Sigma, U.S.A.
- Oxacillin: Sigma, U.S.A.
- Penicillin: Sigma, U.S.A.
- Tetracycline: Sigma, U.S.A.

Aquacide II: Merck, Germany

Bovine serum albumin: Sigma, U.S.A.

Bradford reagent: Bio-Rad, U.S.A.

Bromophenol blue: Merck, Germany

Calcium chloride dihydrate: BDH Prolabo chemicals, UK

Cellobiose: Sigma, U.S.A.

Coomassie® Brilliant Blue R-250: Merck, U.S.A.

Corn starch: Knorr, Thailand

Cyclodextrins (α-, β-, γ-): Bio Research Corporation of Yokohama, Japan

di-Potassium hydrogen phosphate: Merck, Germany

Ethyl acetate: BDH Prolabo chemicals, UK

Glucose luquicolor (Glucose oxidase kit): Human, Germany

Glycerol: Merck, Germany

Glycine: Bio-Rad, U.S.A.

Hexadecane: Sigma, U.S.A.

Hydrochloric acid: Carlo Erba Reagents, France

Iodine: Baker chemical, U.S.A.

Isopropyl-β-D-1-thiogalactoside (IPTG): Vivantis, U.S.A.

Magnesium sulfate: BDH Prolabo chemicals, UK

Mannitol salt agar: Becton Dickinson and Company, U.S.A.

Methanol deuterium: Merck, Germany

Methyl glucopyranoside: Sigma, U.S.A.

β-Mercaptoethanol: Fluga, Switzerland

N,N'-Methylene-bis-acrylamide: Sigma, U.S.A.

N,N,N',N'-Tetramethyl-1,2-diaminoethane (TEMED): Bio-Rad, U.S.A.

Oligosaccharides:

- Glucose: BDH Prolabo chemicals, UK
- Maltoheptaose: Tokyo Chemical Industry, Japan
- Maltohexaose: Sigma, U.S.A.
- Maltopentaose: Sigma, U.S.A.
- Maltose: Sigma, U.S.A.
- Maltotetraose: Sigma, U.S.A.
- Maltotriose: BioChemika, Japan

Phenolphthalein: Sigma, U.S.A.

Pierce™ BCA protein assay kit: Bio-Rad, U.S.A.

Potassium dihydrogen phosphate: Merck, Germany

Potassium iodine: Mallinckrodt, U.S.A.

Resazurin dye: Sigma, U.S.A.

Sodium carbonate anhydrous: BDH Prolabo chemicals, UK

Sodium chloride: Merck, Germany

Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS): Sigma, U.S.A.

Sodium dodecyl sulfate: Vivantis, U.S.A.

Sodium hydroxide: Merck, Germany

Standard protein marker: Bio-Rad, U.S.A.

Sulfuric acid: Carlo ERBA Reagents, France

Soluble potato starch: Sigma, U.S.A.

Tris base: Vivantis, U.S.A.

Tris hydrochloride: Vivantis, U.S.A.

Triton^{TM} X-100: ITW Companies, Germany

Tryptone: Becton Dickinson and Company, U.S.A.

Yeast extract: Becton Dickinson and Company, U.S.A.

3.3 Enzymes and bacterial strains

Rhizopus sp. glucoamylase: Fluka, Switzerland

Saccharomyces cerevisiae α-glucosidase: Toyobo Enzymes, Japan

Escherichia coli ATCC 25922: National Center for Genetic Engineering and Biotechnology, Thailand

Staphylococcus aureus ATCC 25923: National Center for Genetic Engineering and Biotechnology, Thailand

A406R-*CgAM* recombinant cell (a pET-19b based plasmid containing the A406R-mutated amylomaltase gene from *Corynebacterium glutamicum* ATCC 13032 in *E. coli* strain BL21 (DE3) host cell): Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand

pBC recombinant cell (a pET-19b based plasmid containing the CGTase gene with signal peptide sequence from *Bacillus circulans* A11 in *E. coli* strain BL21 (DE3) host cell): Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand

3.4 Bacterial cultivation and enzyme production

3.4.1 Starter inoculum

The pBC transformed *E. coli* strain BL21 (DE3) (GenBank accession no. AF302787) (Kaulpiboon et al., 2010) was transferred from 50% glycerol stock into Luria Bertani (LB) medium containing 100 µg/mL ampicillin and grown overnight at 37 °C with rotary shaking (160-180 rpm).

3.4.2 Enzyme production

One percent (v/v) of the starter inoculum was transferred into 300 mL LB medium in 1,000 mL Erlenmeyer flask and cultured at 37 °C with rotary shaking at 250 rpm. When the turbidity of the culture at O.D. 660 nm reached 0.6, IPTG was added to final concentration of 0.2 mM to induce CGTase gene expression and cultivation was continued at 37 °C for 24 hours. After cultivation, bacterial cells were removed by refrigerated centrifugation at 4,800 rpm for 45 minutes at 4 °C. Culture broth with crude CGTase was collected and kept at 4 °C for further purification.

3.5 Purification of CGTase

The pBC CGTase from culture broth was purified by two steps of purification; starch adsorption (Kato & Horikoshi, 1984) and DEAE-Toyopearl 650M column chromatography.

3.5.1 Starch adsorption

Corn starch was oven dried at 60 °C for 3 hours and cooled to room temperature. It was gradually sprinkled into stirring culture broth of crude CGTase to make a final concentration of 5% (w/v) at 4 °C. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 4,800 rpm for 45 minutes at 4 °C and washed twice with TB1 buffer (10 mM Tris-HCl pH 8.5 containing 10 mM $CaCl_2$). The adsorbed CGTase was eluted from the starch cake with 0.2 M maltose in TB1 buffer (3×50 mL for starting broth of 1,000 mL) by stirring for 30 minutes. The eluted CGTase was collected by centrifugation at 4,800 rpm for 60 minutes at 4 °C. The CGTase solution was concentrated using Aquacide II and dialyzed for 24 hours against three changes of TB1 buffer at 4 °C before subjecting to the next purification step.

3.5.2 DEAE-Toyopearl 650M column chromatography

The DEAE-Toyopearl 650M column was equilibrated with TB2 buffer (10 mM Tris-HCl, pH 8.0). The CGTase solution from starch adsorption was applied to DEAE-Toyopearl 650M column. The unbound proteins were eluted from

the column with equilibration buffer (TB2). After the column had been washed thoroughly with the TB2 buffer, the bound proteins were eluted from the column with a linear salt gradient of 0 to 0.2 M NaCl in the TB2 buffer. Fractions of 2 mL were continuously collected. The protein and CGTase dextrinizing activity profiles of the eluted fractions were monitored by measuring the absorbance at 280 and 600 nm, respectively. Fractions with CGTase dextrinizing activity were pooled for further characterization.

3.6 Assay of CGTase activity

3.6.1 Dextrinizing activity

The dextrinizing activity of CGTase was assayed by measuring the decrease in absorbance of the starch-iodine complex at 600 nm (modified from Fuwa, 1954). Enzyme sample (50 µL) was incubated with 0.15 mL of 0.2% (w/v) soluble potato starch in 0.2 M phosphate buffer, pH 6.0 at 40 °C for 10 minutes. The reaction was halted with 2 mL of 0.2 M HCl and 0.25 mL of iodine reagent (0.02% (w/v) I_2 in 0.2% (w/v) KI) was added. The mixture was adjusted to a final volume of 5 mL with distilled water and the absorbance at 600 nm was measured. For control tube, HCl was added before the enzyme sample. One unit of enzyme was defined as the amount of enzyme which produced 10% reduction in the intensity of blue color of the starch-iodine complex per minute under the described conditions.

3.6.2 Coupling activity

3.6.2.1 Glucose oxidase assay

Glucose oxidase assay measures the enzymatic activity in changing β-CD to glucose by the glucose oxidase kit (Miwa, Okudo, Maeda, & Okuda, 1972). The reaction mixture (100 µL) contained 40 µL of 1% (w/v) β-CD, 40 µL of 1% (w/v) cellobiose, 5 µL of the enzyme and 15 µL of 0.2 M phosphate buffer, pH 6.0. The reaction was performed at 50 °C for 5 minutes and halted by boiling for 5 minutes. After that, the reaction mixture was incubated with 10 µL of glucoamylase (stock 30 units/mg/mL) in 0.2 M phosphate buffer, pH 6.0 at 40 °C for 30 minutes and halted by boiling for 5 minutes. Then, 10 µL aliquot from the reaction mixture was added into 1 mL of glucose oxidase reagent. The glucose oxidase

reaction was incubated at 37 °C for 5 minutes and then the absorbance at 500 nm was measured. The glucose concentration was calculated from the equation (3.1);

$$\text{Glucose concentration (μmol/mL)} = 5.55 \times \frac{A_{Sample}}{A_{Std.}} \quad (3.1)$$

The absorbance of the standard solution ($A_{Std.}$) was measured by using 10 μL of a standard glucose (5.55 μmol/mL) with 1 mL of glucose oxidase reagent. One unit of coupling activity was defined as the amount of enzyme producing 1 μmol of glucose per minute under the assay conditions used.

3.6.2.2 Phenolphthalein assay

This assay measures the disappearance of β-CD in the reaction mixture by the phenolphthalein method which was slightly modified from Goel and Nene (1995). A 250 μL of β-CD as standard or sample solution (in which CGTase was preincubated with β-CD and alcohol acceptor) was incubated with 750 μL of phenolphthalein solution for 15 minutes. The decrease in absorbance at 550 nm caused by the complexion of the dye with β-CD was measured. Conversion of ΔA_{550} to μmoles β-CD was quantitated from the β-CD phenolphthalein calibration curve (Appendix E). The disappearance of β-CD in the reaction mixture was calculated from the difference between β-CD concentration at 0 and 24 hours incubation with CGTase and acceptors.

Phenolphthalein solution was prepared with 1 mL of 4 mM phenolphthalein solution in absolute ethanol, 100 mL of 125 mM Na_2CO_3 solution in distilled water and 4 mL ethanol. The solution was prepared freshly before starting the experiment. 0 to 0.25 mM β-CD standard was prepared.

3.7 Protein determination by Bradford protein assay

Protein concentration was determined by Bradford's method, using bovine serum albumin as the standard protein (Bradford, 1976). Enzyme sample (100 μL) was mixed with 1 mL of Bradford Coomassie brilliant blue reagent and left for 5 minutes before determining the absorbance at 595 nm (Appendix F).

3.8 Polyacrylamide gel electrophoresis (PAGE)

3.8.1 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

The starch degrading activity of CGTase was determined from the migration of enzyme on a non-denaturing gel. The gel was consisted of 7.5% (w/v) separating and 5.0% (w/v) stacking gels and carried out under Tris-glycine, pH 8.0 as electrode buffer. The enzyme samples were mixed with sample buffer and loaded into wells. The electrophoresis was performed at constant current of 12 mA per slab at room temperature on a Mini-Gel electrophoresis unit from cathode (-) towards anode (+) until the dye front reached the bottom of the gel. Then, the gel was cut into two parts for protein and dextrinizing activity staining.

3.8.2 Detection of proteins and CGTase activity

3.8.2.1 Protein staining

After electrophoresis, proteins on native gel were visualized by Coomassie brilliant blue staining. Gel was stained with 0.1% (w/v) of Coomassie® brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes. The slab gel was destained with a solution of 10% (v/v) methanol and 10% (v/v) acetic acid for 2 hours, followed by several changes of destaining solution until gel background was clear.

3.8.2.2 Dextrinizing activity staining

For dextrinizing activity staining, the native gel was soaked with 2% (w/v) soluble starch in 0.2 M phosphate buffer, pH 6.0 at 40 °C for 10 minutes. The gel was then quickly rinsed several times with distilled water and 10 mL of I_2 staining reagent (0.2% (w/v) I_2 in 2.0% (w/v) KI) was added for color development at room temperature. The clear zone on the blue background represents the starch degrading activity of the CGTase.

3.8.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing gel was carried with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels with Tris-glycine buffer, pH 8.0 containing 0.1% (w/v) SDS as electrode buffer. Samples were treated with sample buffer and

boiled for 10 minutes prior to loading to the gel. The electrophoresis was run from cathode (-) towards anode (+) at constant current of 20 mA per slab at room temperature on a Mini-Gel electrophoresis unit. After electrophoresis, proteins in the gel were visualized by protein staining as previously described. The molecular weights of the standard proteins used were myosin (250 kDa), phosphorylase (150 kDa), β-galactosidase (100 kDa), bovine serum albumin (75 kDa) and ovalbumin (50 kDa).

3.9 Characterization of CGTase

3.9.1 Molecular weight determination

The molecular weight of purified CGTase was determined by SDS-PAGE. The relative mobility (R_f) value of the standard proteins was plotted against their log molecular weights to obtain a calibration curve. The R_f values are calculated from the migration distance of the protein on the gel divided by the migration distance of the dye front. The molecular weight of the purified CGTase can be extrapolated from this calibration curve.

3.9.2 Effect of temperature on CGTase activity

The effect of temperature on CGTase activity was examined in 0.2 M phosphate buffer, pH 6.0 at various temperatures in the range of 30-80 °C. The activity was determined by coupling activity as described in Section 3.6.2.1. The results were shown as a percentage of the relative activity. The temperature at which maximum activity was observed for each reaction was set as 100%.

3.9.3 Effect of temperature on CGTase stability

The effect of temperature on CGTase stability was determined at 25, 40, 50 and 60 °C. The enzyme was preincubated at various temperatures for 0-120 minutes before determination of enzyme activity under the standard assay condition as described in Section 3.6.2.1. The results were shown as a percentage of the relative activity. The highest activity was defined as 100%.

3.9.4 Effect of pH on CGTase activity

The effect of pH on CGTase activity was determined at 50 °C at various pHs. The different types of buffers at concentration of 0.2 M were used:

acetate buffer (pH 3.0-5.0); phosphate buffer (pH 5.0-7.0) and Tris-HCl (pH 7.0-9.0). The activity was determined by coupling activity as described in Section 3.6.2.1. The results were shown as a percentage of the relative activity. The pH at which maximum activity was observed for each reaction was set as 100%.

3.9.5 Effect of pH on CGTase stability

CGTase was preincubated in 10 mM of various pH buffers: acetate buffer (pH 3.0-5.0), phosphate buffer (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and Glycine-NaOH (pH 9.0-11.0) at 4 and 50 °C for 24 hours. The remaining CGTase activity was determined by coupling activity as described in Section 3.6.2.1. The results were shown as a percentage of the relative activity. The highest remaining activity was defined as 100%.

3.9.6 Donor specificity

Several glucose-rich donors were investigated for transglycosylation activity by CGTase. The reaction mixtures (250 µL) containing final concentration of 0.64% (w/v) cellobiose, 0.64% (w/v) different glycosyl donors (α-CD, β-CD, γ-CD) and 75 U/mL CGTase in 20 mM phosphate buffer pH 6.0 were incubated at 50 °C for 24 hours. Then, 5 µL aliquot was withdrawn and glucose concentration was determined by the glucose oxidase method (Section 3.6.2.1). Samples were analyzed using thin layer chromatography (TLC) (System I). The intensity of alkyl glycoside product spots was quantitated relative to glucose spot on the same TLC plate. The highest intensity of alkyl glycoside spots was set to 100%.

3.10 Transglycosylation reaction

To determine the synthetic ability of CGTase and an appropriate alcohol acceptor, tranglycosylation reaction with varying types and alcohol contents were performed using β-CD as a glycosyl donor. The reaction mixture (250 µL) consisted of final concentration of 0.64% (w/v) β-CD, various concentrations and types of alcohols (methanol, ethanol: 10-50% (v/v), 1-propanol: 5-40% (v/v), 1-butanol, 1-pentanol, isopentanol: 5-25% (v/v)) and CGTase (final dextrinizing activity of 75 U/mL) in 20 mM phosphate buffer pH 6.0. The reaction mixture was incubated at 50 °C for 24 hours. The reaction mixture was halted by boiling for 5 minutes.

The coupling activity which measured the amount of β-CD disappearance was determined by the phenolphthalein method as described in Section 3.6.2.2. In addition, an aliquot from each reaction mixture was withdrawn and analyzed by TLC (System II) as described in Section 3.11.

3.11 Determination of alkyl glycoside products by thin layer chromatography (TLC)

System I (for determination of donor specificity)

Reaction products were analyzed by applying samples on thin layer chromatography (TLC) silica gel 60 F_{254} (20 cm in height), resolved once in a system composing of 1-butanol/ethanol/water (5:5:3, v/v) for finding the best donor in the synthesis of glycoside products. The TLC chromatograms were visualized by dipping with concentrated sulfuric acid/methanol (1:9, v/v) followed by heating at 110 °C for 20 minutes. The intensity of synthesized product spots was quantitated by a scanning densitometer with Quantity One® 1-D analysis program. Glucose spot (100 µg) was set as standard value.

System II (for identification of alkyl glycoside products)

Alkyl glycoside products were analyzed by TLC. System II composed of ethyl acetate/acetic acid/water (3:1:1, v/v) was used for running a TLC plate. After running, the TLC plate was visualized in the same procedure as System I. The intensity of alkyl glycoside product spots was evaluated by methyl-α-D-glucopyranoside (MG_1) standard curve which was performed from the intensity of standard MG_1 on TLC plate (Appendix G).

3.12 Optimization of alkyl glycoside production

3.12.1 Optimization of alcohol concentration

Alcohol concentration ranging from 5-50% (v/v) in appropriate buffer type and pH from Section 3.9.4 was used to dissolve 0.64% (w/v) β-CD and mixed with 75 U/mL CGTase for 24 hours at a proper temperature from Section 3.9.2. Then, the reaction mixture was runned on TLC plate (System II) as described in

Section 3.11 to find the optimal concentration of each alcohol in the synthesis of alkyl glycosides.

3.12.2 Optimization of β-CD concentration

β-CD was selected as an appropriate donor for the synthesis of alkyl glycosides by transglycosylation reaction. The amount of β-CD which was varied from a final concentration of 0.3-1.8% (w/v), was dissolved in a buffer solution with pH which presented the highest activity from Section 3.9.4. Then, the β-CD solution was mixed with an optimal concentration of alcohol from Section 3.12.1 and 75 U/mL CGTase. The mixture was incubated at the temperature that gave the highest activity from Section 3.9.2 for 24 hours. The optimal concentration of β-CD was determined by TLC analysis (System II) as described in Section 3.11.

3.12.3 Optimization of enzyme concentration

To find the optimal concentration of CGTase, alcohol acceptor concentration (Section 3.12.1) and β-CD substrate concentration (Section 3.12.2) which were selected from the previous investigation were incubated with 50-500 units of CGTase at the appropriate pH (Section 3.9.4) and temperature (Section 3.9.2) for 24 hours with continuous shaking. The optimal enzyme concentration was decided from the amount of alkyl glycoside products from TLC analysis (System II) as described in Section 3.11.

3.12.4 Optimization of incubation time

The reaction mixture consisted of an optimal concentration of alcohol (Section 3.12.1) was incubated with appropriate β-CD (Section 3.12.2) and CGTase (Section 3.12.3) under optimal pH (Section 3.9.4) and temperature (Section 3.9.2) for transglycosylation reaction. The reaction was incubated for various times (24, 48, 72, 96, 120, 144 and 168 hours). The suitable incubation time for the production of alkyl glycosides was chosen from TLC analysis (System II) as described in Section 3.11.

3.12.5 Optimization of temperature

The optimum temperature for synthesis of alkyl glycosides was determined in reaction mixture consisted of an optimum concentration of alcohol, β-CD and CGTase as reported in Section 3.12.1, 3.12.2 and 3.12.3, respectively. The reaction mixture was performed at optimal pH (Section 3.9.4), incubation time

(Section 3.12.4) and various temperatures (30, 40, 50, 60 and 70 °C). The optimal temperature for the production of alkyl glycosides was decided from TLC analysis (System II) as described in Section 3.11.

3.12.6 Optimization of pH

The reaction mixture was performed by incubation of an optimal concentration of alcohol acceptor, β-CD donor and CGTase at best incubation time and temperature from the previous Section in buffers with pH ranging from 4.0-9.0. Different inorganic substances were used as buffers e.g. acetate (pH 5.0-6.0), phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.0-8.0). The optimal pH for the production of alkyl glycosides was judged from TLC analysis (System II) as described in Section 3.11.

3.13 Large scale production of alkyl glycosides

Large scale production of alkyl glycosides was performed under the optimum conditions. In the initial experiments, transglycosylation reaction was performed in the reaction mixture of 250 µL. To obtain the higher yield of products, large scale production of alkyl glycosides was prepared in a total volume of 250 mL in 1,000 mL Erlenmeyer flask. After the incubation was completed, the reaction mixture was halted by boiling for 5 minutes and concentrated by a centrifugal evaporator at 45 °C before purification of alkyl glycosides by preparative thin layer chromatography (PLC).

3.14 Purification of alkyl glycosides by preparative thin layer chromatography (PLC)

The reaction mixture from the previous step was applied on a PLC plate. The PLC was allowed to run with the solvent System II until the solvent front reached to top. After the preparative plate was dried, one lane of the reaction mixture on the plate was then gently cut to detect the position of alkyl glycoside products using sulfuric acid/methanol (1:9 by vol) developing solution. The partial plate was heated at 110 °C for 20 minutes. Each product from PLC which had approximately the same

R_f values was scraped separately. The products were extracted from silica gel by dissolving with methanol for 30 minutes at room temperature and then centrifuged at 4,800 rpm for 1 hour at 25 °C. Methanol was further removed by a centrifugal evaporator at 45 °C until the products were completely dry.

3.15 Structural analysis of alkyl glycosides

3.15.1 Digestion with amylolytic enzymes

The structure of alkyl glycosides was preliminarily characterized by incubating with amylolytic enzymes. The dried alkyl glycoside products at different R_f value (2 mg) from Section 3.14 were mixed and dissolved in 0.2 M acetate buffer pH 5.5. Then, the reaction was incubated with 20 U/mL of *Rhizopus* sp. glucoamylase (E.C. 3.2.1.3) and *S. cerevisiae* α-glucosidase (E.C. 3.2.1.20) at 37 °C for 24 hours. After incubation, the reaction was halted by boiling for 5 minutes. Then, 10 µL aliquot from the reaction was analyzed by TLC (System II) as described in Section 3.11.

3.15.2 Mass spectrometry (MS) analysis

A mass spectrometry device determines the molecular mass of chemical compounds by separating ionic molecules according to their mass-to-charge ratio (m/z). The molecular mass was calculated by the equation (3.2);

$$\frac{(\text{molecular mass} + \text{number of protons})}{\text{charge}} = \text{mass-to-charge ratio (m/z)} \qquad (3.2)$$

The alkyl glycoside products were dissolved in a 50% (v/v) methanol solution and subjected into mass spectrometry device. Electrospray Ionization Time of Flight Mass Spectrometry (ESI-TOF-MS) profile was recorded on a Bruker micrOTOF mass spectrometer at the Department of Biology, Graduated School of Science, Osaka City University (Osaka, Japan). The compounds were ionized by electrospray ionization on the positive-ion mode using capillary voltage of 5,000 volts. The 4 liters per minute flow of nitrogen gas at a temperature of 150 °C was used to nebulize the analytic solution to droplets using nebulizer pressure at 1 bar.

3.15.3 Nuclear magnetic resonance (NMR) analysis

Structural identification of the synthesized alkyl glycosides by CGTase was performed by ^{1}H- and ^{13}C-NMR with Bruker AVANCE III HD 600 NMR Spectrometer at the Department of Biology, Graduated School of Science, Osaka City University (Osaka, Japan). The operation was 600 MHz at ambient temperature. The 2 mg of dried alkyl glycoside products were dissolved in 1 mL of methanol deuterium (CD_3OD). The chemical shift values were measured with sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard.

3.16 Characterization of alkyl glycoside products

3.16.1 Emulsification activity

The final concentration (0.5 mg/mL) of purified alkyl glycosides was prepared by diluting with 0.5 mM Tris-HCl buffer containing 0.05 M $MgSO_4$ (pH 8.0). The 4 mL of solution was mixed with 100 µL of the n-hexadecane substrate to form an oil-in-water emulsion by vigorous mixing on vortex for 2 minutes. The emulsification activity was obtained after the reaction was left to stand for 10 minutes. The turbidity of emulsion was determined by spectrophotometer at 540 nm. Commercial non-ionic surfactants such as Triton^{TM} X-100, methyl-α-D-glucoside (MG_1) were also studied. The highest turbidity resulted from the presence of surfactant sample was set as 100% emulsification activity. The blank used only contained the Tris-HCl buffer.

3.16.2 Emulsification stability

The turbidity of emulsion performed was determined at 10, 20, 30, 40, 50 and 60 minutes. The absorbance at 540 nm was calculated as logarithm function. The dissociation constant (K_d) was calculated from slope obtained from plotting the time versus log $OD_{540.}$ The lowest K_d value, the best stable of emulsion formed, was set as 100% emulsification stability.

3.16.3 Antibacterial activity

Cultures of the following microorganisms were used in the study: gram-positive *Staphylococcus aureus* ATCC 25923 and gram-negative *Escherichia coli* ATCC 25922.

3.16.3.1 Disc diffusion technique

Disc diffusion technique was used to screen the susceptibility of bacteria to an antibacterial agent. Filter paper discs (6 mm in diameter) were impregnated with a 10 µL of the alkyl glycosides (concentration 5 mg/disc). The inoculum density for a susceptibility test was adjusted equivalent to a 1×10^8 CFU/mL (0.5 McFarland standard). An air-dried disc was placed on the surface of *S. aureus*-inoculated mannitol salt agar and *E. coli*-inoculated LB agar. Positive controls such as 10 µg/disc ampicillin, 10 µg/disc penicillin, 30 µg/disc tetracycline and 1 µg/disc oxacillin were also tested as well as sterile water as a negative control. The plates were incubated at 37 °C for 24 hours. The clear zone of inhibition was calculated by measuring the diameter of the inhibition zone surrounding the paper disc. The readings were taken in three different fixed directions and the average value of inhibition zone was tabulated.

3.16.3.2 Evaluation of minimal inhibitory concentration (MIC)

The MIC value was determined by microdilution technique in liquid LB medium by incubating the bacteria in LB broth with variable amounts of the sample tested. This technique utilizes the microdilution method in a 96-well microplate. Briefly, the 6 mg starter concentration of alkyl glycosides in 50 µL of sterile water was diluted as a two-fold serial dilution in the microplate. The 0.5 McFarland cultures of *S. aureus* and *E. coli* were diluted with LB broth in a 1:200 (v/v) ratio. Then, 50 µL of culture was added in the 96-well microplate. After 18 hours of incubation at 37 °C, 10 µL of resazurin solution was added to each well and incubated at 37 °C for 2 hours. The MIC value of alkyl glycoside products was defined as the lowest concentration of the alkyl glycosides that prevented a color change of resazurin from blue to pink in the 96-well microplate. Resazurin, a blue dye, becomes pink when is reduced to resorufin by oxidoreductase within a medium of viable bacteria. The MIC value was done in triplicate experiments and expressed as mg/mL.

3.16.3.3 Evaluation of minimal bactericidal concentration (MBC)

The MBC test determines the lowest concentration at which an antibacterial agent will kill a whole bacterium. The MBC is determined using a series of steps, undertaken after a MIC test has been completed. Briefly, the MBC test was determined by sub-culturing each well in which no visible growth occurred from a previous MIC test to an agar medium. Then, the agar medium was incubated at 37 °C for 24 hours, and bacterial growth was evaluated. The lowest concentration of alkyl glycosides showing no growth on the agar plate was determined as the MBC value. This experiment was tested in triplicate and the MBC value was recorded as mg/mL.

3.16.4 Examination of bacterial insoluble protein solubility

3.16.4.1 Preparation of *Corynebacterium glutamicum* insoluble amylomaltase protein from *E. coli* BL21 (DE3) transformants

The recombinant A406R *C. glutamicum* amylomaltase from *E. coli* strain BL21 (DE3) transformants was selected as the insoluble protein model (Nimpiboon et al., 2016). The transformants were grown in LB medium containing 100 µg/mL ampicillin at 37 °C until the absorbance at 600 nm of the culture medium reached 0.4, then the final concentration of 0.4 mM IPTG was added. After 2 hours of IPTG induction, the cell pellets with insoluble amylomaltase protein were harvested by centrifugation at 4,800 rpm at 4 °C for 45 minutes.

3.16.4.2 Analysis of amylomaltase protein solubility

One percent (w/v) of alkyl glycoside product was added to 50 mM Tris-HCl buffer at pH 7.4 and used as a sonication buffer to break the cell pellets. The sonication was performed using a 5 mm diameter microtip in an ice bath with 50% amplitude for 3 cycles of 5 minutes pulse and 5 minutes pause. Then, the soluble and insoluble proteins obtained were incubated at 30 °C for 2 hours following by centrifugation at 8,000 rpm at 4 °C for 2 hours. The property of the added alkyl glycoside in the solubilization of insoluble proteins was determined from the amount of the target amylomaltase protein in the soluble fraction on the 10% SDS-PAGE and its starch transglycosylation activity of potato starch and maltose acceptor, using the iodine detection method as described in Section 3.16.4.3. The concentration of alkyl glycoside was varied in 1, 3 and 5% (w/v) to achieve the best amylomaltase solubility.

In addition, other commercial ionic and non-ionic surfactants such as sodium dodecyl sulfate (SDS) and Triton™ X-100, which are commonly used to inhibit the aggregation of insoluble proteins, were also investigated as comparators.

3.16.4.3 Starch transglycosylation activity of amylomaltase

The activity of amylomaltase was assayed by the starch transglycosylation reaction. This assay measures the disappearance of starch-iodine complex caused by the transferring glucosyl unit from starch polysaccharide to maltose using the iodine solution method (Nimpiboon et al., 2016). The 100 µL of enzyme solution was incubated with 250 µL of 0.2% (w/v) soluble potato starch, 50 µL of 1% (w/v) maltose and 0.6 mL of 50 mM Tris-HCl buffer, pH 7.4. The reaction was performed at 30 °C for 10 minutes and halted by boiling for 10 minutes. Then, 100 µL aliquots were withdrawn and mixed with 1 mL iodine solution (0.02% (w/v) I_2 in 0.2% KI (w/v)) and the absorbance at 600 nm was measured. One unit of starch transglycosylation assay was defined as the amount of enzyme that produces a one percent reduction in the absorbance of the starch-iodine complex per minute under the described conditions.

3.16.4.4 Protein determination by bichinoninic acid assay

The amount of protein was measured by the Pierce™ BCA protein assay kit based on bicinchoninic acid method, using bovine serum albumin as a standard (Smith et al., 1985). The 25 µL of sample was mixed with 200 µL of the working bicinchoninic reagent in a 96-well microplate. After incubation at 37 °C for 30 minutes, the absorbance at 562 nm was measured on microplate spectrophotometer (Appendix H).

CHAPTER 4
FINDINGS

4.1 Purification of CGTase

4.1.1 Preparation of crude enzyme

The cells were cultivated from 2.4 liters of LB medium as described in Section 3.4.2. After cultivation, the cells were removed by centrifugation at 4,800 rpm for 45 minutes at 4 °C. The obtained supernatant was used as crude CGTase. The total dextrinizing activity and protein of crude CGTase were measured to be 1.06×10^6 units and 595.20 mg protein, respectively (Table 4.1).

4.1.2 Enzyme purification steps

4.1.2.1 Starch adsorption

The crude enzyme was purified by starch adsorption as described in Section 3.5.1. Starch adsorption step, total activity of the enzyme was 7.56×10^5 units of dextrinizing activity and the recovered proteins were 69.95 mg protein. Therefore, the specific activity of the enzyme from this step was 1.08×10^4 units/mg protein by calculating from the equation (4.1);

$$\text{Specific activity (unit/mg protein)} = \frac{\text{Total enzymatic activity}}{\text{Total protein}} \qquad (4.1)$$

The obtained yield of the enzyme from starch adsorption step was 71% yield with 6-fold purification (Table 4.1).

4.1.2.2 DEAE-Toyopearl 650M column chromatography

The partially purified enzyme from starch adsorption was concentrated by Aquacide II and dialyzed before loaded to DEAE-Toyopearl 650M column as described in Section 3.5.2. The column was first equilibrated with TB2. Then, the unbound proteins were eluted from the column by the equilibration buffer. The bound proteins were eluted by a linear salt gradient from 0 to 0.2 M NaCl in the same buffer (Figure 4.1). The fractions with dextrinizing activity were pooled and assayed. For this step, the enzyme was purified to 7-fold purification with 43% yield

and the specific activity was 1.30×10^4 units/mg protein (Table 4.1). The purified enzyme from DEAE-Toyopearl 650M column was kept at 4 °C for future experiment.

4.2 Polyacrylamide gel electrophoresis (PAGE)

The CGTase from each step of purification was monitored by two types of electrophoresis.

4.2.1 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

Purification of CGTase was determined by native polyacrylamide gel electrophoresis (Native-PAGE) and stained for both protein and dextrinizing activity as described in Section 3.8.1. By Native-PAGE analysis, protein staining revealed that the enzyme was highly purified through the first starch adsorption step. A single protein band was observed on the protein staining gel which was consistent with activity band of the enzyme as shown in Figure 4.2, Lane 2. Although the result showed only one protein band on the native gel of starch adsorption step, it was unclear on SDS-PAGE with the smeared bands (Figure 4.3, Lane 2). So, DEAE-Toyopearl 650M column chromatography should be performed in the next step.

4.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The CGTase from each step of purification was also examined for its purity and protein pattern by SDS-PAGE as described in Section 3.8.3. By SDS-PAGE analysis, the crude enzyme showed many bands of protein. When the crude enzyme was first purified by starch adsorption, the result showed one major band and smeared bands on the SDS gel (Figure 4.3, Lane 2). To purify a CGTase protein to homogeneity, the DEAE-Toyopearl 650M column chromatography was used in the second step. After purified through the DEAE column, a single band of CGTase was observed (Figure 4.3, Lane 3).

Table 4.1 Purification of CGTase from the pBC transformant cell

Fraction	Total volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude CGTase	2400	443.02	1.06×10^{6}	0.248	595.20	1.78×10^{3}	1	100
5% Starch adsorption	250	3026.48	7.56×10^{5}	0.280	69.95	1.08×10^{4}	6	71
DEAE-Toyopearl 650M	90	4795.92	4.32×10^{5}	0.370	33.30	1.30×10^{4}	7	43

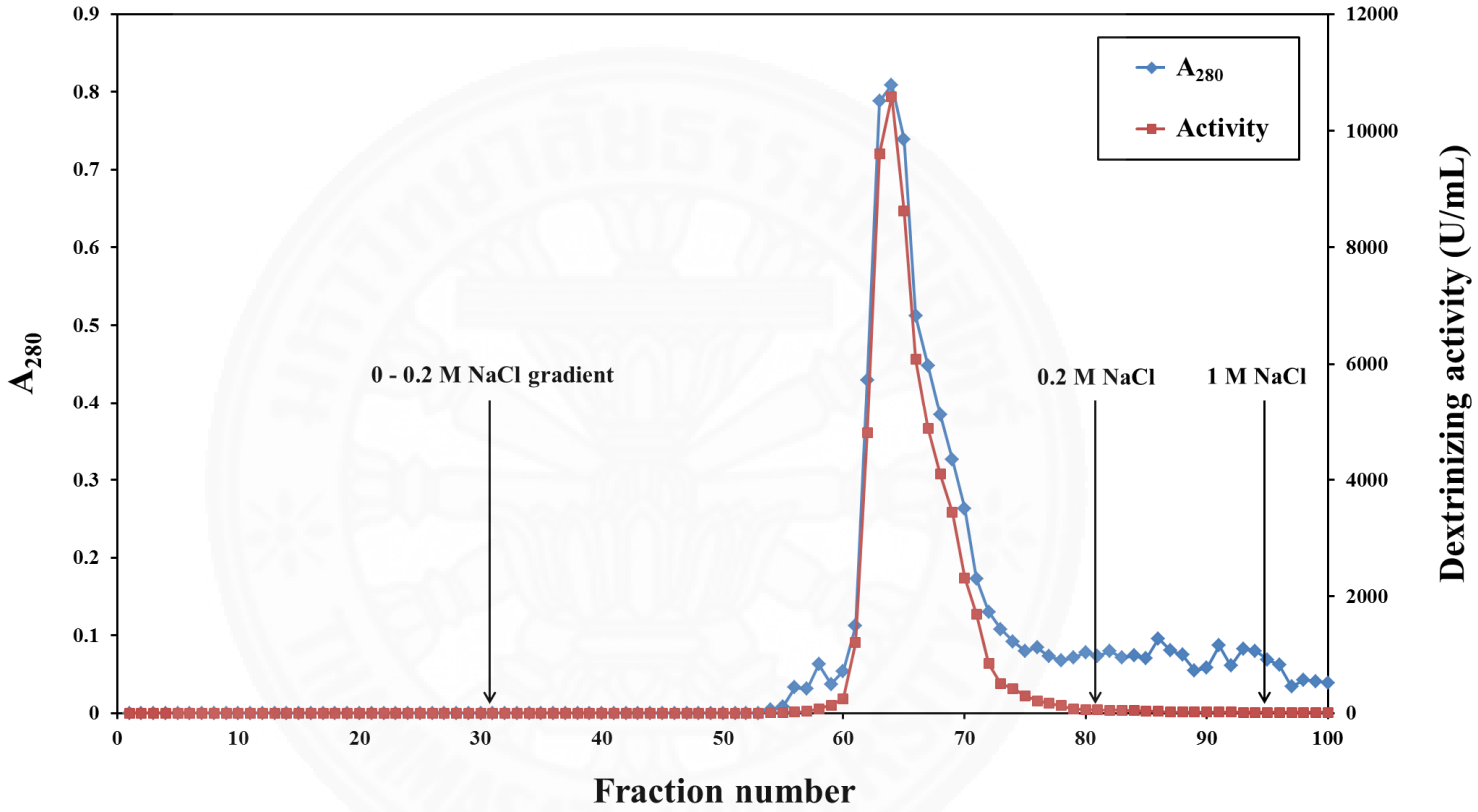


Figure 4.1 Purification profile of pBC recombinant CGTase by DEAE-Toyopearl 650M column

The partially purified CGTase was applied to DEAE-Toyopearl 650M column (7×2 cm) and eluted with 10 mM Tris-HCl, pH 8.0 containing 0 to 0.2 M NaCl gradient at a flow rate of 1 mL per minute. Fractions of 2 mL were collected. The arrows indicate where elution of the bound proteins started with different elution buffers.

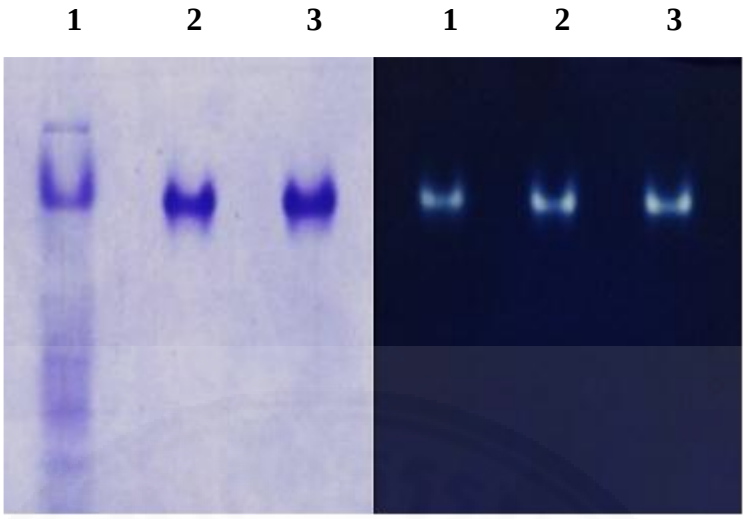


Figure 4.2 7.5% Non-denaturing PAGE of CGTase from each purification step

(A): Coomassie brilliant blue staining

Lane 1: Crude enzyme (50 µg)

Lane 2: Starch adsorption (10 µg)

Lane 3: DEAE-Toyopearl 650M column (10 µg)

(B): Dextrinizing activity staining

Lane 1: Crude enzyme (0.02 U)

Lane 2: Starch adsorption (0.02 U)

Lane 3: DEAE-Toyopearl 650M column (0.02 U)

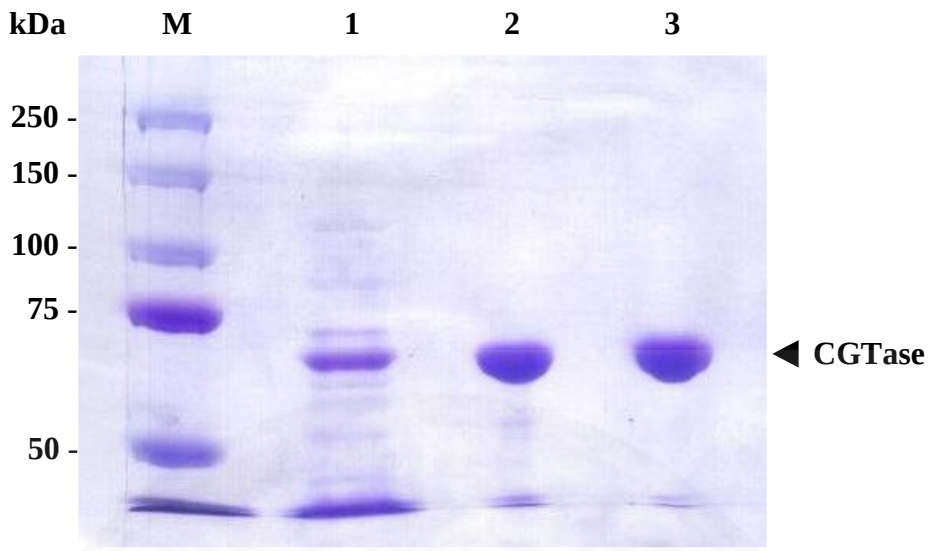


Figure 4.3 7.5% SDS-PAGE of CGTase from each purification step

Lane M: Protein marker (5 µL)

Lane 1: Crude enzyme (50 µg)

Lane 2: Starch adsorption (20 µg)

Lane 3: DEAE-Toyopearl 650M column (10 µg)

The arrow indicates a molecular weight of CGTase (72 kDa).

4.3 Characterization of CGTase

4.3.1 Molecular weight determination

The molecular weight of CGTase was determined by SDS-PAGE as described in Section 3.9.1. The standard curve of protein markers was constructed from the molecular weight of the standard proteins and their R_f values. The relative mobility of CGTase was 0.66, and the molecular weight of enzyme was estimated to be about 72 kDa by 7.5% SDS-PAGE (Figure 4.4).

4.3.2 Effect of temperature on CGTase activity

The effect of temperature on CGTase activity was examined in the range of 30-80 °C. The enzyme activity was determined as described in Section 3.9.2. The highest enzyme activity was at 50 °C which is defined as 100% activity. In addition, the enzyme could retain its activity over 80% at a temperature in the range of 30-60 °C (Figure 4.5).

4.3.3 Effect of temperature on CGTase stability

The effect of temperature on CGTase stability was determined at 25, 40, 50 and 60 °C. The enzyme was preincubated at various temperatures for 0-120 minutes before its activity was determined under the standard assay condition as described in Section 3.9.3. The enzyme showed high stability at 25, 40 and 50 °C for 120 minutes. At 60 °C, the enzyme showed low stability and mostly lost the activity after incubation for 120 minutes (Figure 4.6).

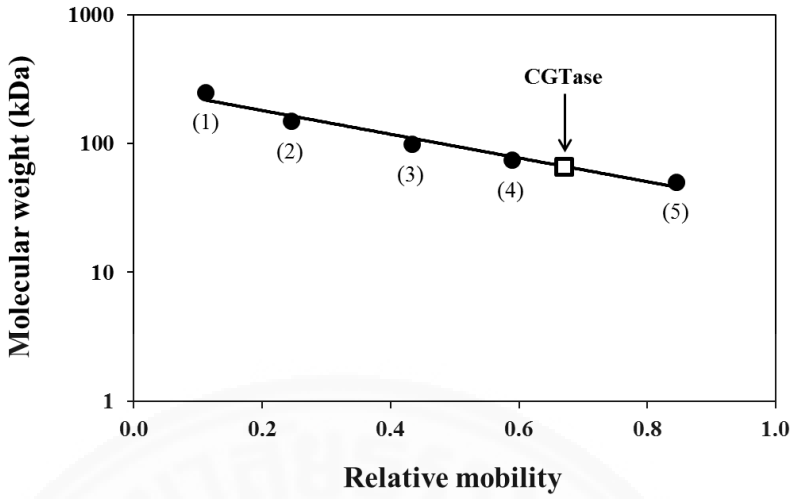


Figure 4.4 Calibration curve for molecular weight of CGTase by 7.5% SDS-PAGE

(1) = 250,000 Da (Myosin)

(2) = 150,000 Da (Phosphorylase)

(3) = 100,000 Da (β-galactosidase)

(4) = 75,000 Da (Bovine serum albumin)

(5) = 50,000 Da (Ovalbumin)

The arrow indicates a determined molecular weight of CGTase (72 kDa).

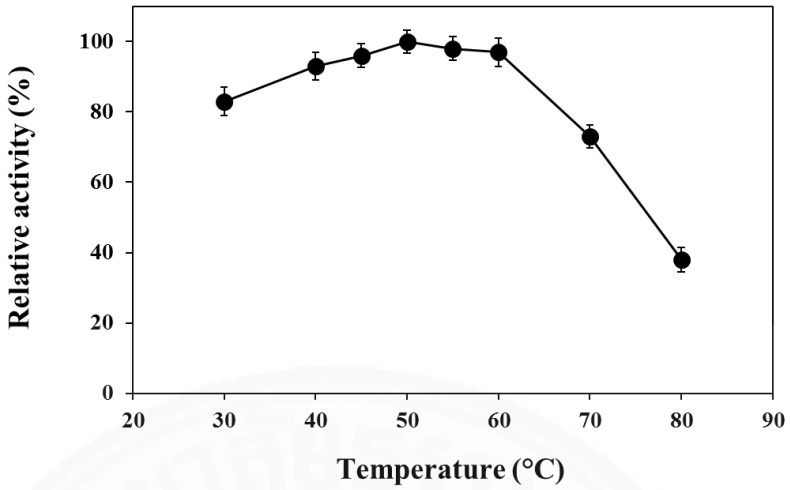


Figure 4.5 Effect of temperature on CGTase activity

The activity of CGTase in 0.2 M phosphate buffer, pH 6.0 was measured at various temperatures. The highest activity was defined as 100%. Data are shown as the mean ± SD and derived from three independent repeats.

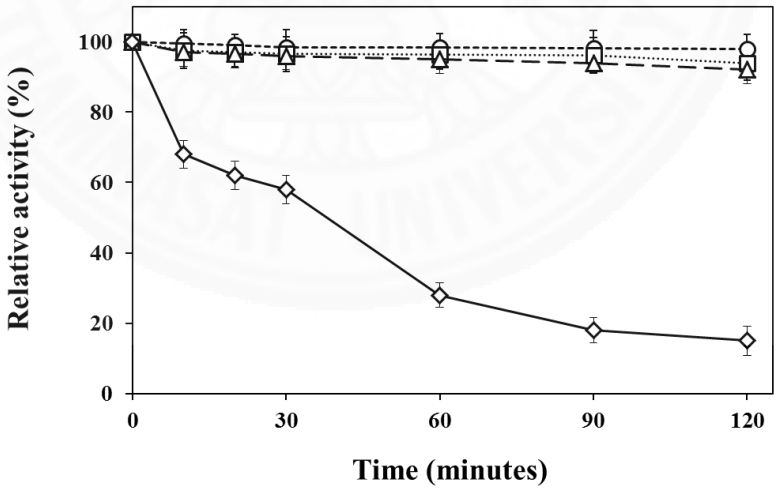


Figure 4.6 Effect of temperature on CGTase stability

The CGTase in 0.2 M phosphate buffer, pH 6.0 was preincubated at temperatures 25 (○), 40 (□), 50 (△) and 60 °C (◇) for 120 minutes, followed by measuring the residual activity. The highest activity was defined as 100%. Data are shown as the mean ± SD and derived from three independent repeats.

4.3.4 Effect of pH on CGTase activity

The effect of pH on CGTase activity was determined at 50 °C at various pHs. The buffers at concentration of 0.2 M were used: acetate buffer (pH 3.0-5.0), phosphate buffer (pH 5.0-7.0) and Tris-HCl (pH 7.0-9.0). The activity was determined by coupling reaction as described in Section 3.9.4. The result found that the optimum pH of CGTase was at pH 6.0 which was defined as 100%. The enzyme showed over 75% of the activity at pH 5.0 while low activity was observed at pH values below 4.0 and above 8.0 (Figure 4.7).

4.3.5 Effect of pH on CGTase stability

CGTase was preincubated in 10 mM of various pH buffers: acetate buffer (pH 3.0-5.0), phosphate buffer (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and Glycine-NaOH (pH 9.0-11.0) at 4 and 50 °C for 24 hours. The residual activity of the enzyme was determined by coupling reaction as described in Section 3.9.5. The result found that the enzyme showed high stability at pH 5.0-11.0 (4 °C) and pH 5.0-10.0 (50 °C) (Figure 4.8).

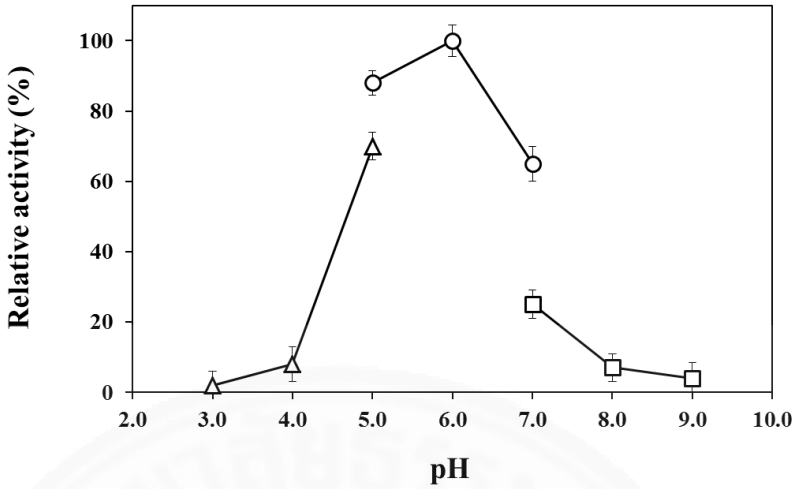


Figure 4.7 Effect of pH on CGTase activity

The activity of CGTase was determined by coupling activity at different pHs as the following: acetate buffer (pH 3.0-5.0; △), phosphate buffer (pH 5.0-7.0; ○) and Tris-HCl (pH 7.0-9.0; □). The highest activity was defined as 100%. Data are shown as the mean ± SD and derived from three independent repeats.

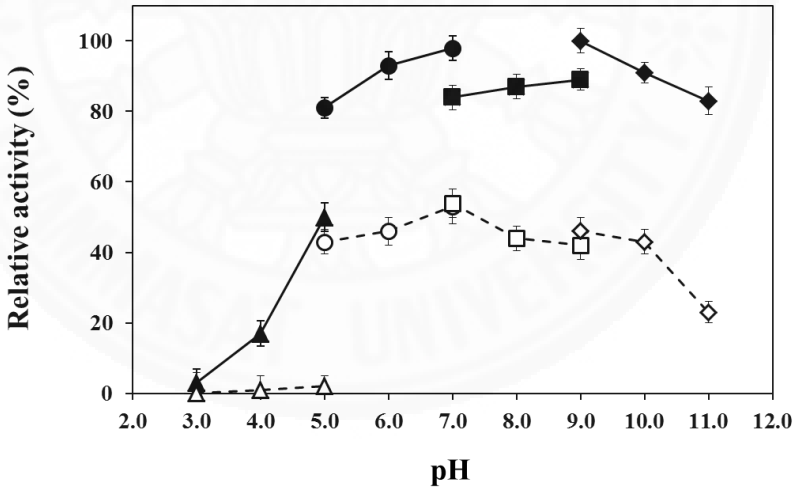


Figure 4.8 Effect of pH on CGTase stability

The CGTase was preincubated at 4 (solid line) and 50 °C (dash line) for 24 hours in 10 mM of various pHs as the following: acetate buffer (pH 3.0-5.0; ▲,△), phosphate buffer (pH 5.0-7.0; ●,○), Tris-HCl (pH 7.0-9.0; ■,□) and Glycine-NaOH (pH 9.0-11.0; ◆,◇). The residual activity was measured and the highest activity was defined as 100%. Data are shown as the mean ± SD and derived from three independent repeats.

4.3.6 Donor specificity

In order to investigate the donor specificity of CGTase on transglycosylation reaction, the reaction with cellobiose as an acceptor was performed. The various cyclodextrins (α-CD, β-CD and γ-CD) were selected as candidate glycosyl donors (Section 3.9.6). Donor specificity of CGTase was determined by coupling activity (glucose oxidase assay) and TLC analysis (System I) as described in Section 3.6.2.1 and 3.11, respectively. The result of coupling activity was expressed as a percentage of the highest activity, which was determined as 100%. The results indicated that relative activities of α-, β- and γ-CD donors were 96, 100 and 92.5%, respectively (Figure 4.9). Furthermore, the TLC result showed that many product spots were observed in the reaction mixtures at 24-hour incubation time (Figure 4.10, Lanes 7, 9 and 11). In contrast, at 0-hour incubation (control), no any transglycosylation products were observed on the TLC plate (Figure 4.10, Lanes 6, 8 and 10). The intensities of the product spots were also quantified using Quantity One® 1-D analysis software of densitometer. When α-, β- and γ-CD donors were used with cellobiose acceptor, the concentration of obtained transglycosylation products were 567.21, 588.71 and 544.89 μg/mL, respectively, compared to standard glucose spot from the same TLC plate. This finding concluded that descending order of preferred glycosyl donor substrate was β-CD>α-CD>γ-CD. Thus, the highest specificity of CGTase on glycosyl donor substrate was exerted by β-CD which was chosen as a glycosyl donor for transglycosylation to alcohol acceptor by CGTase further. The summary of the characteristics of pBC recombinant CGTase was shown in Table 4.2.

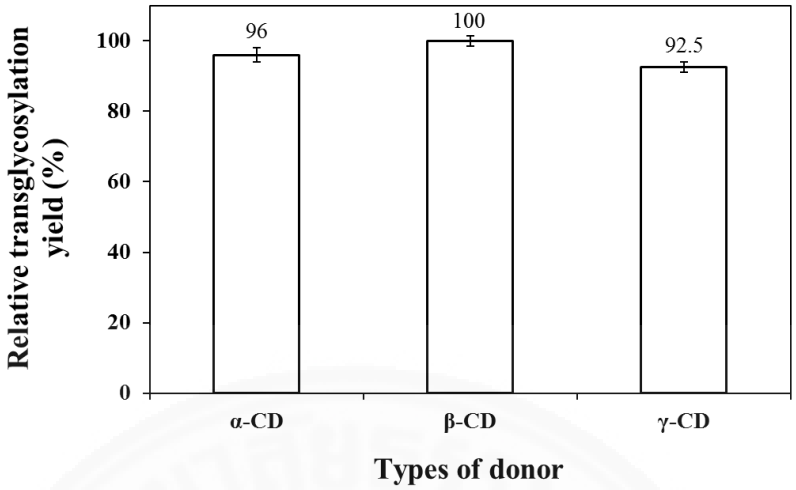


Figure 4.9 Relative transglycosylation yield of CGTase using cellobiose as acceptor with different glycosyl donors

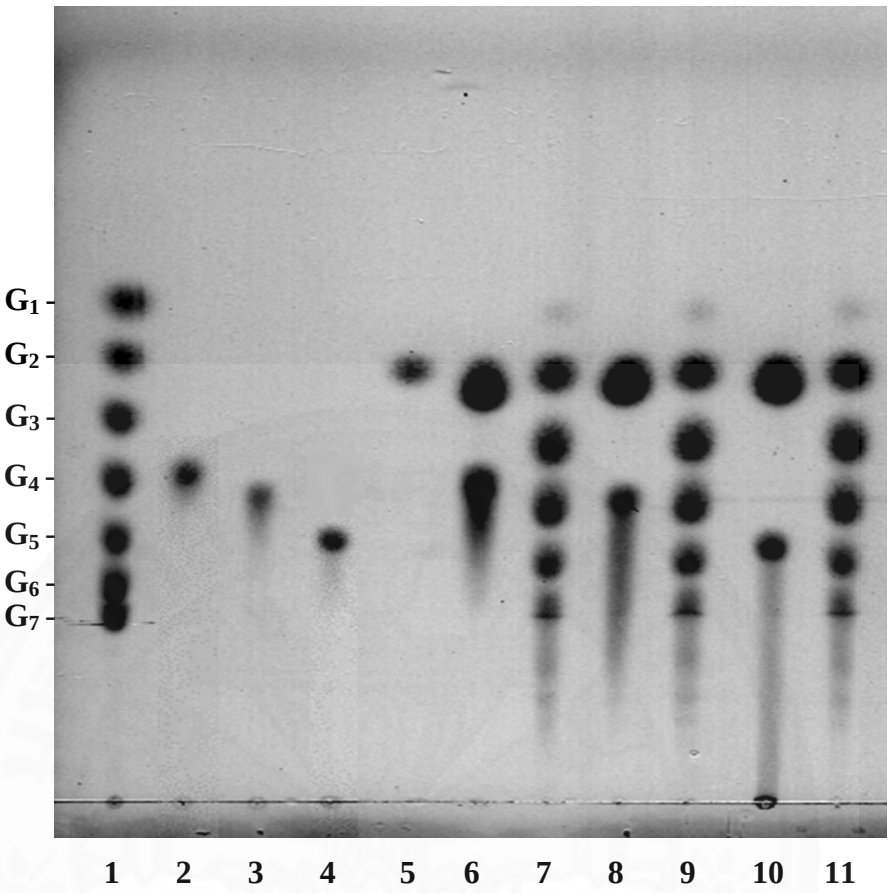


Figure 4.10 TLC analysis of the transglycosylation products of CGTase incubated with cellobiose and various donors. TLC condition was System I, 1-butanol/ethanol/ water with a 5:5:3 (v/v) ratio.

Lane 1: Standard G_1-G_7 mixed (100 µg)
Lane 2: Standard α-CD (10 µg)
Lane 3: Standard β-CD (10 µg)
Lane 4: Standard γ-CD (10 µg)
Lane 5: Standard cellobiose (10 µg)
Lane 6-7: Reaction mixture of α-CD as a donor, 0 and 24 hours
Lane 8-9: Reaction mixture of β-CD as a donor, 0 and 24 hours
Lane 10-11: Reaction mixture of γ-CD as a donor, 0 and 24 hours

Table 4.2 Summary of the characteristics of the pBC recombinant CGTase

Characteristic	**pBC recombinant CGTase**
Molecular weight	72 kDa
Optimum temperature	50 °C
Temperature stability	Retained > 90% activity (25-50 °C, 120 minutes)
Optimum pH	6.0
pH stability	5.0-11.0 (4 °C) and 5.0-10.0 (50 °C)
Donor specificity	β-CD

4.4 Effect of alcohol structure and concentration on the production of alkyl glycosides

4.4.1 Determination of CGTase coupling activity by phenolphthalein assay

The effect of alcohols on coupling activity of CGTase was investigated in buffer and in a mixture of buffer and alcohol solution with varying alcohol chain lengths and concentration at the optimum temperature 50 °C and pH 6.0 of the enzyme for 24 hours as described in Section 3.10. In a single phase reaction, the alcohol used here were 10-50% (v/v) methanol, ethanol, 5-40% (v/v) 1-propanol, 5-10% (v/v) 1-butanol. However, for 15-25% (v/v) 1-butanol and 5-25% (v/v) 1-pentanol and isopentanol, they were used in a two phase system due to their solubility in water. Following incubation, the reaction was halted by boiling and assayed by phenolphthalein assay as described in section 3.6.2.2. The coupling activity of CGTase in these alcohols was determined in relative to that in 20 mM phosphate buffer, pH 6.0 which was set as 100%. As shown in Figure 4.11, when the alkyl length and concentration of alcohol increased, the activity of the enzyme was dramatically decreased. However, in a two phase system of reaction mixtures, the activities of enzyme uncorrelated with alkyl length and concentration as compared to single phase experiments. The activity of CGTase was highest in system of 10 % (v/v) methanol, followed by 5 % (v/v) 1-pentanol, 10% (v/v) ethanol, 5% (v/v) 1-propanol, 25% (v/v) 1-butanol and 10% (v/v) isopentanol, respectively. These results showed that the alcohol structure and concentration had a significant effect on CGTase activity.

4.4.2 Determination of alkyl glycoside products by TLC analysis

The effect of alcohols on alkyl glycoside synthesis by CGTase was determined by TLC method and the product spot intensities were measured with Quantity One® 1-D analysis program as described in Section 3.11. The quantity of alkyl glycosides was determined from the intensity of product spots as µg/mL, compared to standard methyl-α-D-glucopyranoside (MG_1) spot on the same TLC plate. Only product spots from each alcohol acceptor which gave the higher R_f value than glucose, was quantitated to determine acceptor specificity because different

numbers of products were detected from each alcohol on TLC plate (data not shown). The results reflected that the production yield was highest in 30% (v/v) methanol, followed by 10% (v/v) 1-pentanol, 20% (v/v) isopentanol, 20% (v/v) ethanol, 10% (v/v) 1-propanol and 5% (v/v) 1-butanol, respectively (Figure 4.12).

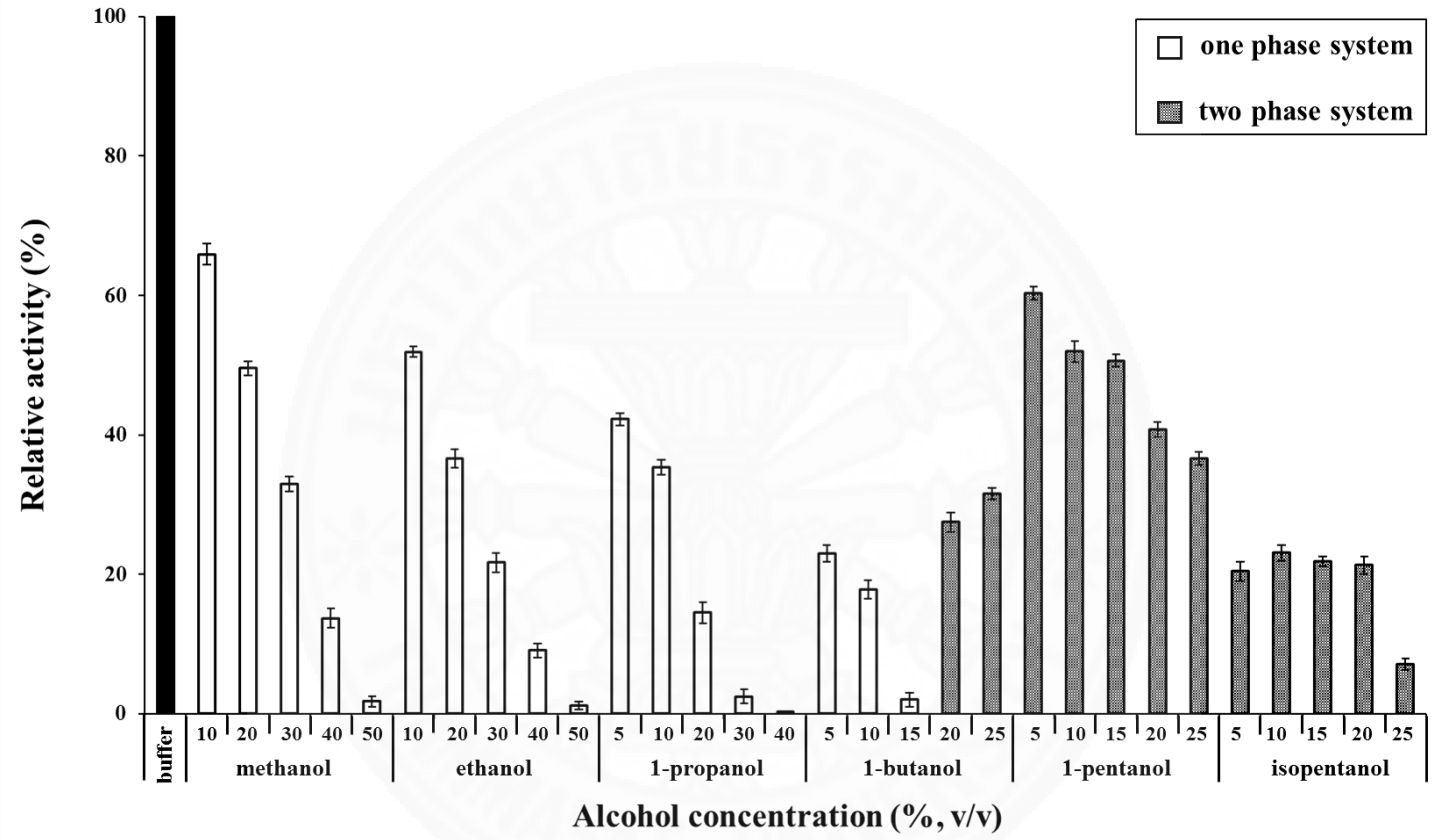


Figure 4.11 Relative coupling activity of CGTase in alcohol solvent mixtures

Residual activity (%) was relative to the coupling activity of CGTase incubated with 0.64% (w/v) β-CD and 5-50% (v/v) alcohols in 20 mM phosphate buffer solution (pH 6.0) at 50 °C for 24 hours. Data are shown as the mean ± SD and derived from three independent repeats.

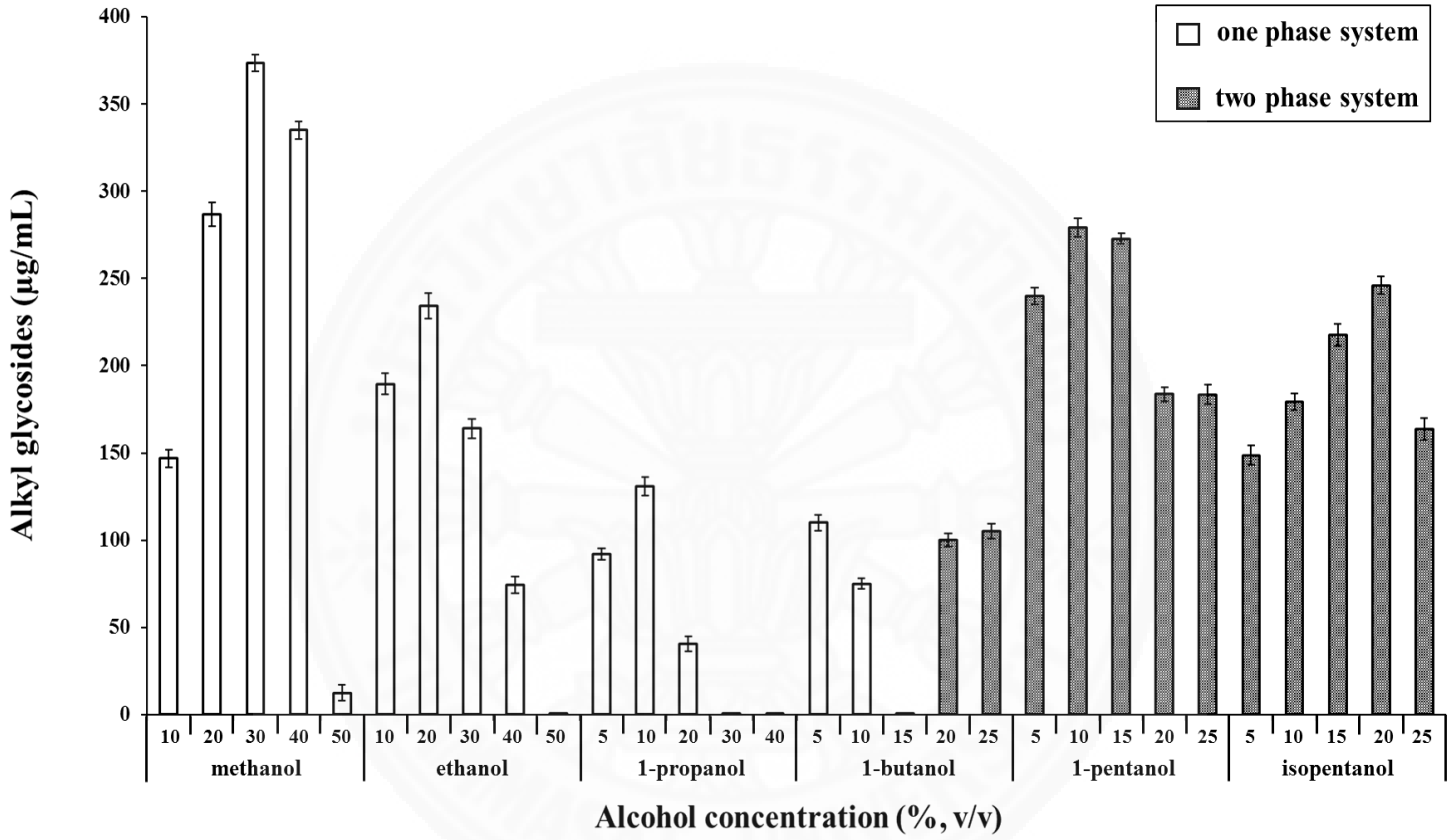


Figure 4.12 Alkyl glycoside products from transglycosylation reaction of β-CD to various alcohol acceptors

Alkyl glycosides of each spot were quantitated relative to standard MG_1 spot in the same TLC plate. Data are shown as the mean ± SD and derived from three independent repeats.

4.5 Synthesis of alkyl glycosides and detection of the products

Attempts were made to synthesize alkyl glycosides and to investigate the capability of CGTase in its transglycosylation activity on various types of alkyl alcohol. Different types and concentrations of alcohols were chosen as glycosyl acceptor substrates as described in Section 3.10. Products from each reaction mixture were analyzed by TLC (System II). In order to assure that the product observed as a spot on TLC was alkyl glycoside products by the action of CGTase, the reaction mixture without alcohol acceptors and the reaction mixture containing both substrates at appropriate concentration which gave the highest yield of alkyl glycosides as reported in Section 4.4.2 were incubated with CGTase for 24 hours. The control experiments of each alcohol acceptor were also performed by incubating CGTase with the substrates at 0 hour.

At 0 hour incubation time, only β-CD spots which slightly moved from the origin were seen (Figure 4.13, Lanes 4, 6, 8, 10, 12, 14 and 16). After 24 hours incubation of CGTase with β-CD donor and various concentrations of alkyl alcohol acceptors, expected alkyl glycoside spots were observed (at R_f value of 0.35-0.80). In addition, glucose and other oligosaccharides (at R_f value of 0.03-0.28) which resulted from hydrolysis reaction, were also observed (Figure 4.13, Lanes 5, 7, 9, 11, 13, 15 and 17). The transfer products of alcohols were distinguished from other oligosaccharides by higher R_f values (Table 4.3). Methanol gave at least one product while ethanol gave at least two products. When 1-propanol, 1-butanol, 1-pentanol and isopentanol were used as acceptor, three alkyl glycosides were detected. In the control condition where alcohols were not added, only oligosaccharides which ran slower than those of alkyl glycosides were observed (Figure 4.13, Lane 5). The results showed that CGTase from pBC recombinant cell could transfer glucose residues from β-CD to alkyl alcohols giving alkyl glycosides.

To study the effect of alkyl alcohol length and its structure on biological properties of the synthesized alkyl glycosides, the reaction mixtures of three types of primary alcohol acceptor (1-propanol, 1-pentanol and isopentanol) with β-CD donor under CGTase catalysis were optimized by varying several parameters involved in the reaction.

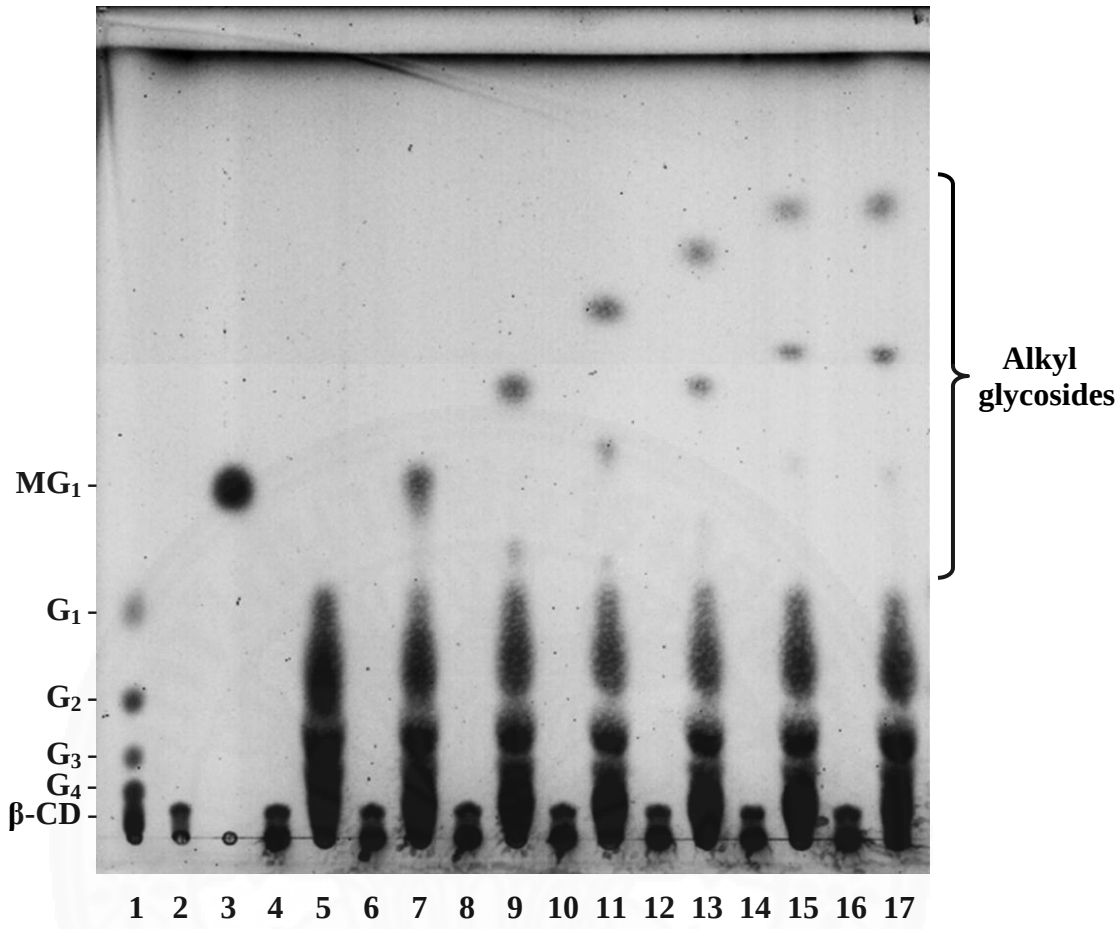


Figure 4.13 TLC analysis of the transglycosylation products from β-CD to various alcohols by CGTase. TLC condition was System II, ethyl acetate/acetic acid/water, 3:1:1 (v/v).

Lane 1:	Standard G_1-G_7 mixed (25 µg)
Lane 2:	Standard β-CD (10 µg)
Lane 3:	Standard MG_1 (25 µg)
Lane 4-5:	Reaction mixture without alcohol acceptor, 0 and 24 hours
Lane 6-7:	Reaction mixture with 30% (v/v) methanol, 0 and 24 hours
Lane 8-9:	Reaction mixture with 20% (v/v) ethanol, 0 and 24 hours
Lane 10-11:	Reaction mixture with 10% (v/v) 1-propanol, 0 and 24 hours
Lane 12-13:	Reaction mixture with 5% (v/v) 1-butanol, 0 and 24 hours
Lane 14-15:	Reaction mixture with 10% (v/v) 1-pentanol, 0 and 24 hours
Lane 16-17:	Reaction mixture with 20% (v/v) isopentanol, 0 and 24 hours

Table 4.3 R_f values from TLC analysis (System II) of standard saccharides and the reaction products

Standard/Product			$\boldsymbol{R_f}$
Standards	Saccharides	Glucose	0.28
		Maltose	0.17
		Maltotriose	0.1
		β-CD	0.03
		MG_1	0.45
Reaction products top ↓ bottom	Methanol	I	0.45
	Ethanol	I	0.57
		II	0.37
	1-Propanol	I	0.67
		II	0.51
		III	0.35
	1-Butanol	I	0.74
		II	0.58
		III	0.40
	1-Pentanol	I	0.80
		II	0.62
		III	0.53
	Isopentanol	I	0.80
		II	0.61
		III	0.52

4.6 Optimization of alkyl glycoside production

In order to find the appropriate conditions for the production of alkyl glycosides, several parameters involved in the reaction including substrates concentration, enzyme concentration, incubation time, temperature and pH were optimized. The reaction was performed as described in Section 3.12 and reaction products were analyzed by TLC (System II). The optimum conditions were considered from the total amount of all alkyl glycoside products. The total amount of alkyl glycoside products was calculated by comparing with MG_1 standard curve.

4.6.1 Effect of alcohol concentration

To determine the effect of acceptors on the production of alkyl glycosides, various concentrations of alcohol (5-40% (v/v) 1-propanol, 5-25% (v/v) 1-pentanol and 5-25% (v/v) isopentanol) and 0.64% (w/v) β-CD donor in 20 mM phosphate buffer pH 6.0 were incubated with 75 U/mL CGTase at 50 °C for 24 hours as described in Section 3.12.1. The results showed that the reaction mixtures with 10% (v/v) 1-propanol (Figure 4.14.1), 10% (v/v) 1-pentanol (Figure 4.15.1) and 20% (v/v) isopentanol (Figure 4.16.1) could synthesize the highest alkyl glycosides. Thus, 10% (v/v) 1-propanol, 10% (v/v) 1-pentanol and 20% (v/v) isopentanol were chosen to be the optimum alcohol concentration for the synthesis of propyl-, pentyl- and isopentyl- glycosides.

4.6.2 Effect of β-CD concentration

In order to investigate the effect of the concentration of a β-CD glycosyl donor, the condition as mentioned in Section 3.12.2 with various β-CD concentrations was used. The results found that the product yield was varied with various β-CD concentrations. The highest yield of alkyl glycosides was presented in the reaction mixtures with 1.5% (w/v) β-CD concentration for 1-propanol and 1-pentanol acceptors and 1.2% (w/v) β-CD concentration for isopentanol acceptor (Figures 4.14.2, 4.15.2 and 4.16.2). Thus, the β-CD concentration of 1.5% (w/v) and 1.2% (w/v) should be chosen to use as the optimum concentration in the synthesis of propyl- and pentyl- glycosides, and isopentyl glycosides, respectively.

4.6.3 Effect of enzyme concentration

The optimization of the enzyme concentration for the production of alkyl glycosides at higher yield was performed by varying amounts of CGTase unit as described in Section 3.12.3. Various amounts of CGTase concentration (50-500 U/mL, determined by dextrinizing activity) were tested. The amount of alkyl glycosides increased rapidly when enzyme concentration increased from 50-250 U/mL and reached the maximum yield at 500 U/mL CGTase (Figures 4.14.3, 4.15.3 and 4.16.3). Thus, this enzyme concentration was chosen to be the optimum concentration for the production of three alkyl glycosides.

4.6.4 Effect of incubation time

The incubation time was varied from 24-168 hours. After the experiment had been carried out as described in Section 3.12.4, the reaction mixtures were analyzed by TLC. The results showed that the production of alkyl glycosides was clearly affected by incubation time. The highest yield of alkyl glycosides was obtained at an incubation time of 96, 168 and 168 hours for using 1-propanol, 1-pentanol and isopentanol as acceptors, respectively (Figures 4.14.4, 4.15.4 and 4.16.4).

4.6.5 Effect of temperature

The effect of temperature on the synthesis of alkyl glycosides was determined as described in Section 3.12.5. The temperatures used were in the range of 30-70 °C. The synthesized product was detected by TLC. The results showed that at the temperature 50 °C, CGTase could synthesize the highest alkyl glycosides (Figures 4.14.5, 4.15.5 and 4.16.5). Thus, the optimal temperature of 50 °C was chosen for the production of alkyl glycosides.

4.6.6 Effect of pH

The effect of pH on CGTase reaction for the production of alkyl glycosides was investigated. The six conditions of different pH values in three kinds of buffer solution were employed. The three diverse buffers consisting of acetate buffer (pH 5.0-6.0), phosphate buffer (pH 6.0-7.0) and Tris-HCl buffer (pH 7.0-8.0) with the same concentration were used as the main environment of the reaction mixture. The protocol was done as shown in Section 3.12.6. The maximum alkyl glycoside was produced at the reaction pH of 6.0 (Figures 4.14.6, 4.15.6 and 4.16.6).

Therefore, the optimum conditions for the production of propyl-, pentyl- and isopentyl- glycosides by recombinant CGTase from *B. circulans* A11 were incubation of 1.5% (w/v) β-CD with 10% (v/v) 1-propanol, or 10% (v/v) 1-pentanol, and 1.2% (w/v) β-CD with 20% (v/v) isopentanol using 500 U/mL CGTase in 20 mM phosphate buffer, pH 6.0 at 50 °C for 96, 168 and 168 hours, respectively (Table 4.4). After optimization, the total yield of alkyl glycosides was massively increased as compared to the yield obtained before optimization.

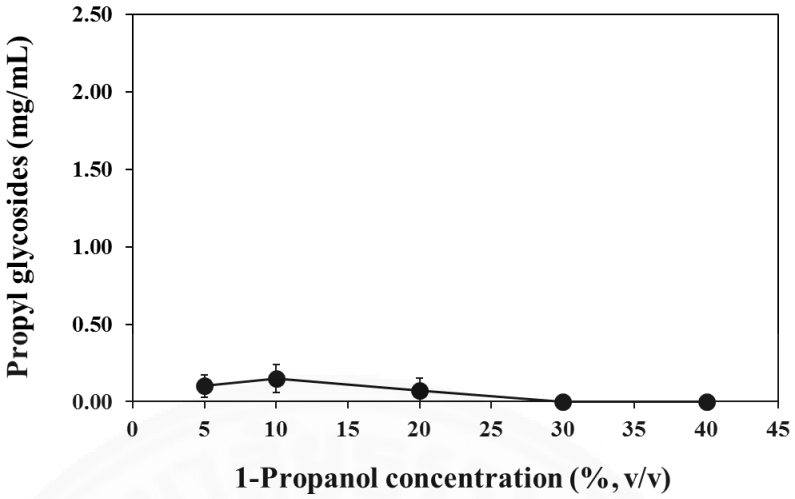


Figure 4.14.1 Effect of 1-propanol concentration on the production of propyl glycosides

The reaction mixture containing 0.64% (w/v) β-CD and various amounts of 1-propanol concentration (5-40%, v/v) was incubated with 75 U/mL CGTase at 50 °C, pH 6.0 for 24 hours.

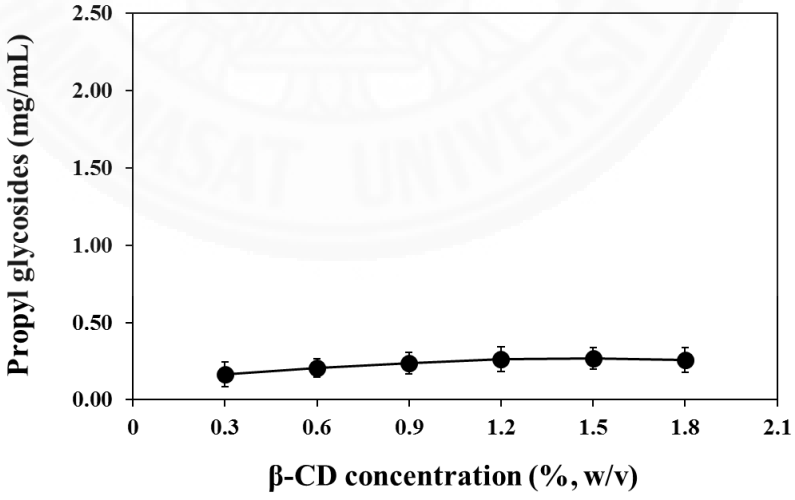


Figure 4.14.2 Effect of β-CD concentration on the production of propyl glycosides

The reaction mixture containing 10% (v/v) 1-propanol, various amounts of β-CD (0.3-1.8%, w/v) was incubated with 75 U/mL CGTase at 50 °C, pH 6.0 for 24 hours.

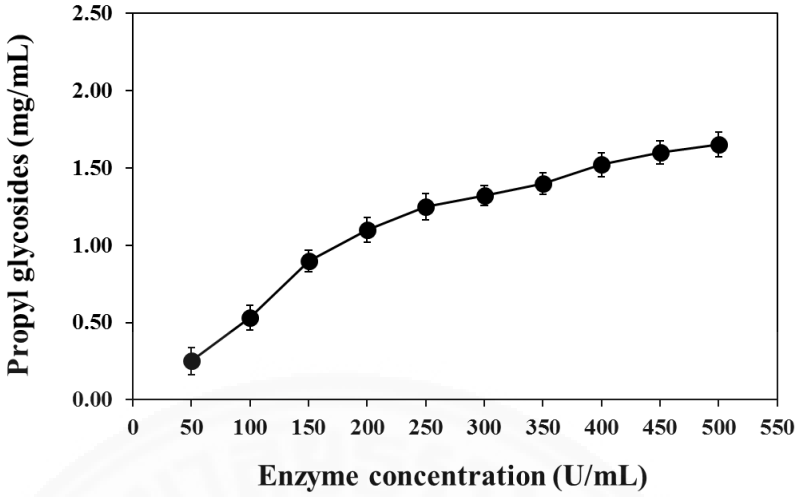


Figure 4.14.3 Effect of enzyme concentration on the production of propyl glycosides

The reaction mixture containing 10% (v/v) 1-propanol and 1.5% (w/v) β-CD was incubated with CGTase (50-500 U/mL), at 50 °C, pH 6.0 for 24 hours.

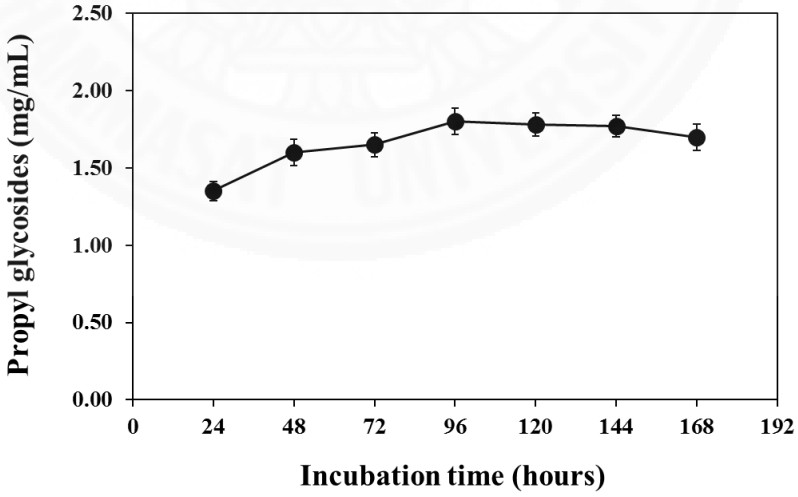


Figure 4.14.4 Effect of incubation time on the production of propyl glycosides

The 500 U/mL CGTase was incubated with a mixture containing 10% (v/v) 1-propanol and 1.5% (w/v) β-CD at 50 °C, pH 6.0 for 24-168 hours.

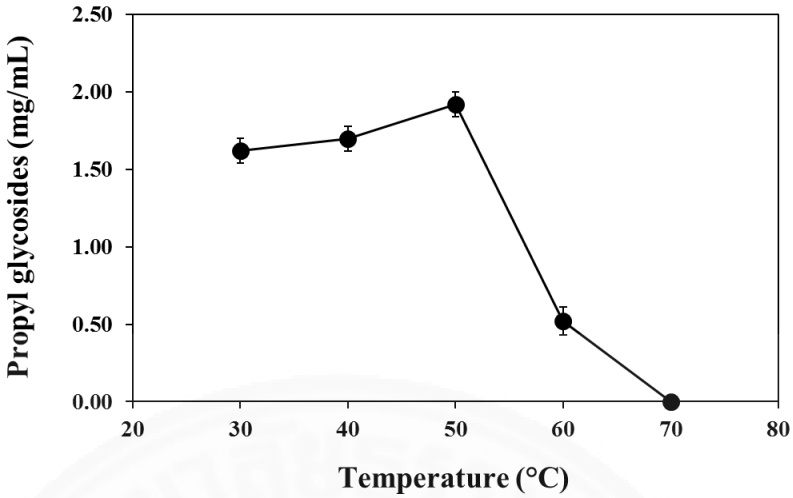


Figure 4.14.5 Effect of temperature on the production of propyl glycosides

The CGTase (500 U/mL) was incubated with a mixture containing 10% (v/v) 1-propanol and 1.5% (w/v) β-CD at indicated temperatures for 96 hours.

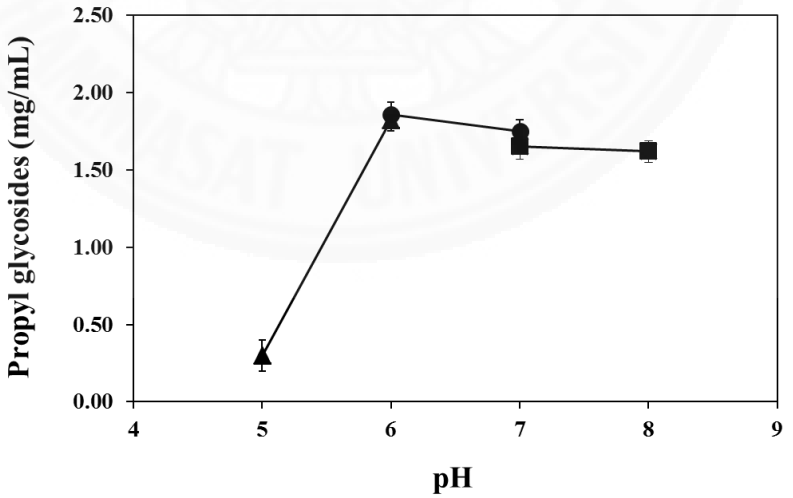


Figure 4.14.6 Effect of pH on the production of propyl glycosides

The CGTase (500 U/mL) was incubated with a mixture containing 10% (v/v) 1-propanol and 1.5% (w/v) β-CD at different pH values at 50 °C for 96 hours. (Acetate buffer; ▲, Phosphate buffer; ● and Tris-HCl buffer; ■)

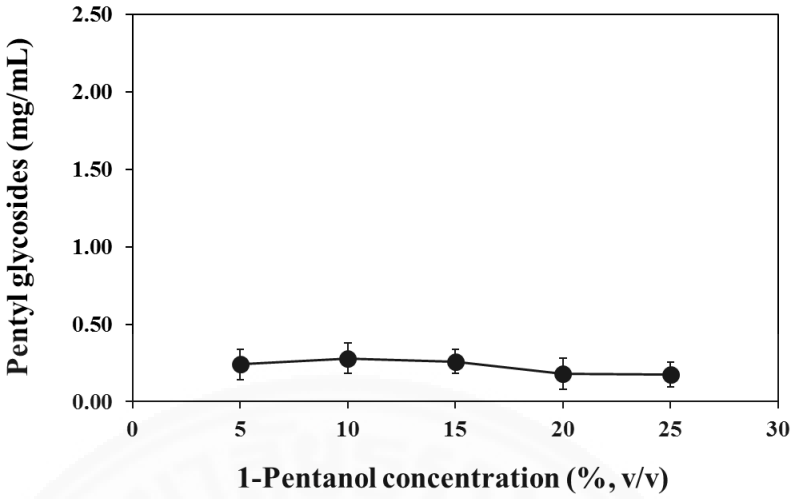


Figure 4.15.1 Effect of 1-pentanol concentration on the production of pentyl glycosides

The reaction mixture containing 0.64% (w/v) β-CD and various amounts of 1-pentanol concentration (5-25%, v/v) was incubated with 75 U/mL CGTase at 50 °C, pH 6.0 for 24 hours.

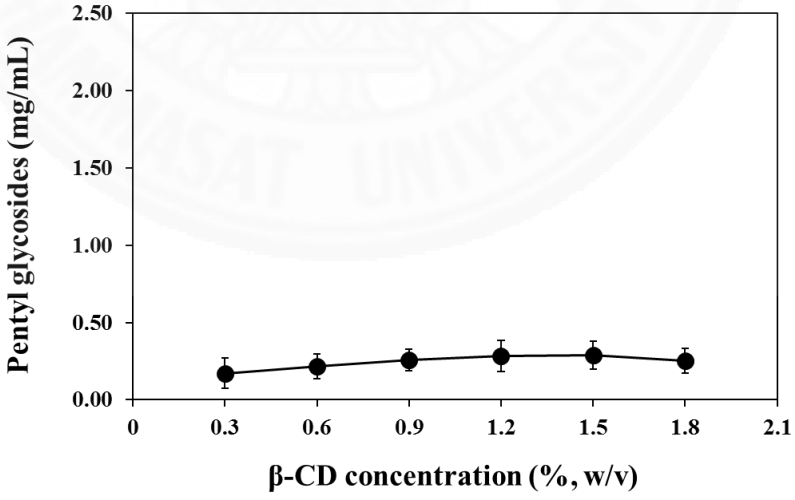


Figure 4.15.2 Effect of β-CD concentration on the production of pentyl glycosides

The reaction mixture containing 5% (v/v) 1-pentanol, various amounts of β-CD (0.3-1.8%, w/v) was incubated with 75 U/mL CGTase at 50 °C, pH 6.0 for 24 hours.

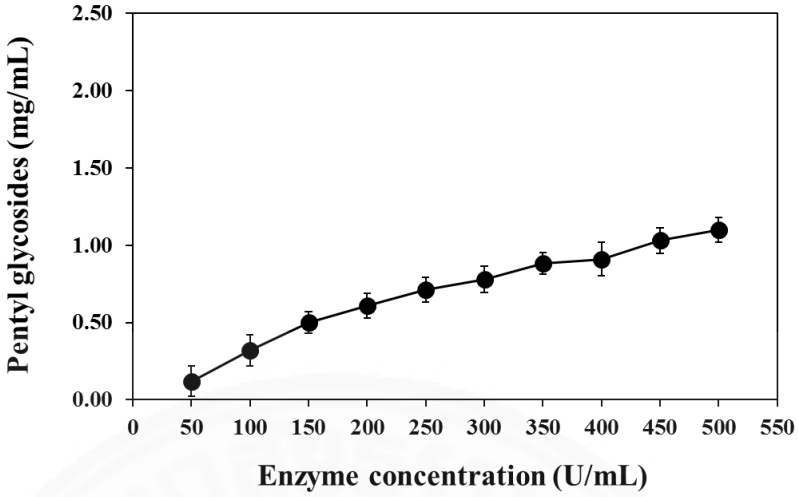


Figure 4.15.3 Effect of enzyme concentration on the production of pentyl glycosides

The reaction mixture containing 5% (v/v) 1-pentanol and 1.5% (w/v) β-CD was incubated with CGTase (50-500 U/mL), at 50 °C, pH 6.0 for 24 hours.

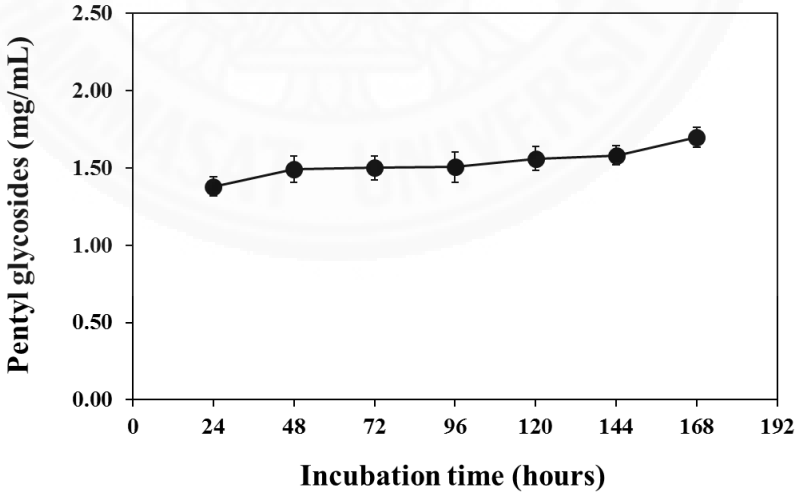


Figure 4.15.4 Effect of incubation time on the production of pentyl glycosides

The 500 U/mL CGTase was incubated with a mixture containing 5% (v/v) 1-pentanol and 1.5% (w/v) β-CD at 50 °C, pH 6.0 for 24-168 hours.

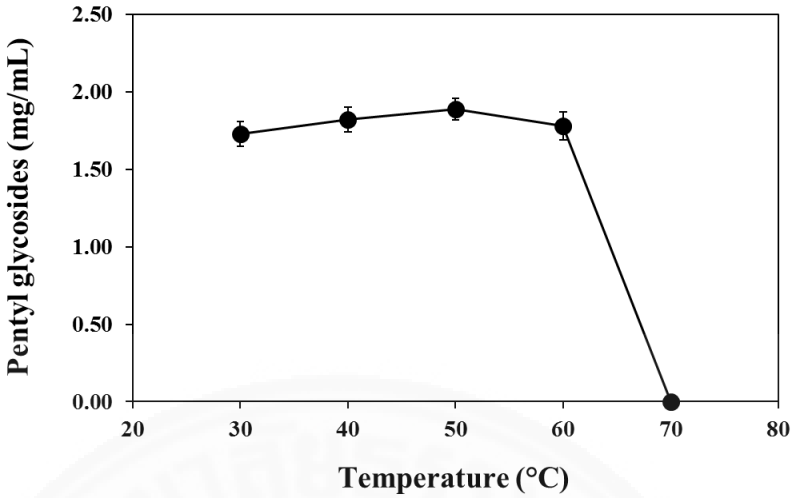


Figure 4.15.5 Effect of temperature on the production of pentyl glycosides

The CGTase (500 U/mL) was incubated with a mixture containing 5% (v/v) 1-pentanol and 1.5% (w/v) β-CD at indicated temperatures for 168 hours.

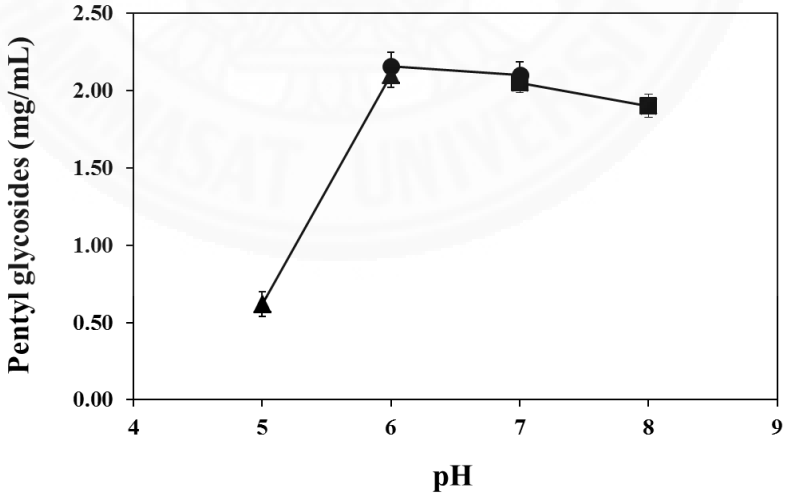


Figure 4.15.6 Effect of pH on the production of pentyl glycosides

The CGTase (500 U/mL) was incubated with a mixture containing 5% (v/v) 1-pentanol and 1.5% (w/v) β-CD at different pH values at 50 °C for 168 hours. (Acetate buffer; ▲, Phosphate buffer; ● and Tris-HCl buffer; ■)

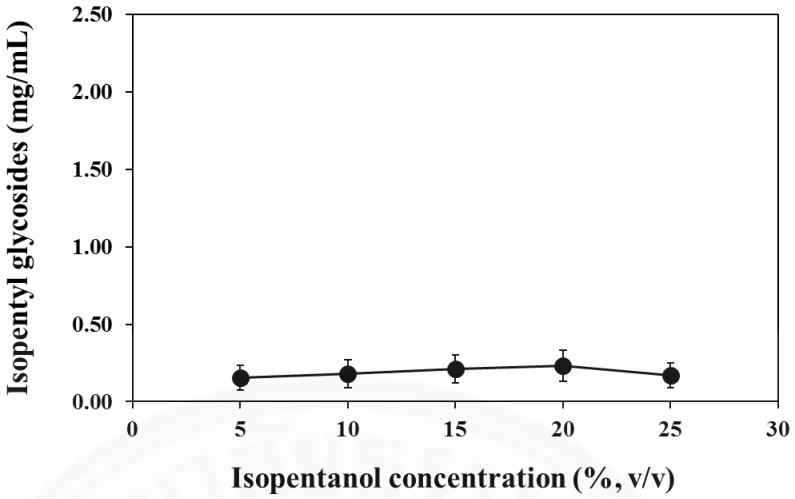


Figure 4.16.1 Effect of isopentanol concentration on the production of isopentyl glycosides

The reaction mixture containing 0.64% (w/v) β-CD and various amounts of isopentanol concentration (5-25%, v/v) was incubated with 75 U/mL CGTase at 50 °C, pH 6.0 for 24 hours.

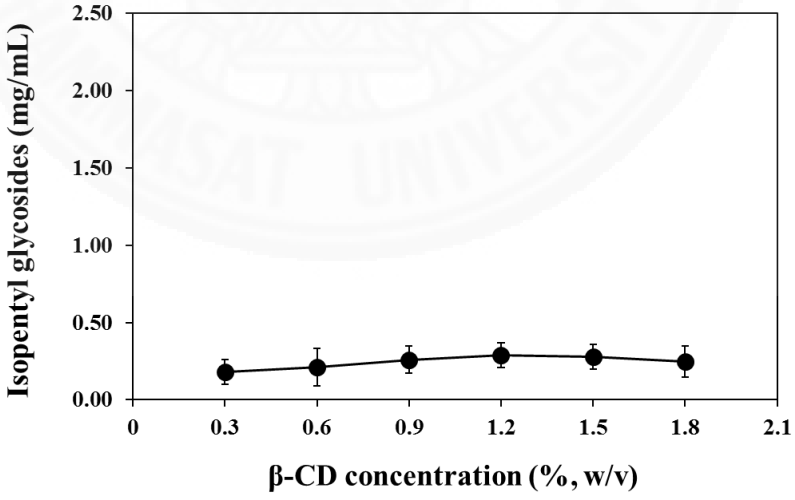


Figure 4.16.2 Effect of β-CD concentration on the production of isopentyl glycosides

The reaction mixture containing 20% (v/v) isopentanol, various amounts of β-CD (0.3-1.8%, w/v) was incubated with 75 U/mL CGTase at 50 °C, pH 6.0 for 24 hours.

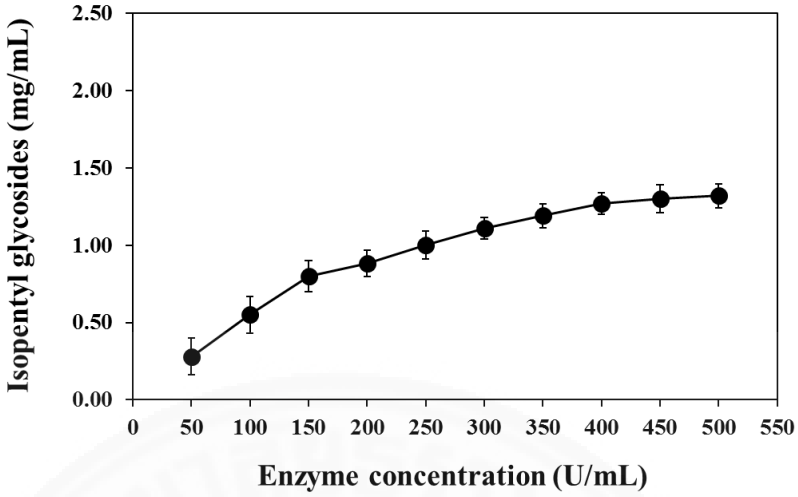


Figure 4.16.3 Effect of enzyme concentration on the production of isopentyl glycosides

The reaction mixture containing 20% (v/v) isopentanol and 1.2% (w/v) β-CD was incubated with CGTase (50-500 U/mL), at 50 °C, pH 6.0 for 24 hours.

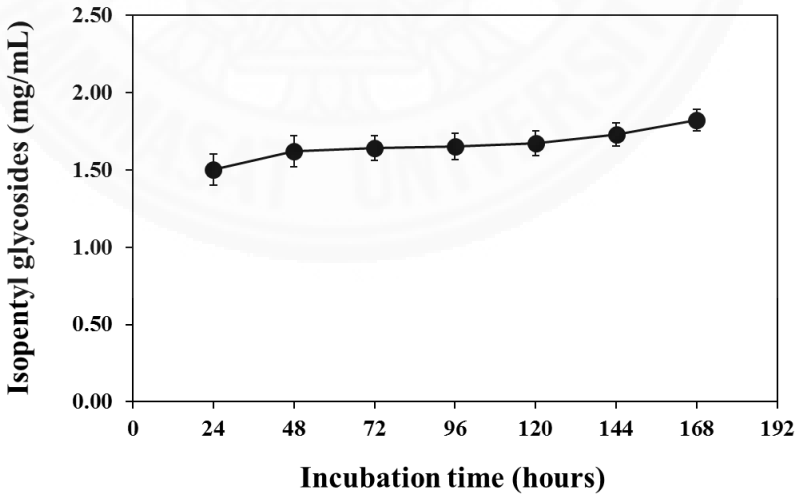


Figure 4.16.4 Effect of incubation time on the production of isopentyl glycosides

The 500 U/mL CGTase was incubated with a mixture containing 20% (v/v) isopentanol and 1.2% (w/v) β-CD at 50 °C, pH 6.0 for 24-168 hours.

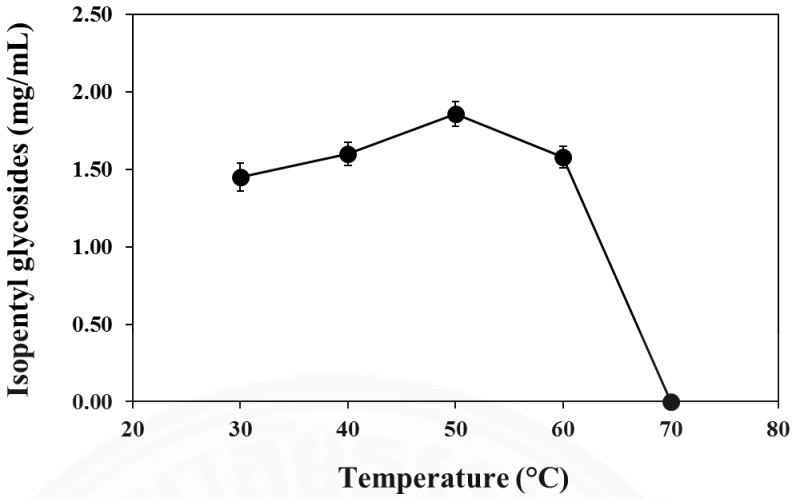


Figure 4.16.5 Effect of temperature on the production of isopentyl glycosides

The CGTase (500 U/mL) was incubated with a mixture containing 20% (v/v) isopentanol and 1.2% (w/v) β-CD at indicated temperatures for 168 hours.

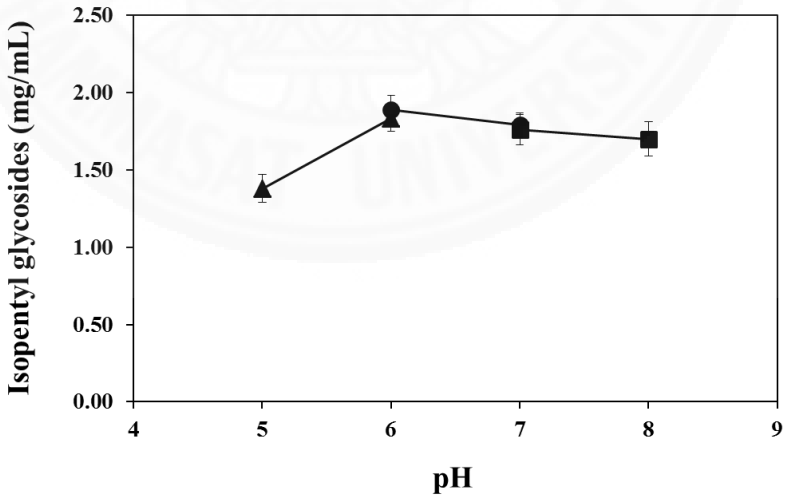


Figure 4.16.6 Effect of pH on the production of isopentyl glycosides

The CGTase (500 U/mL) was incubated with a mixture containing 20% (v/v) isopentanol and 1.2% (w/v) β-CD at different pH values at 50 °C for 168 hours. (Acetate buffer; ▲, Phosphate buffer; ● and Tris-HCl buffer; ■)

Table 4.4 Summary of the optimum conditions for the syntheses of alkyl glycosides by pBC recombinant CGTase

Parameter	**Propyl glycosides ($ProG_n$)**	**Pentyl glycosides ($PenG_n$)**	**Isopentyl glycosides ($IsoPenG_n$)**
Substrate concentration			
- Acceptor	10% (v/v) 1-propanol	10% (v/v) 1-pentanol	20% (v/v) isopentanol
- Donor	1.5% (w/v) β-CD	1.5% (w/v) β-CD	1.2% (w/v) β-CD
Enzyme unit	500 U/mL	500 U/mL	500 U/mL
Incubation time	96 hours	168 hours	168 hours
Temperature	50 °C	50 °C	50 °C
pH	6.0 (phosphate buffer)	6.0 (phosphate buffer)	6.0 (phosphate buffer)

4.7 Large scale production and purification of alkyl glycosides

After the reaction conditions had been optimized, the large scale of the reaction was prepared by increasing the reaction volume from 250 µL to 250 mL. After the incubation was completed, the reaction mixture was halted by boiling for 5 minutes as described in Section 3.13. Then, 30 µL of the reaction was analyzed by TLC (System II). Although the volume of reaction was increased to 250 mL, the amount of alkyl glycosides was still high when compared with the small scale reaction mixture (data not shown). Alkyl glycoside products from large scale production were then purified using PLC as described in Section 3.14. The two main products of each alkyl glycoside at the different R_f value were separately scraped from PLC and dissolved with methanol. The purity of each glycoside product was checked again on the TLC plate. As shown in Figure 4.17, the transglycosylation reactions of propyl-, pentyl- and isopentyl- glycoside syntheses were in Lanes 3, 6 and 9, respectively. After PLC purification, the two expected products of each glycoside were clearly separated at individual R_f value on TLC (Figure 4.17, Lanes 4-5, 7-8 and 10-11).

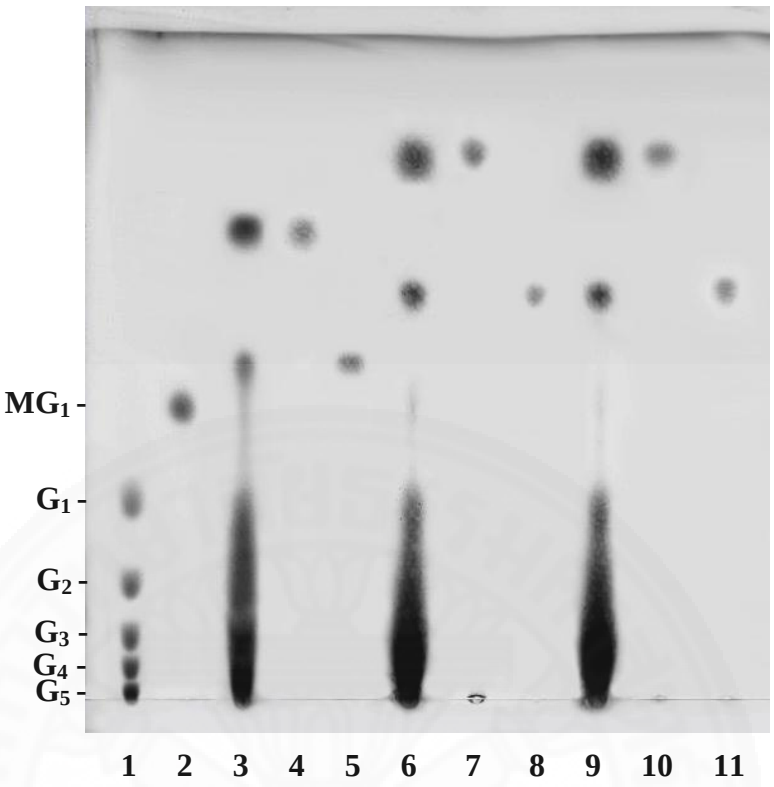


Figure 4.17 TLC analysis of the alkyl glycosides isolated from PLC. TLC condition was System II, ethyl acetate/acetic acid/water, 3:1:1 (v/v).

Lane 1: Standard G_1-G_7 mixed (25 µg)
Lane 2: Standard MG_1 (25 µg)
Lane 3: Transglycosylation reaction for the synthesis of propyl glycosides
Lane 4: The expected propyl glucoside ($ProG_1$) (at R_f value = 0.67) separate from PLC
Lane 5: The expected propyl maltoside ($ProG_2$) (at R_f value = 0.51) separate from PLC
Lane 6: Transglycosylation reaction for the synthesis of pentyl glycosides
Lane 7: The expected pentyl glucoside ($PenG_1$) (at R_f value = 0.80) separate from PLC
Lane 8: The expected pentyl maltoside ($PenG_2$) (at R_f value = 0.62) separate from PLC
Lane 9: Transglycosylation reaction for the synthesis of isopentyl glycosides
Lane 10: The expected isopentyl glucoside ($IsoPenG_1$) (at R_f value = 0.80) separate from PLC
Lane 11: The expected isopentyl maltoside ($IsoPenG_2$) (at R_f value = 0.61) separate from PLC

4.8 Structural analysis of alkyl glycosides

4.8.1 Digestion with amylolytic enzymes

To preliminarily investigate the structure of synthesized alkyl glycosides, the two main products of each purified alkyl glycoside were dissolved in 0.2 M acetate buffer pH 5.5, followed by digesting with glucoamylase and α-glucosidase as described in Section 3.15.1. These amylolytic enzymes were used to confirm that the products from the action of CGTase composed of α-configuration of glycosyl units. As shown in Figure 4.18, when $ProG_n$, $PenG_n$ and $IsoPenG_n$ were treated with glucoamylase, the alkyl glycosides with lower R_f values ($ProG_2$, $PenG_2$ and $IsoPenG_2$) disappeared while the intensities of alkyl glycosides with higher R_f values ($ProG_1$, $PenG_1$ and $IsoPenG_1$) and glucose spots increased (Figure 4.18, Lanes 4, 8 and 12). This finding suggests that the glycosidic linkage between the glucose units of lower R_f products ($ProG_2$, $PenG_2$ and $IsoPenG_2$) was hydrolyzed. In addition, the treatment of alkyl glycosides with α-glucosidase showed the only spot of glucose on TLC while the spots of both alkyl glycosides disappeared. The result of α-glucosidase treatment suggests that α-glucosidase digested the α-linkage between the alkyl chain and the glucosyl unit. From overall results, it could be summarized that the alkyl glycosides with higher R_f value were alkyl monoglucoside, and the alkyl glycosides with lower R_f value were alkyl diglucoside consisting of an α-1,4-glycosidic linkage of disaccharide unit. CGTase is specific for an α-1,4-linkage transfer, so the configuration of the catalyzed product from the enzyme was found to be an α-form.

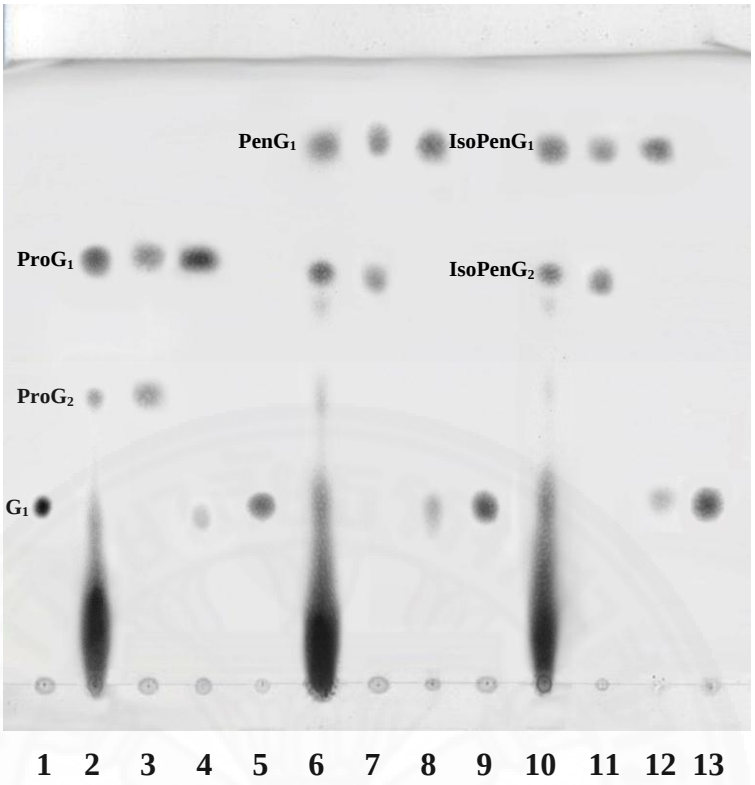


Figure 4.18 TLC analysis of the purified alkyl glycosides treatment with amylolytic enzymes. TLC condition was System II, ethyl acetate/acetic acid/water, 3:1:1 (v/v).

Lane 1: Standard glucose (G_1) (10 µg)
Lane 2: Transglycosylation reaction for the synthesis of propyl glycosides
Lane 3: Mixture of two purified propyl glycosides ($ProG_{1 \text{ and } 2}$)
Lane 4: Mixture of two purified propyl glycosides ($ProG_{1 \text{ and } 2}$) with glucoamylase treatment
Lane 5: Mixture of two purified propyl glycosides ($ProG_{1 \text{ and } 2}$) with α-glucosidase treatment
Lane 6: Transglycosylation reaction for the synthesis of pentyl glycosides
Lane 7: Mixture of two purified pentyl glycosides ($PenG_{1 \text{ and } 2}$)
Lane 8: Mixture of two purified pentyl glycosides ($PenG_{1 \text{ and } 2}$) with glucoamylase treatment
Lane 9: Mixture of two purified pentyl glycosides ($PenG_{1 \text{ and } 2}$) with α-glucosidase treatment
Lane 10: Transglycosylation reaction for the synthesis of isopentyl glycosides
Lane 11: Mixture of two purified isopentyl glycosides ($IsoPenG_{1 \text{ and } 2}$)
Lane 12: Mixture of two purified isopentyl glycosides ($IsoPenG_{1 \text{ and } 2}$) with glucoamylase treatment
Lane 13: Mixture of two purified isopentyl glycosides ($IsoPenG_{1 \text{ and } 2}$) with α-glucosidase treatment

4.8.2 Mass spectrometry (MS)

The molecular weights of synthesized products were elucidated by ESI-TOF mass spectrometer with the positive mode as described in Section 3.15.2. The pseudomolecular ion peak $[M+Na]^+$ of standard methyl-α-D-glucoside (MG_1) displayed at m/z 217 (194 plus 23 of sodium molecule) (Figure 4.19). The molecular weight of synthesized products which used 1-propanol as an acceptor appeared at m/z 245 (222 plus 23 of sodium molecule), m/z 407 (384 plus 23 of sodium molecule) and m/z 569 (546 plus 23 of sodium molecule) (Figure 4.20). These results corresponded to the size of propyl glucoside ($ProG_1$, $C_9H_{18}O_6$), propyl maltoside ($ProG_2$, $C_{15}H_{28}O_{11}$) and propyl maltotrioside ($ProG_3$, $C_{21}H_{38}O_{16}$), respectively. For 1-pentanol as an acceptor, the molecular weights of products were at m/z 273 (250 plus 23 of sodium molecule), m/z 435 (412 plus 23 of sodium molecule) and m/z 597 (574 plus 23 of sodium molecule) (Figure 4.21). These results were equivalent to the molecular weights of pentyl glucoside ($PenG_1$, $C_{11}H_{22}O_6$), pentyl maltoside ($PenG_2$, $C_{17}H_{32}O_{11}$) and pentyl maltotrioside ($PenG_3$, $C_{23}H_{42}O_{16}$), respectively. Finally, the synthesized products using isopentanol acceptor gave the same ion peaks as that obtained from 1-pentanol reaction at m/z 273, 435 and 597 (Figure 4.22). These m/z ratios exhibited that the obtained molecular weights correponeded to the size of isopentyl glucoside ($IsoPenG_1$, $C_{11}H_{22}O_6$), isopentyl maltoside ($IsoPenG_2$, $C_{17}H_{32}O_{11}$) and isopentyl maltotrioside ($IsoPenG_3$, $C_{23}H_{42}O_{16}$), respectively. The theoretical and ESI-TOF-MS analytical masses of alkyl glycosides were shown in Table 4.5.

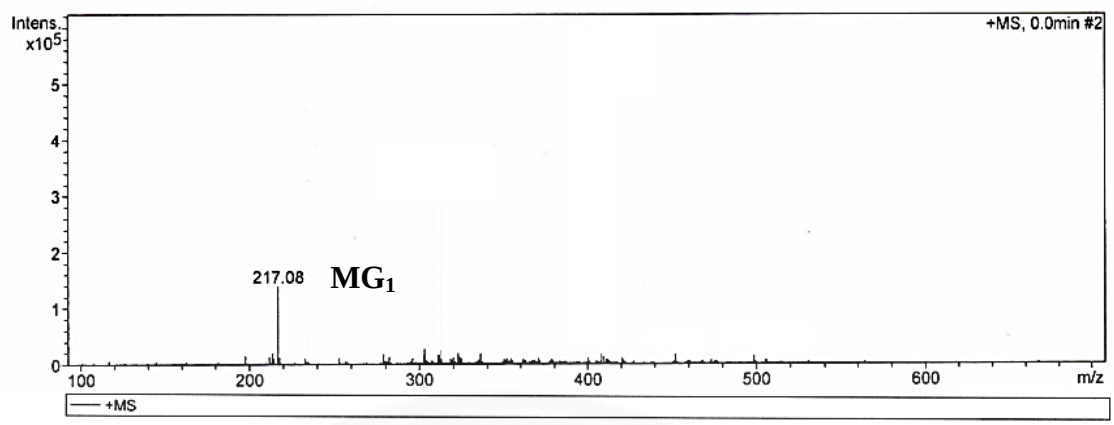


Figure 4.19 ESI-TOF mass spectrum of methyl-α-D-glucoside (MG_1)

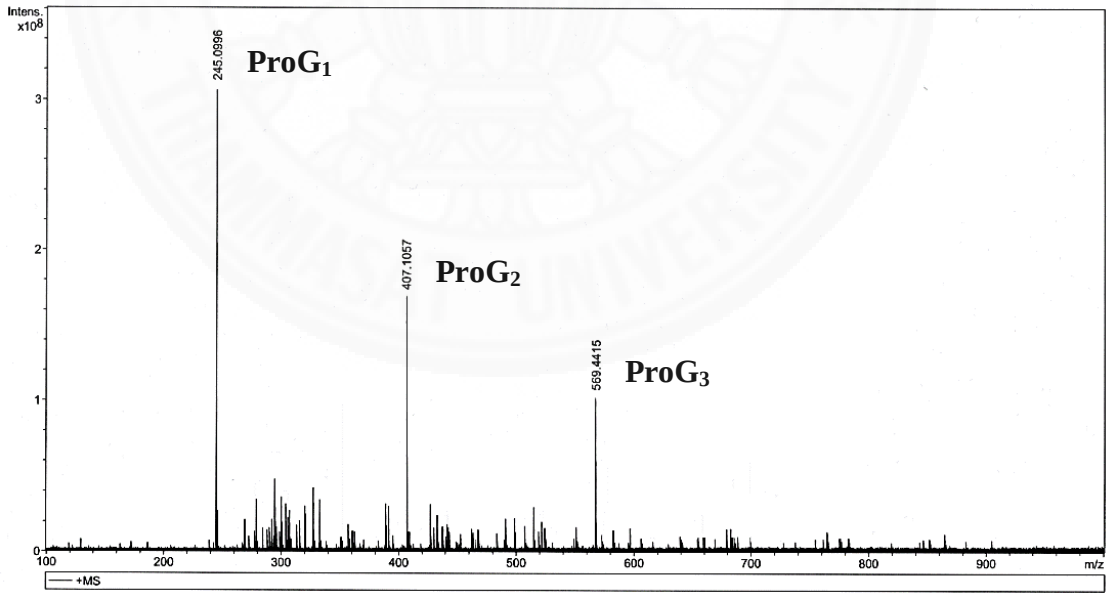


Figure 4.20 ESI-TOF mass spectra of propyl glycosides ($ProG_1$ = propyl glucoside, $ProG_2$ = propyl maltoside and $ProG_3$ = propyl maltotrioside)

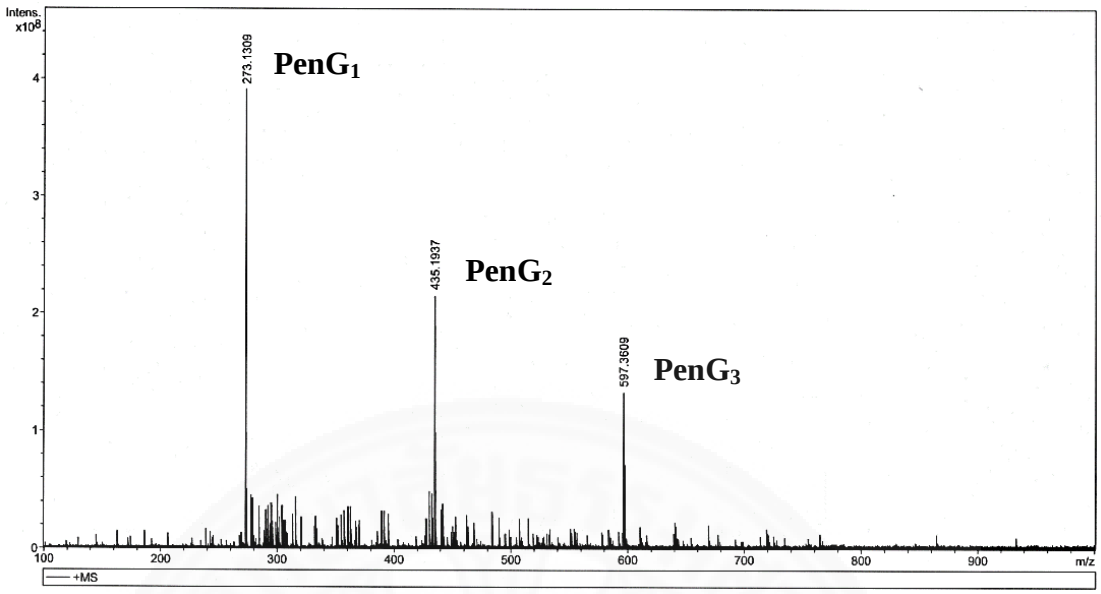


Figure 4.21 ESI-TOF mass spectra of pentyl glycosides ($PenG_1$ = pentyl glucoside, $PenG_2$ = pentyl maltoside and $PenG_3$ = pentyl maltotrioside)

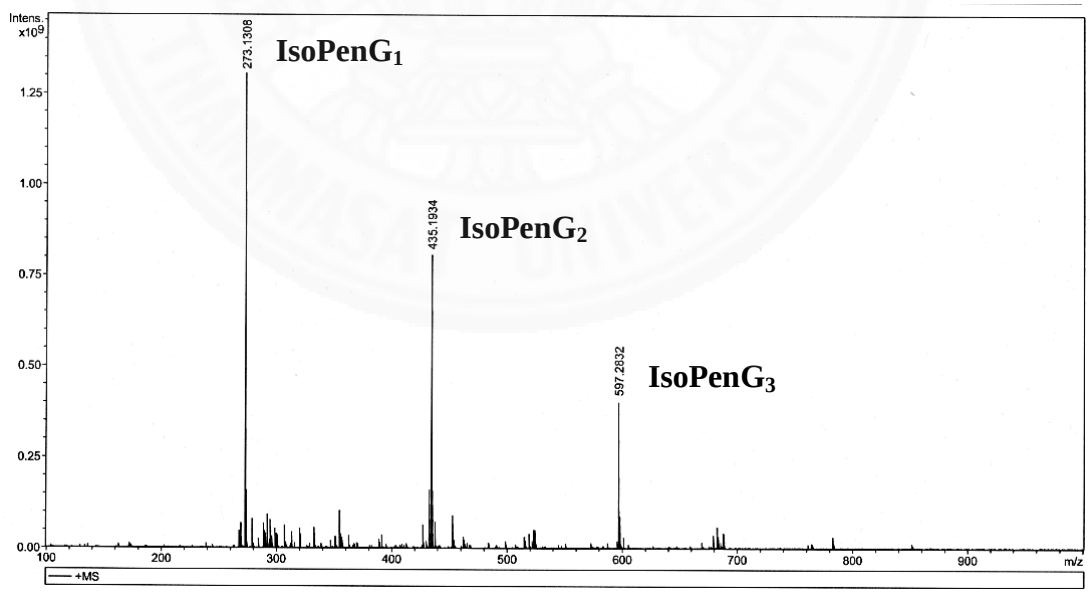


Figure 4.22 ESI-TOF mass spectra of isopentyl glycosides ($IsoPenG_1$ = isopentyl glucoside, $IsoPenG_2$ = isopentyl maltoside and $IsoPenG_3$ = isopentyl maltotrioside)

Table 4.5 The theoretical and ESI-TOF-MS analytical masses of alkyl glycosides

Compound	Mass number (m/z)	
	Calculated[a] $[M+Na]^+$	Determined[b] $[M+Na]^+$
Standard methyl-α-D-glucoside (MG_1)	217.2	217.1
Propyl glucoside ($ProG_1$)	245.3	245.1
Propyl maltoside ($ProG_2$)	407.4	407.1
Propyl maltotrioside ($ProG_3$)	569.5	569.4
Pentyl glucoside ($PenG_1$)	273.3	273.1
Pentyl maltoside ($PenG_2$)	435.4	435.2
Pentyl maltotrioside ($PenG_3$)	597.6	597.4
Isopentyl glucoside ($IsoPenG_1$)	273.3	273.1
Isopentyl maltoside ($IsoPenG_2$)	435.4	435.2
Isopentyl maltotrioside ($IsoPenG_3$)	597.6	597.3

[a] Mass number were calculated from glucose = 180.1559, methanol = 32.0419, 1-propanol = 60.0950, 1-pentanol = 88.1482, isopentanol = 88.1482, Na = 22.9898 and H_2O = 18.0153.

[b] $[M+Na]^+$ were determined by ESI-TOF mass spectrometry.

4.8.3 Nuclear magnetic resonance (NMR)

The structure of alkyl glycosides was analyzed by ^{1}H- and ^{13}C-NMR to identify their configuration and the number of glucosyl units as described in Section 3.15.3. The standard methyl-α-D-glucopyranoside (MG_1) and two main products of each purified alkyl glycoside were dissolved in CD_3OD. The type of glycosidic linkage was investigated from ^{1}H-NMR analysis, based on the coupling constant values of the anomeric protons. As shown in Figures 4.23-4.26 and Table 4.6, the ^{1}H-NMR spectrum of standard MG_1 showed the double signal at 4.66 ppm with coupling constant (*J*) of 3.5 Hz, while $ProG_1$, $PenG_1$ and $IsoPenG_1$ also gave one double signal at 4.79 with coupling constant (*J*) of 3.5 Hz. These results indicated that the type of glycosidic linkage was an α-configuration. Similarly, the ^{1}H-NMR spectrum of $ProG_2$, $PenG_2$ and $IsoPenG_2$ showed the two of doublet signals in the range of 4.81 to 4.84 with the same coupling constant (J = 3.5 Hz) (Figures 4.27-4.29). The results indicated that these alkyl maltosides composed of two α-linkages. One of which was between alkyl group and first glucose unit and another was the inter-glycosidic linkage between first and second glucose.

The ^{13}C-NMR was also used in combination with ^{1}H-NMR to detect the carbon atom of the synthesized compounds. All of the carbon signals were assigned in ppm and compared to NMR spectrum data of alcohols, glucose and standard MG_1 as previously reported (Table 4.7). The standard MG_1 exhibited seven carbon signals and the carbon signals at C-1 of methanol and C1' of original glucose were greatly changed from δ 51.8 to δ 54.11 (+2.31 ppm) and δ 92.77 to δ 99.86 (+ 7.09 ppm) as compared to the standard MG_1 (Figure 4.30). This result was found to be similar to the previous report by de Segura and co-workers (2006) (δ 56.1 at C-1 and δ 100.4 at C-1'). For the synthesized $ProG_{1\text{-}2}$, $PenG_{1\text{-}2}$ and $IsoPenG_{1\text{-}2}$ (Figures 4.31-4.36), the glycosylation in $ProG_1$, $PenG_1$ and $IsoPenG_1$ were confirmed by the downfield chemical shift at C-1 of alkyl group and C-1' of glucose residue. The carbon signals at C-1 of $ProG_1$, $PenG_1$ and $IsoPenG_1$ showed the downfield chemical shifts of 11.2, 6.93 and 7.29 ppm as compared with 1-propanol, 1-pentanol and isopentanol, respectively, and the signals at C-1' showed the downfield chemical shifts of 7.20, 7.26 and 7.34 when compared with sole glucose. These results indicated that the C-1' of the transferred glucosyl residue ($ProG_1$, $PenG_1$ and

$IsoPenG_1$) had attached to C-1 of the 1-propanol, 1-pentanol and isopentanol, respectively. Likewise, the downfield chemical shifts presented at C-1, C-1', C-4' and C-1" in the $ProG_2$, $PenG_2$ and $IsoPenG_2$. These downfield shifts confirmed the characteristic that the $ProG_2$, $PenG_2$ and $IsoPenG_2$ corresponded to alkyl maltopyranoside with an α-1,4-glycosidic linkage.

Based on both NMR and MS analyses, the $ProG_1$ and $ProG_2$ were identified as propyl-α-D-glucopyranoside and propyl-α-D-maltopyranoside, respectively. The $PenG_{1,2}$ and $IsoPenG_{1,2}$ were pentyl-α-D-glucopyranoside, pentyl-α-D-maltopyranoside, isopentyl-α-D-glucopyranoside and isopentyl-α-D-maltopyranoside, respectively. The proposed structure of synthesized alkyl glycosides was shown in Figure 4.37.

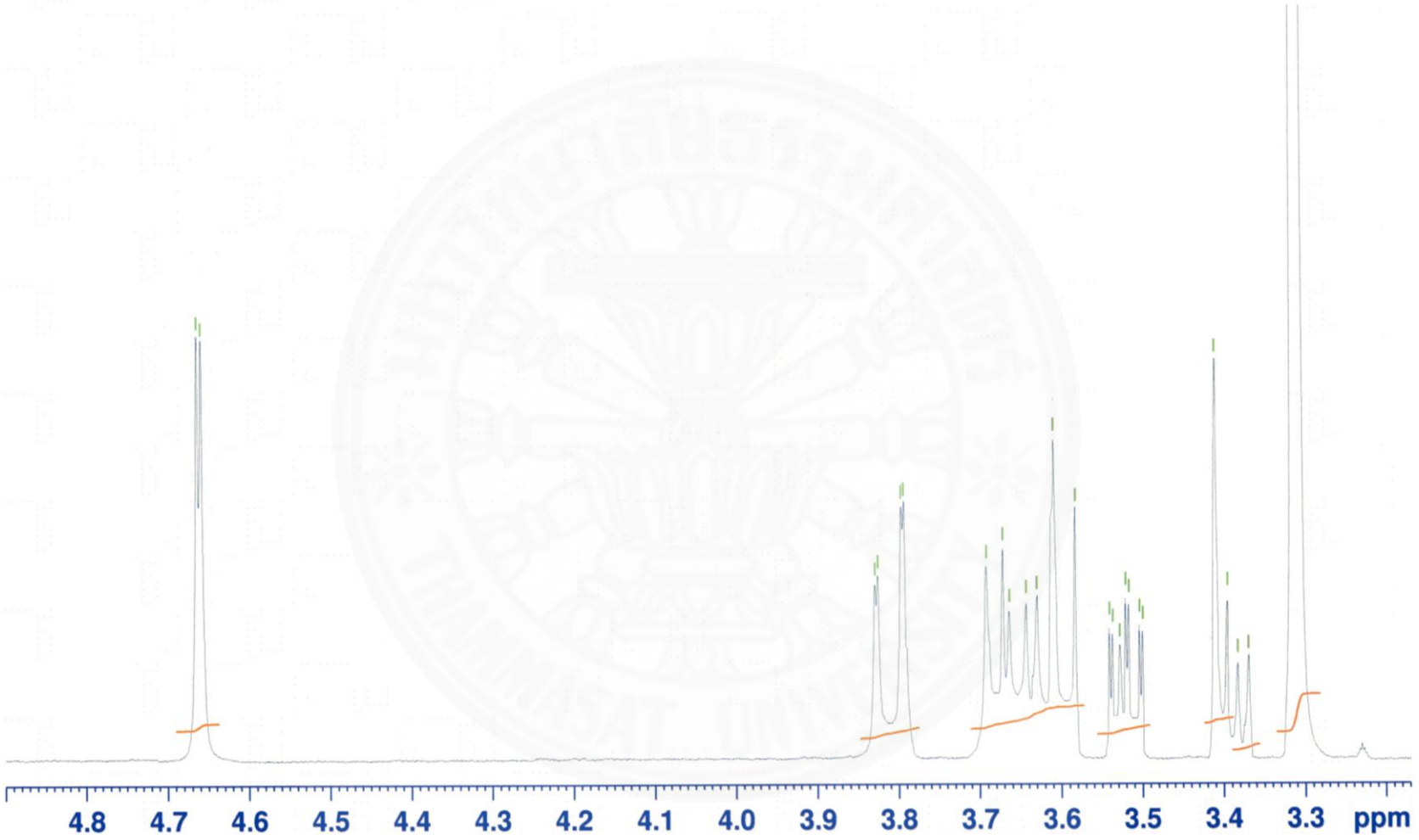


Figure 4.23 ^{1}H-NMR spectrum of methyl-α-D-glucoside (MG_1)

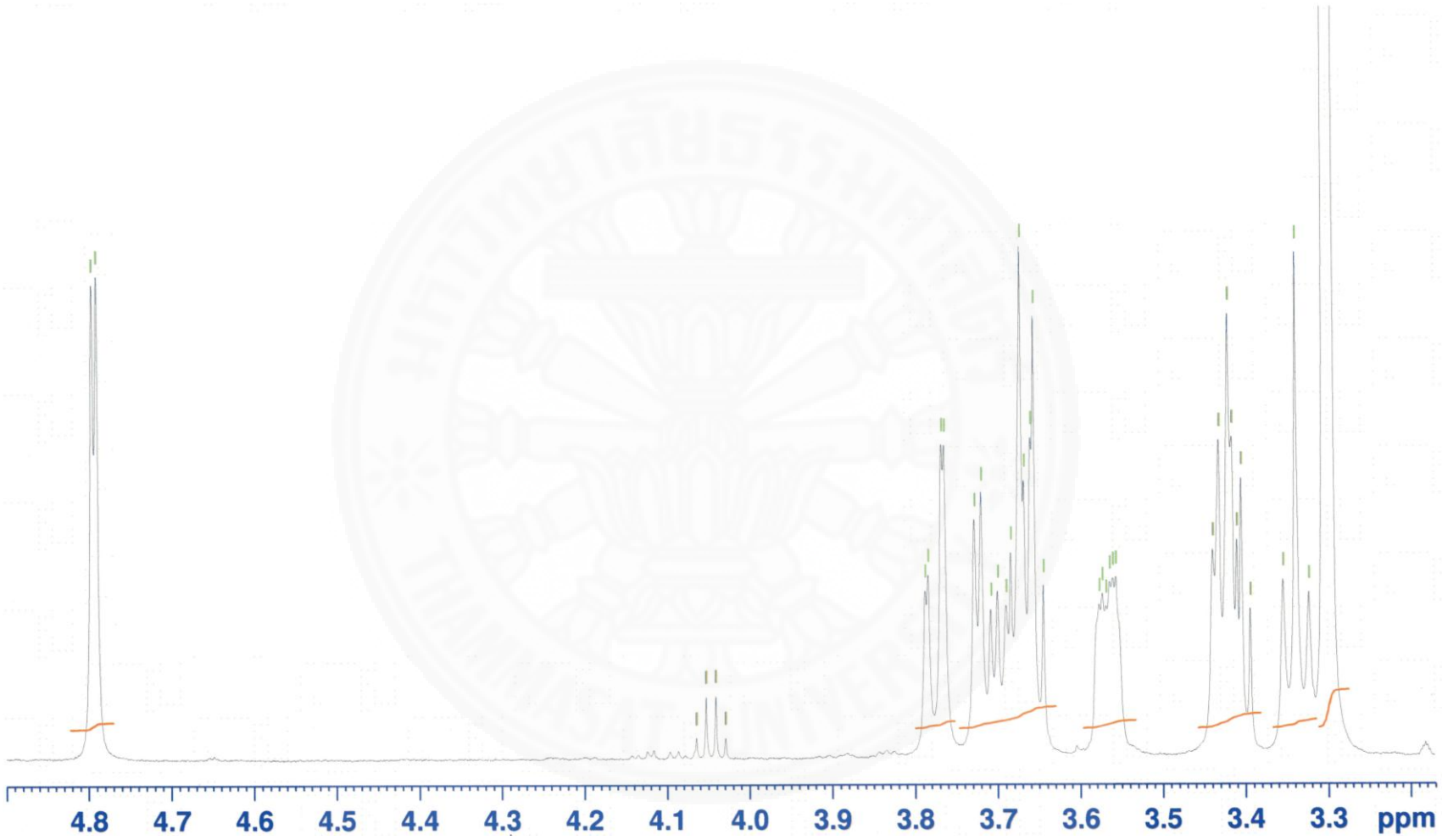


Figure 4.24 ^{1}H-NMR spectrum of propyl glucoside ($ProG_1$)

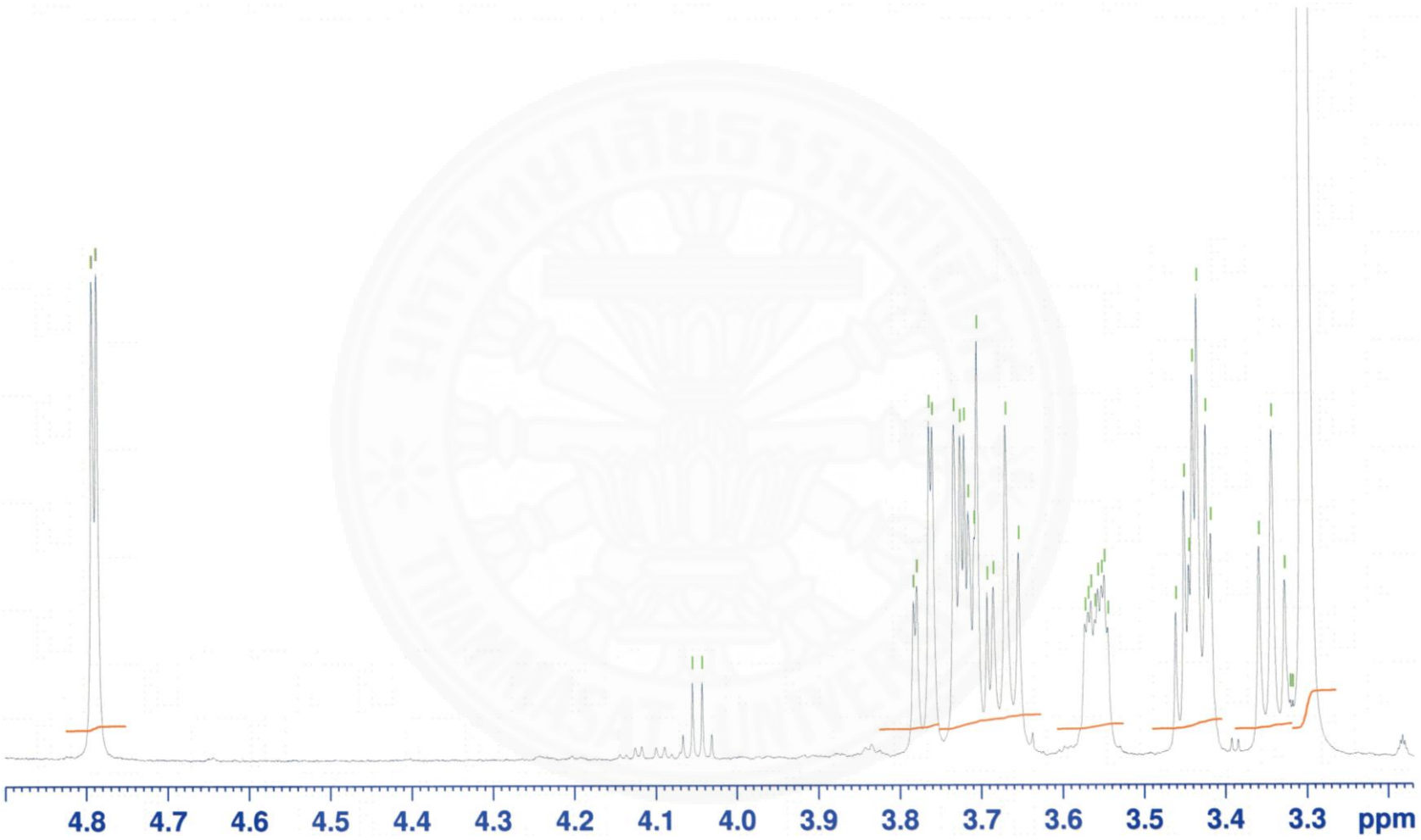


Figure 4.25 ^{1}H-NMR spectrum of pentyl glucoside ($PenG_1$)

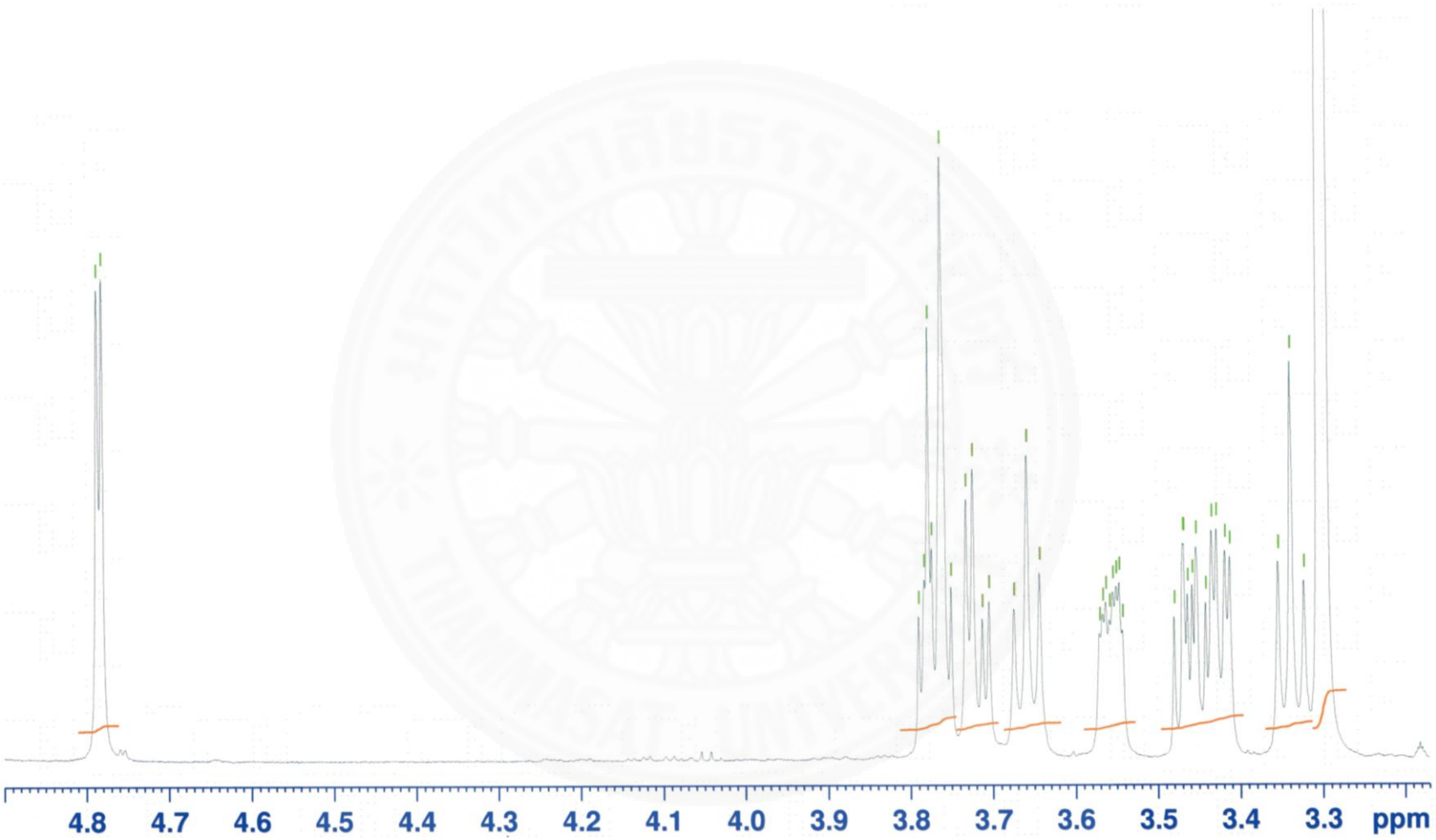


Figure 4.26 ^{1}H-NMR spectrum of isopentyl glucoside (IsoPenG_1)

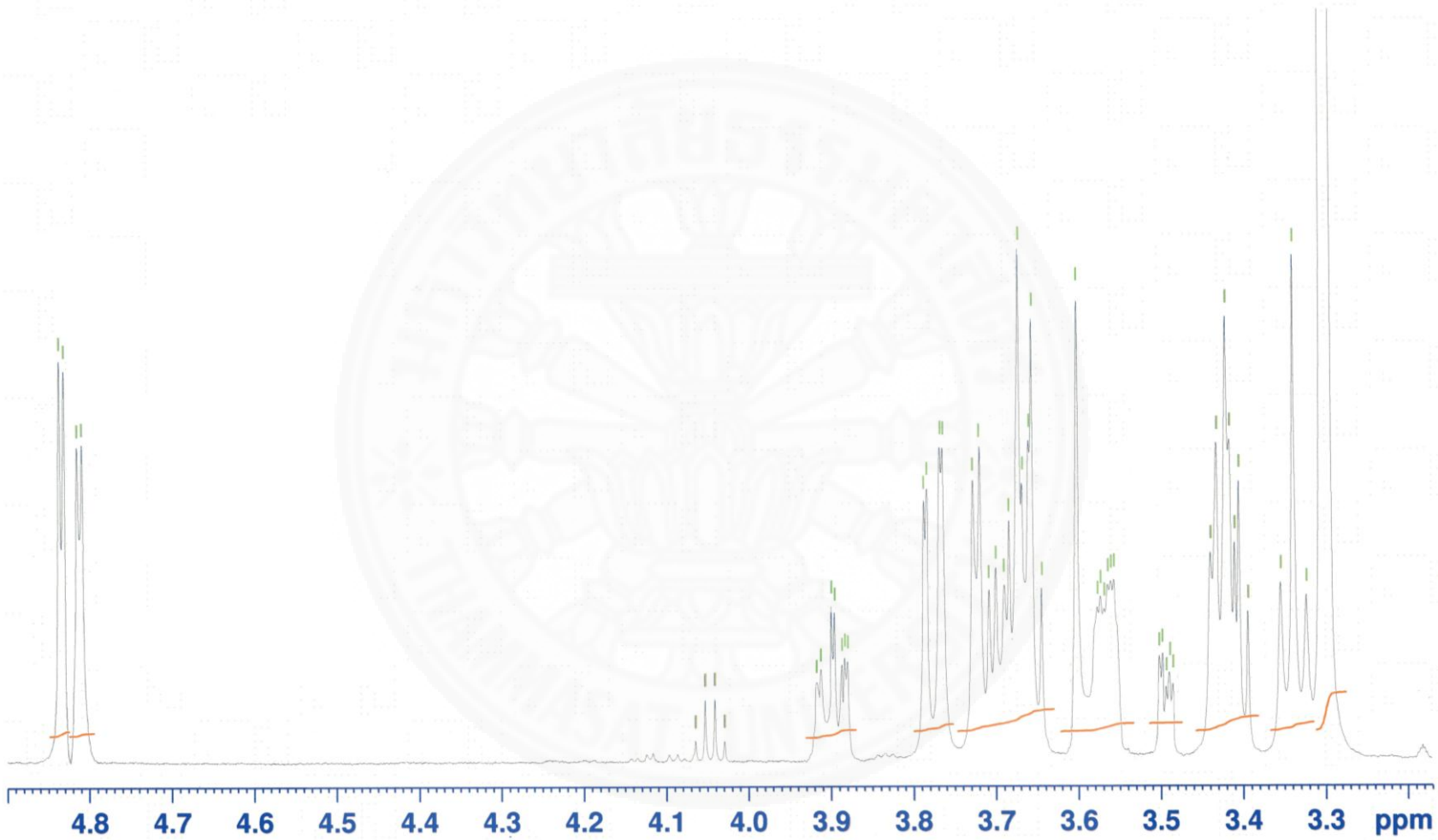


Figure 4.27 ^{1}H-NMR spectrum of propyl maltoside ($ProG_2$)

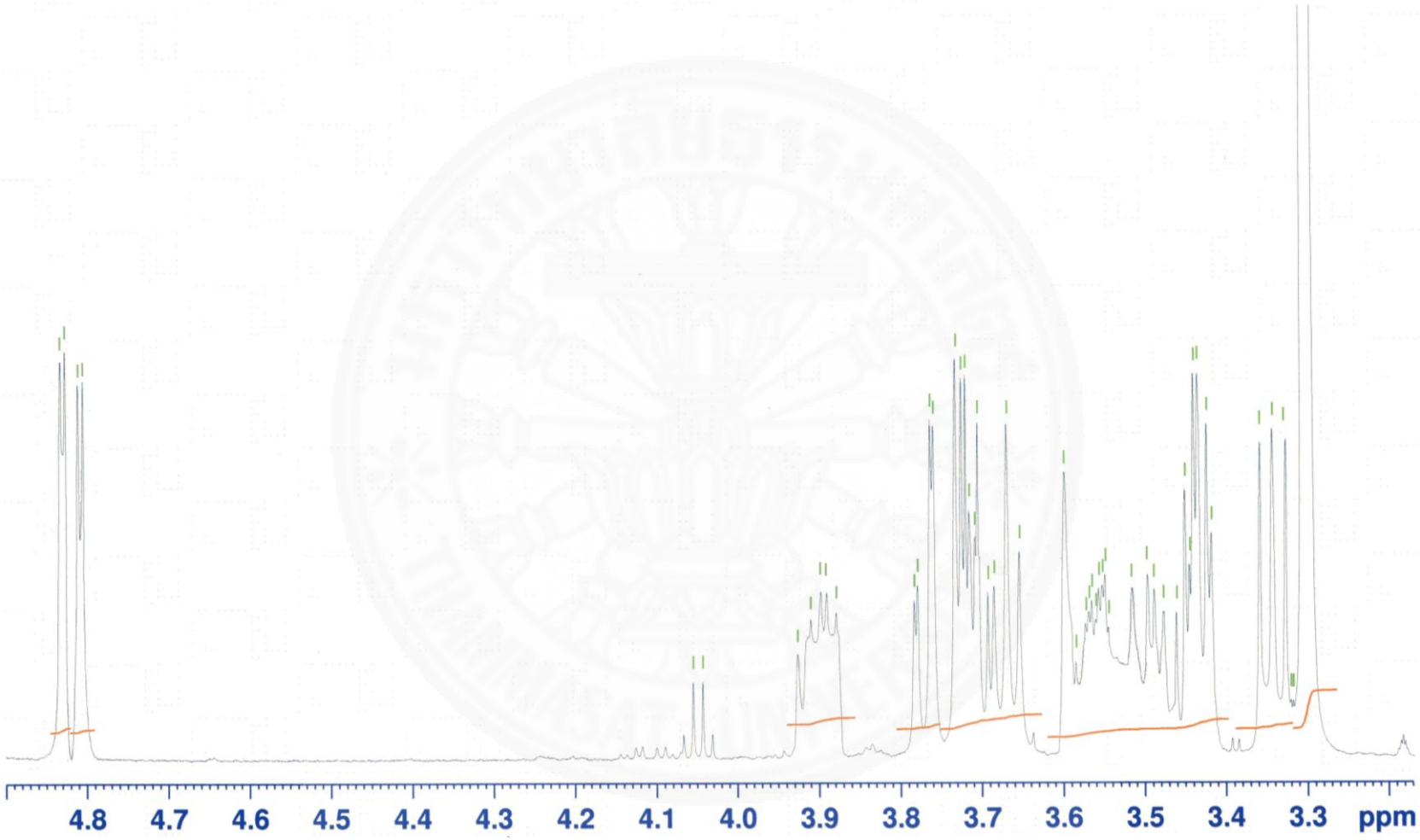


Figure 4.28 ^{1}H-NMR spectrum of pentyl maltoside ($PenG_2$)

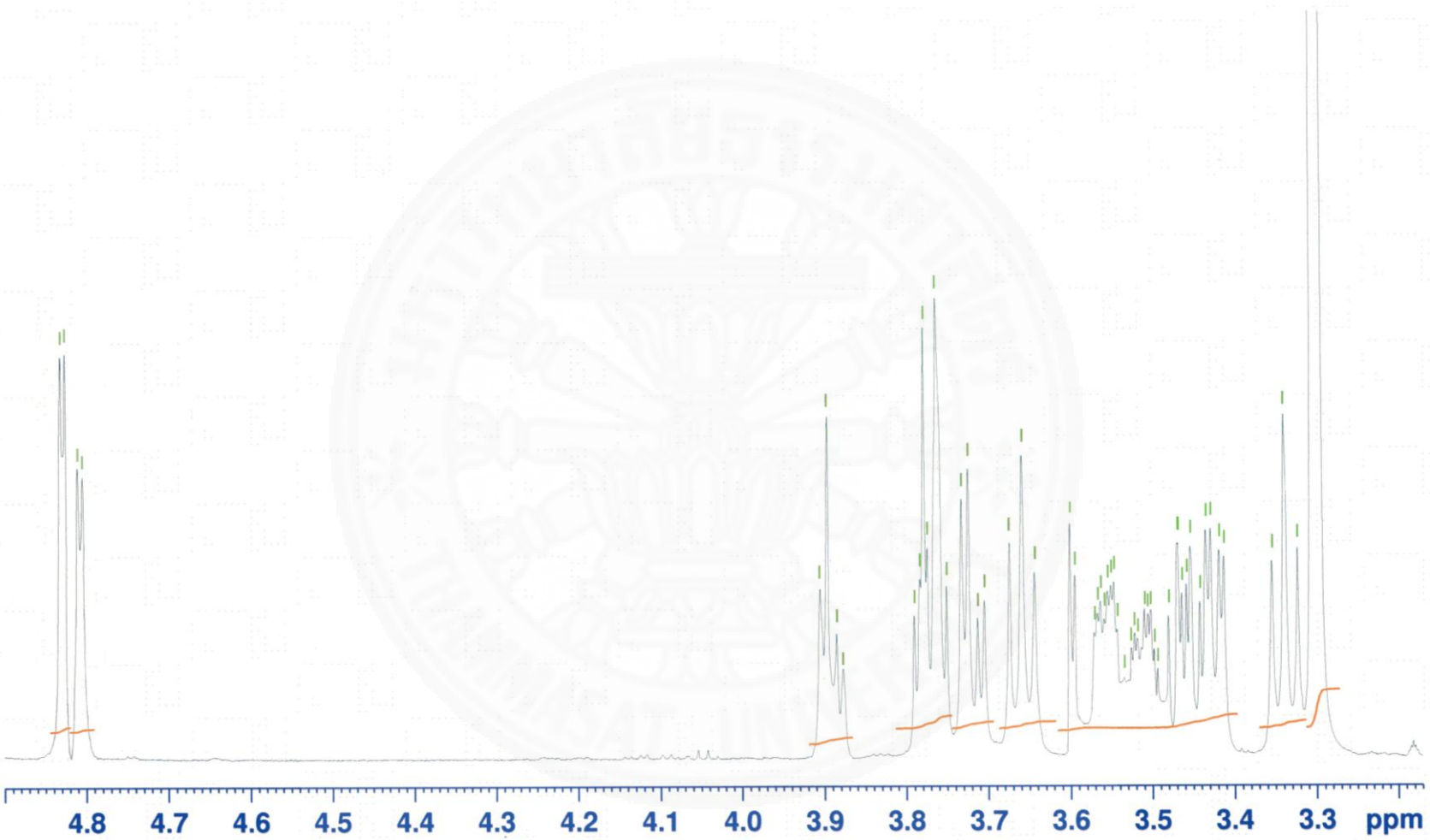


Figure 4.29 ^{1}H-NMR spectrum of isopentyl maltoside (IsoPenG_2)

Table 4.6 ^{1}H-NMR chemical shift values and coupling constants for the glucosidic protons of the synthesized alkyl glycosides

Compound	**Glucosidic protons**	
Standard methyl-α-D-glucoside (MG_1)	4.66 (3.6)	
Propyl glucoside ($ProG_1$)	4.79 (3.5)	
Propyl maltoside ($ProG_2$)	4.81 (3.5)	4.84 (3.5)
Pentyl glucoside ($PenG_1$)	4.79 (3.5)	
Pentyl maltoside ($PenG_2$)	4.81 (3.5)	4.83 (3.5)
Isopentyl glucoside ($IsoPenG_1$)	4.79 (3.5)	
Isopentyl maltoside ($IsoPenG_2$)	4.81 (3.5)	4.83 (3.5)

Values of chemical shift (δ) are given in ppm. Coupling constants (Hz) are shown in parentheses.

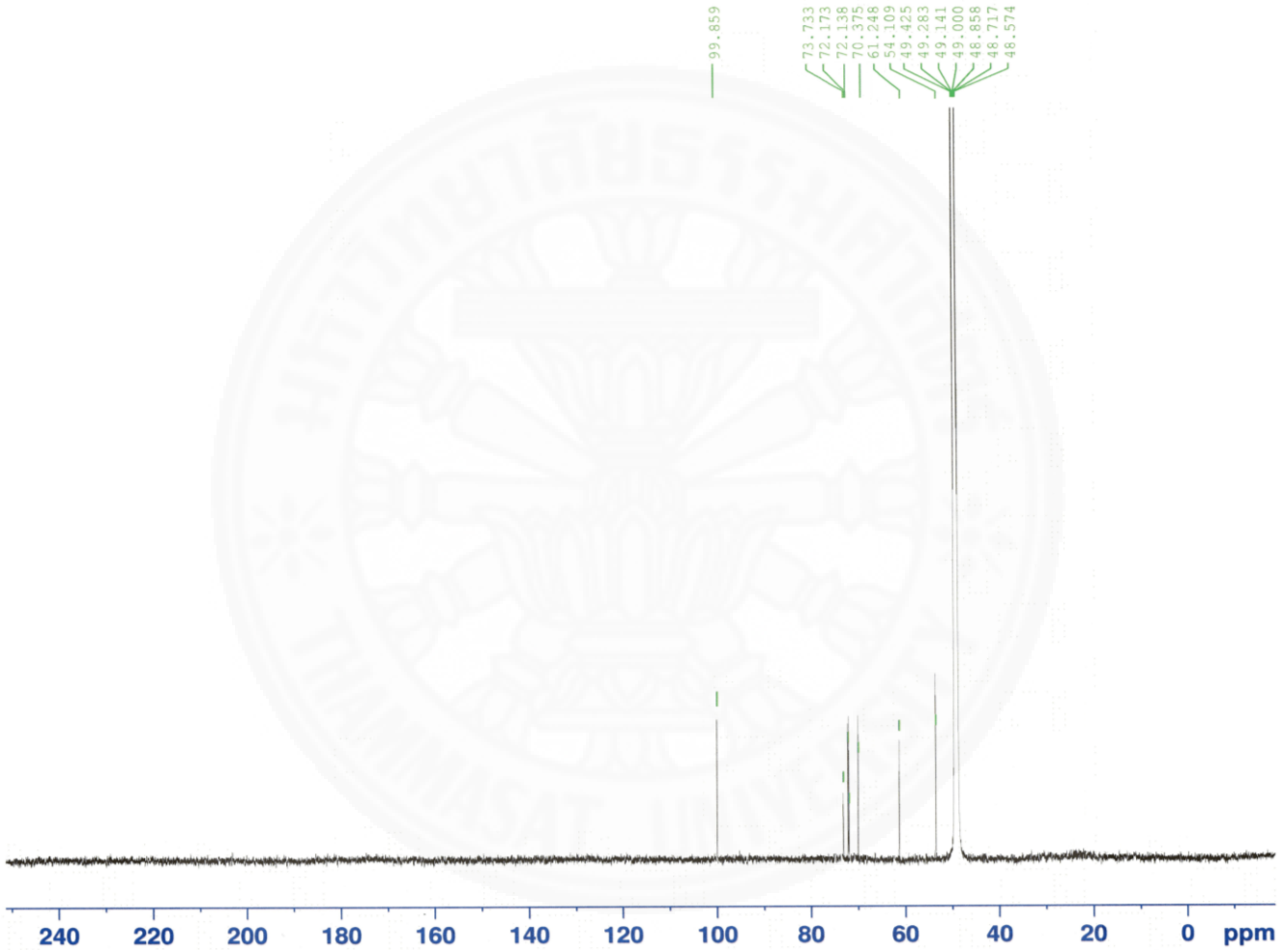


Figure 4.30 ^{13}C-NMR spectrum of methyl-α-D-glucoside (MG_1)

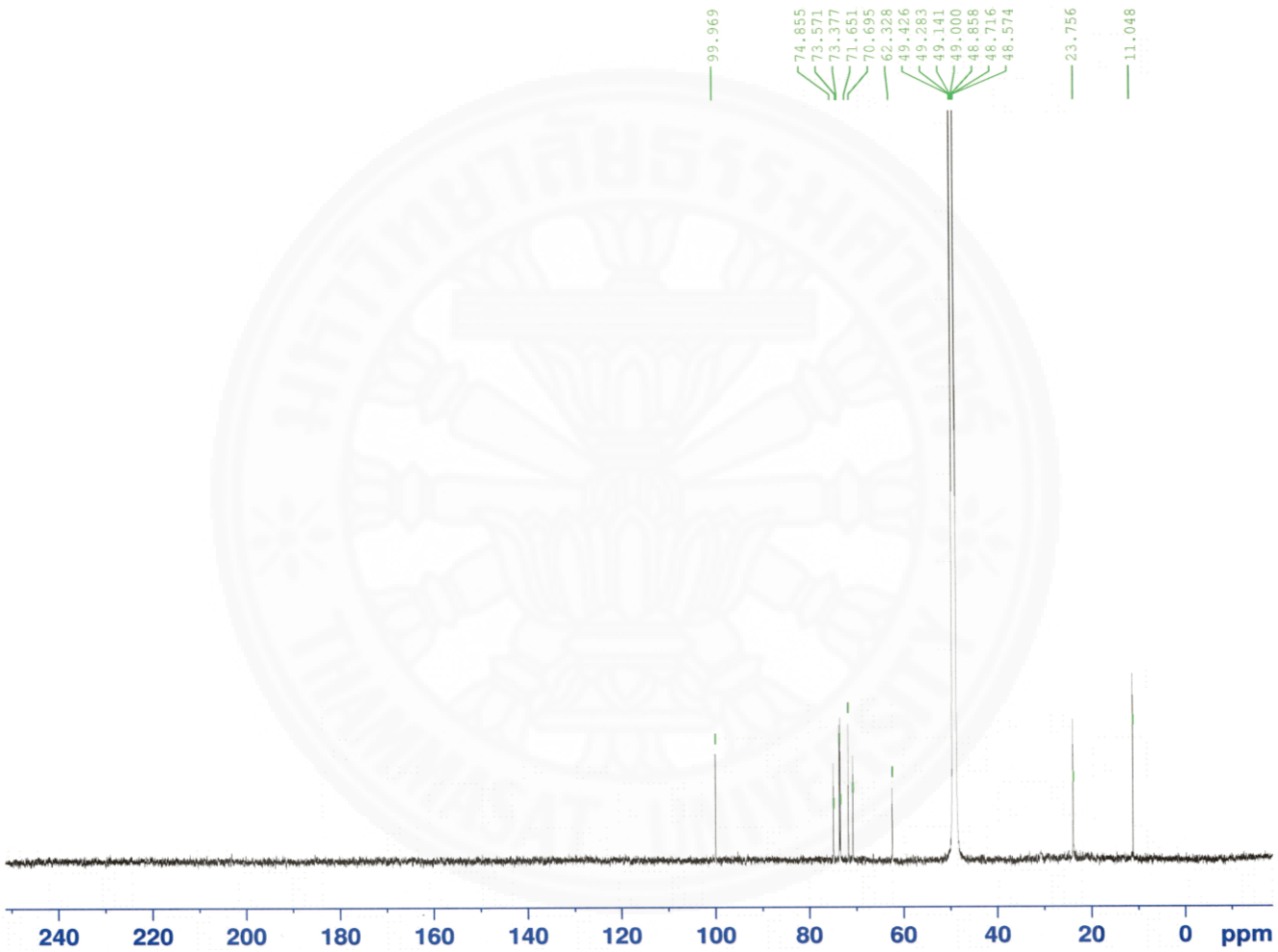


Figure 4.31 ^{13}C-NMR spectrum of propyl glucoside (ProG_1)

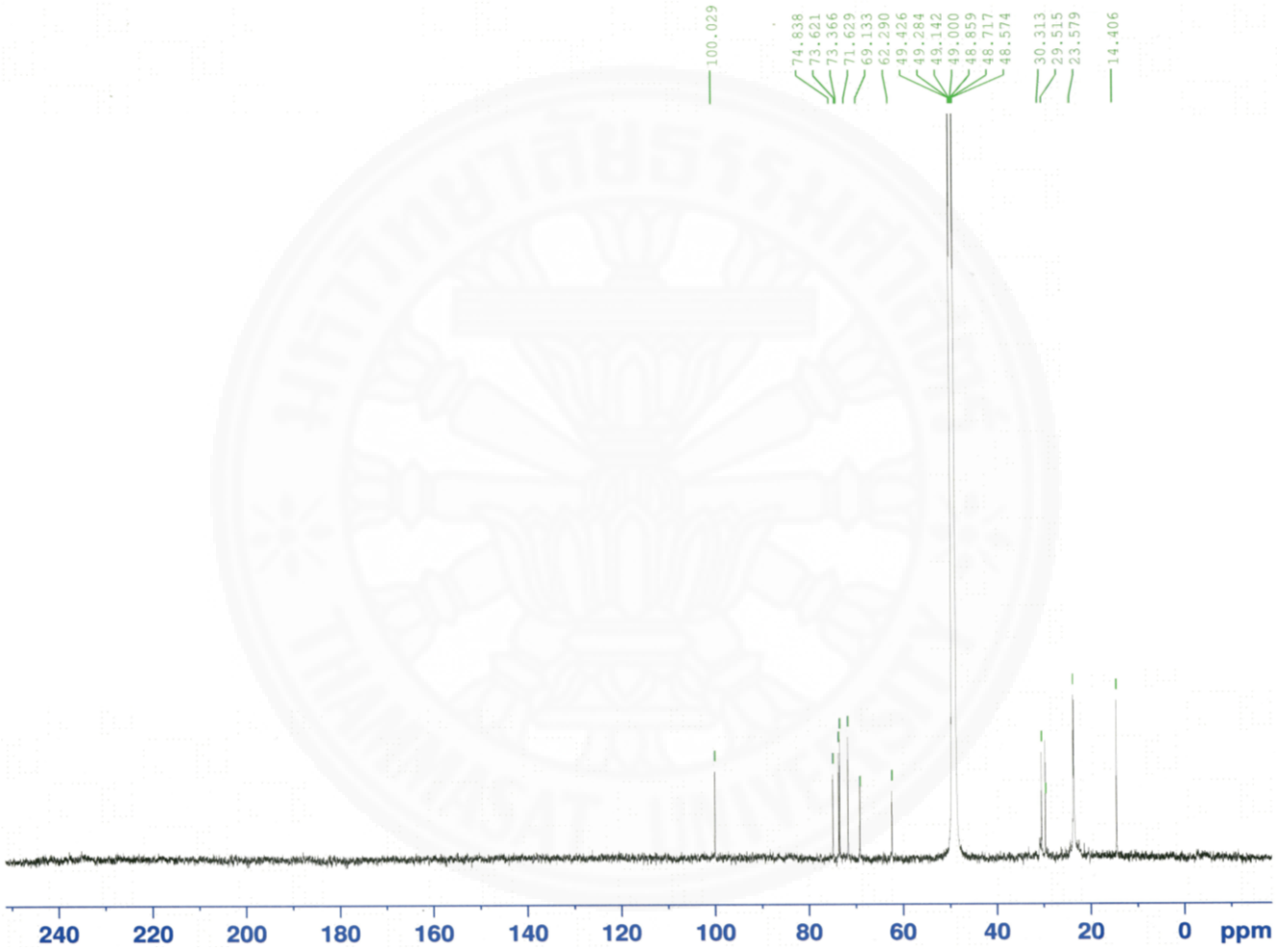


Figure 4.32 ^{13}C-NMR spectrum of pentyl glucoside ($PenG_1$)

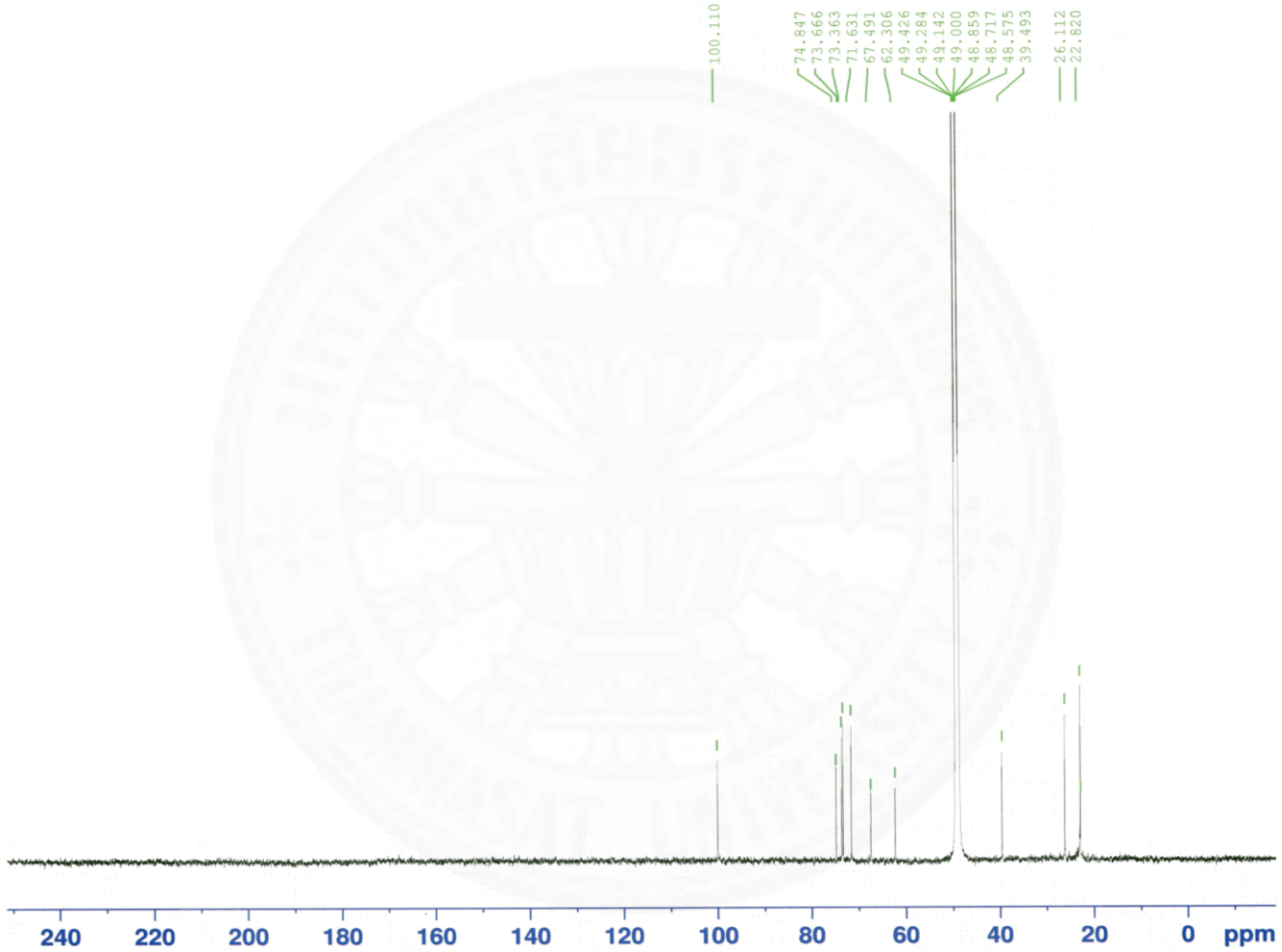


Figure 4.33 ^{13}C-NMR spectrum of isopentyl glucoside (IsoPenG_1)

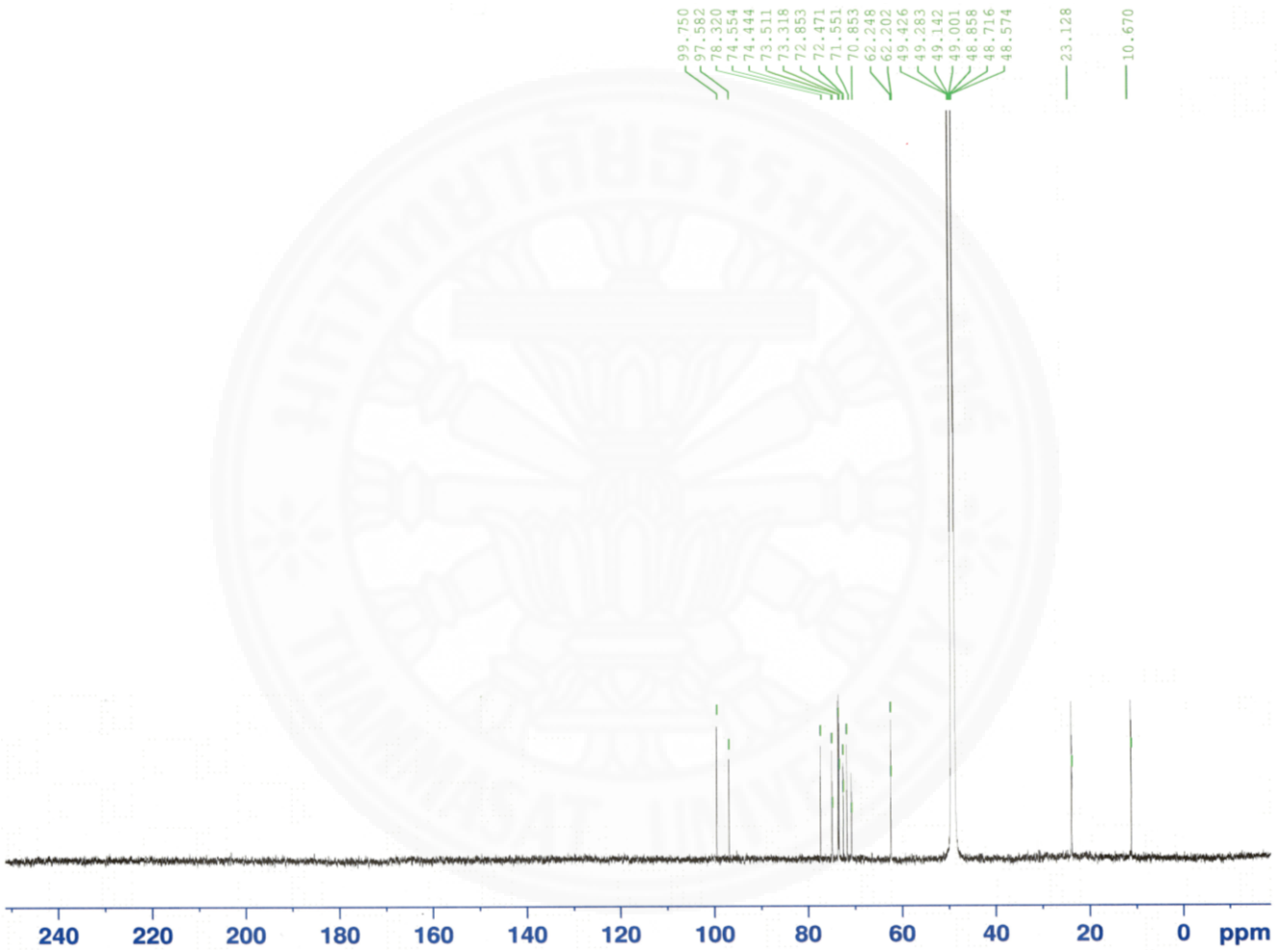


Figure 4.34 ^{13}C-NMR spectrum of propyl maltoside ($ProG_2$)

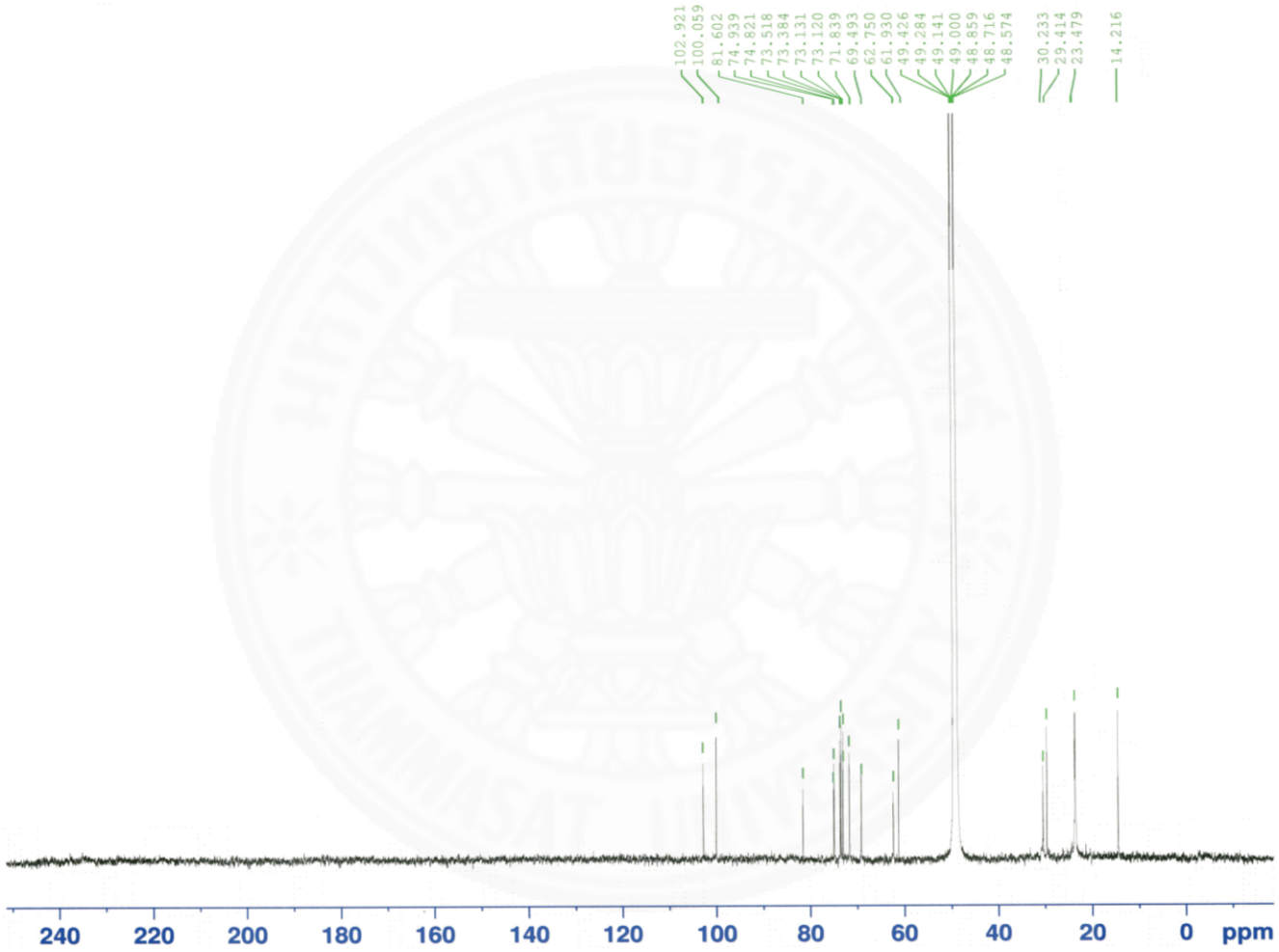


Figure 4.35 ^{13}C-NMR spectrum of pentyl maltoside ($PenG_2$)

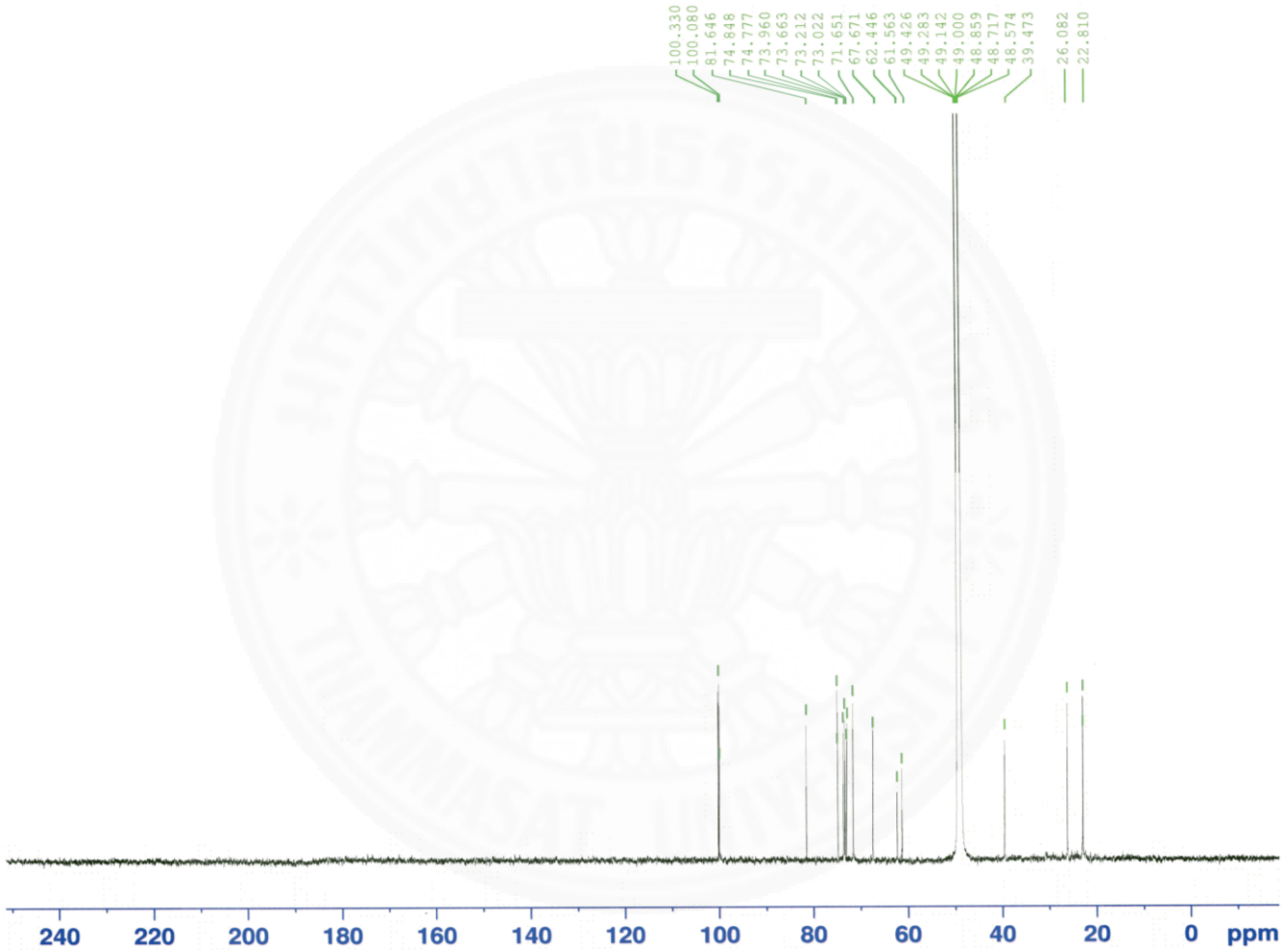


Figure 4.36 ^{13}C-NMR spectrum of isopentyl maltoside (IsoPenG_2)

Table 4.7 ^{13}C-NMR data of propyl glycosides ($ProG_n$), pentyl glycosides ($PenG_n$) and isopentyl glycosides ($IsoPenG_n$)

	Compound												
	Methanol[a]	**1-Propanol[a]**	**1-Pentanol[a]**	**Isopentanol[a]**	**Glucose[b]**	**MG_1[c]**	**Standard MG_1**	**$ProG_1$**	**$ProG_2$**	**$PenG_1$**	**$PenG_2$**	**$IsoPenG_1$**	**$IsoPenG_2$**
C-Alkyl													
1	51.8	59.5	62.2	60.2		56.1	54.11	70.70	70.85	69.13	69.49	67.49	67.67
2		23.5	32.2	41.3				23.76	23.13	30.31	30.23	39.49	39.47
3		10.0	27.9	24.5				11.05	10.67	29.52	29.41	26.11	26.08
4			22.4	22.3						23.58	23.48	22.82	22.81
5			13.7	22.3						14.41	14.22	22.82	22.81
C-Glucose													
1'					92.77	100.4	99.86	99.97	99.75	100.03	100.06	100.11	100.33
2'					72.15	72.4	72.17	73.38	73.32	73.37	73.13	73.36	73.21
3'					73.43	74.5	73.73	74.86	74.44	74.84	74.82	74.85	74.85
4'					70.32	70.5	70.38	71.65	78.32	71.63	81.60	71.63	81.65
5'					72.10	71.0	72.14	73.57	72.85	73.62	73.52	73.67	73.96
6'					61.27	66.0	61.25	62.33	62.20	62.29	62.75	62.31	62.45
1''									97.58		102.92		100.08
2''									72.47		73.12		73.02
3''									74.55		74.94		74.78
4''									71.55		71.84		71.65
5''									73.51		73.38		73.66
6''									62.25		61.93		61.56

Values of chemical shift (δ) are stated in ppm.

[a,b,c] The chemical shift values are taken from [a]Smith, 2011; [b]Roslund, Tähtinen, Niemitz, & Sjöholm, 2008; [c]de Segura, Alcalde, Bernabé, Ballesteros, & Plou, 2006.

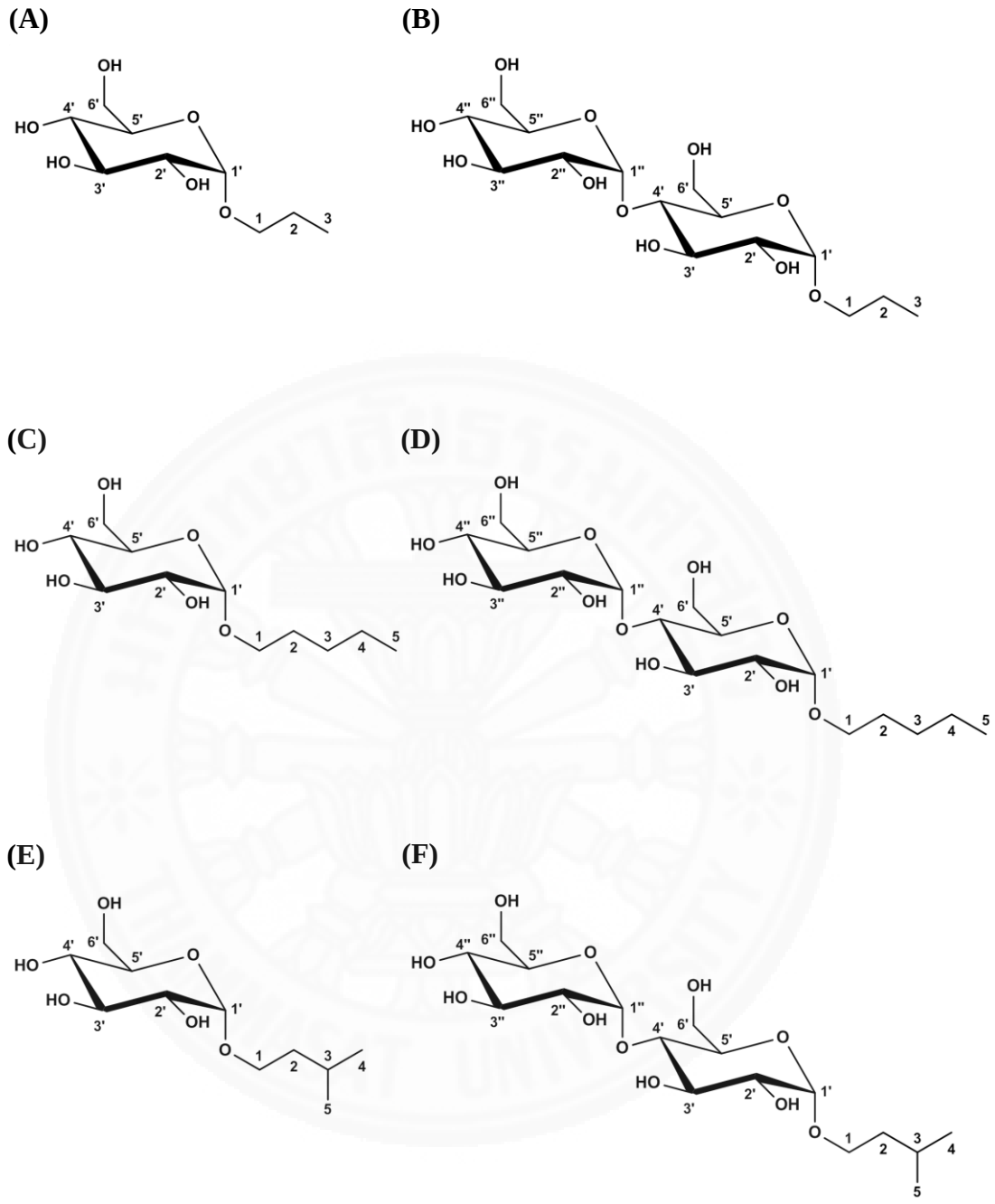


Figure 4.37 Proposed structure of the (A) propyl-α-D-glucopyranoside ($ProG_1$), (B) propyl-α-D-maltopyranoside ($ProG_2$), (C) pentyl-α-D-glucopyranoside ($PenG_1$), (D) pentyl-α-D-maltopyranoside ($PenG_2$), (E) isopentyl-α-D-glucopyranoside ($IsoPenG_1$) and (F) isopentyl-α-D-maltopyranoside ($IsoPenG_2$)

4.9 Investigation of alkyl glycoside properties

4.9.1 Emulsification activity

Emulsification activity of purified alkyl glycosides was determined from their ability to form an oil-in-water emulsion with the n-hexadecane substrate as described in Section 3.16.1. Standard MG_1 and alcohols were also checked for emulsification activity. The highest turbidity was found in Triton^{TM} X-100 and set as 100% emulsification activity. The turbidity of other alkyl glycosides was measured and compared to that with Triton^{TM} X-100. As shown in Figure 4.38, the highest emulsification activity of alkyl glycosides was found in $PenG_2$, followed by $PenG_1$, $IsoPenG_2$, $ProG_2$, $IsoPenG_1$, $ProG_1$ and MG_1, respectively. These results suggested that the emulsification activity of alkyl-α-D-glycosides increased when increasing number of hydrophobic alkyl chain length and hydrophilic moiety from glucose residue. Furthermore, the alkyl-α-D-glycosides with linear alkyl chain showed better emulsification activity than that of branched chain alkyl-α-D-glycosides. These observations could be summarized that the obtained alkyl glycosides in this study showed the effect to form oil-in-water emulsion depending on the number of both alkyl chains and carbohydrate groups as well as the structure of hydrophobic tail.

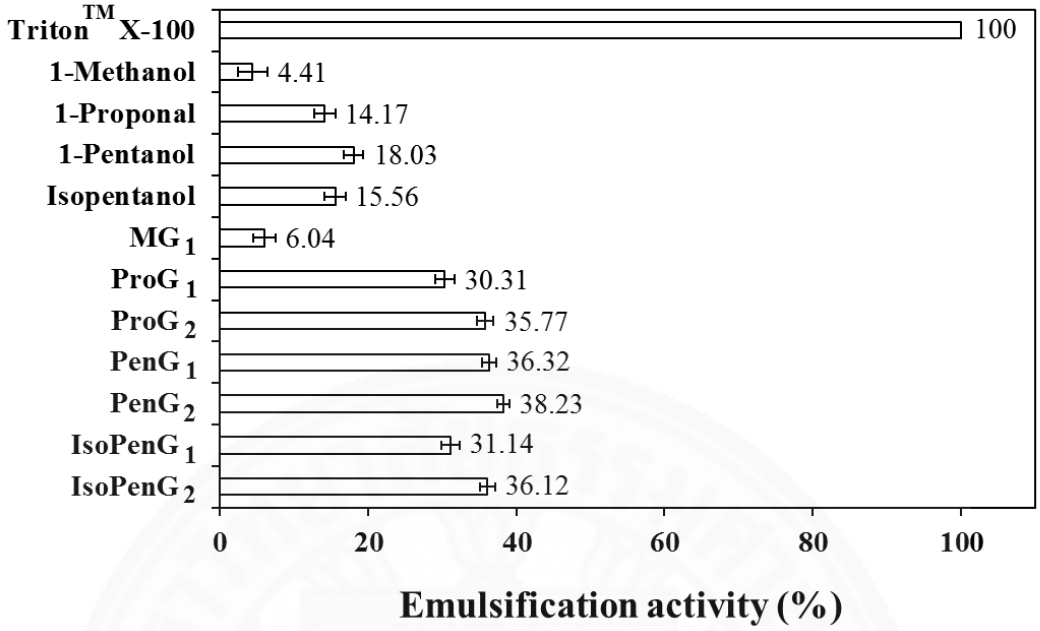


Figure 4.38 Emulsification activities of alcohols, commercial non-ionic surfactants and the synthesized alkyl glycosides

4.9.2 Emulsification stability

The stability of emulsion formed by alkyl glycosides was studied as a function of time. The emulsion of n-hexadecane was performed and the turbidity was measured every 10 minutes for 60 minutes by spectrophotometer at 540 nm as described in Section 3.16.2. The logarithms of the absorbance were plotted versus time and the dissociation constant (K_d) value was calculated from the slope of the graph. The smaller K_d is the greater stability. The emulsification stability of Triton^{TM} X-100 was set as 100% due to its most effective in the stabilization of the oil-in-water emulsion. The addition of alkyl-α-D-glycosides to the emulsion revealed that they were able to stabilize the emulsion over a period of time tested (Figure 4.39 and Table 4.8). PenG_2 exhibited the best ability to stabilize an oil-in-water emulsion. This data indicated that the synthesized alkyl-α-D-glycosides in this study could be used as an emulsion-stabilizing agent. In addition, the results also showed that n-hexadecane formed an emulsion with water in the presence of alcohols but this emulsion rapidly separated after 10 minutes (data not shown).

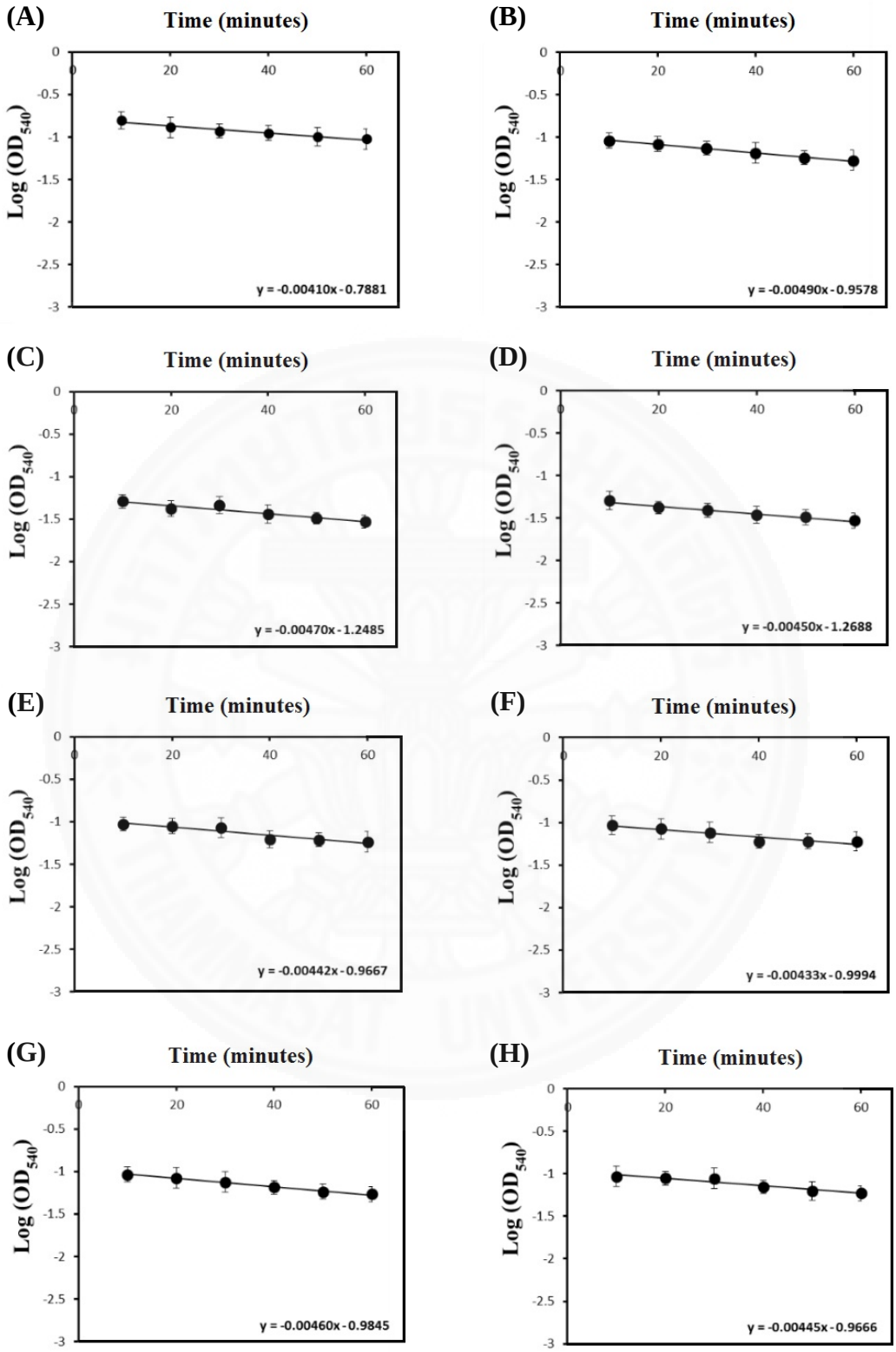


Figure 4.39 Stabilization of n-hexadecane oil emulsion by (A) Triton^{TM} X-100, (B) MG_1, (C) $ProG_1$, (D) $ProG_2$, (E) $PenG_1$, (F) $PenG_2$, (G) $IsoPenG_1$ and (H) $IsoPenG_2$.

Table 4.8 Emulsification stability of commercial non-ionic surfactants and the synthesized alkyl glycosides

Compound	**K_d**	**Emulsification staibility (%)**
Triton^{TM} X-100	0.00410	100
Standard MG_1	0.00490	83.7 ± 1.1
$ProG_1$	0.00470	87.2 ± 0.9
$ProG_2$	0.00450	91.1 ± 1.1
$PenG_1$	0.00442	92.7 ± 1.2
$PenG_2$	0.00433	94.6 ± 0.8
$IsoPenG_1$	0.00460	89.1 ± 1.3
$IsoPenG_2$	0.00445	92.1 ± 1.2

All values are averaged from three replicate experiments.

4.9.3. Antibacterial activity

4.9.3.1 Disc diffusion technique

The antibacterial activity of synthesized alkyl glycosides was preliminarily screened by disc diffusion technique as described in Section 3.16.3.1. Filter paper discs were impregnated with 5 mg of dried alkyl glycosides in 10 µL sterile water and placed on the surface of bacterial inoculated agar. The result was expressed by the diameter of inhibition zone. The standard antibiotic drugs and sterile water were also tested as control groups. As shown in Figure 4.40 and Table 4.9, all synthesized alkyl glycosides showed a moderately antibacterial function against both *S. aureus* and *E. coli* in the range of inhibition zone of 10-16 mm.

4.9.3.2 Minimal inhibitory concentration (MIC)

In order to determine the MIC, various concentrations of alkyl glycosides were prepared in a 96-well microplate by a two-fold serial dilution in multiple steps. Then, 50 µL (dilute 1:200 (v/v)) of the 0.5 McFarland inoculated culture was added in the 96-well microplate. After incubation, the 10 µL of resazurin solution was added to each well for using in the determination of the viability of bacteria as described in Section 3.16.3.2. As a result, the lowest MIC values of each alkyl glycoside were found in $ProG_2$, $PenG_2$ and $IsoPenG_2$ at the same MIC value of 1.88 mg/mL against *S. aureus*, and 1.88, 0.94 and 1.88 mg/mL against *E. coli*, respectively. In the negative control, sterile water had no inhibitory effect on any bacteria tested. The positive controls (antibiotics) showed that they had an ability to inhibit the bacterial growth in Table 4.10.

4.9.3.3 Minimal bactericidal concentration (MBC)

To determine the lowest concentration of each alkyl glycoside that can kill whole bacteria, the well with no visible growth occurred from MIC test was then sub-culturing to an agar medium again as described in Section 3.16.3.3. The results showed that the lowest MBC values against both *S. aureus* and *E. coli* were found in $ProG_2$, $PenG_2$ and $IsoPenG_2$ as compared to $ProG_1$, $PenG_1$ and $IsoPenG_1$, respectively (Table 4.11). From MIC and MBC results, it showed that the alkyl maltopyranoside had a higher antibacterial activity than alkyl glucopyranoside and was the most effective agent for inhibiting of *E. coli* growth.

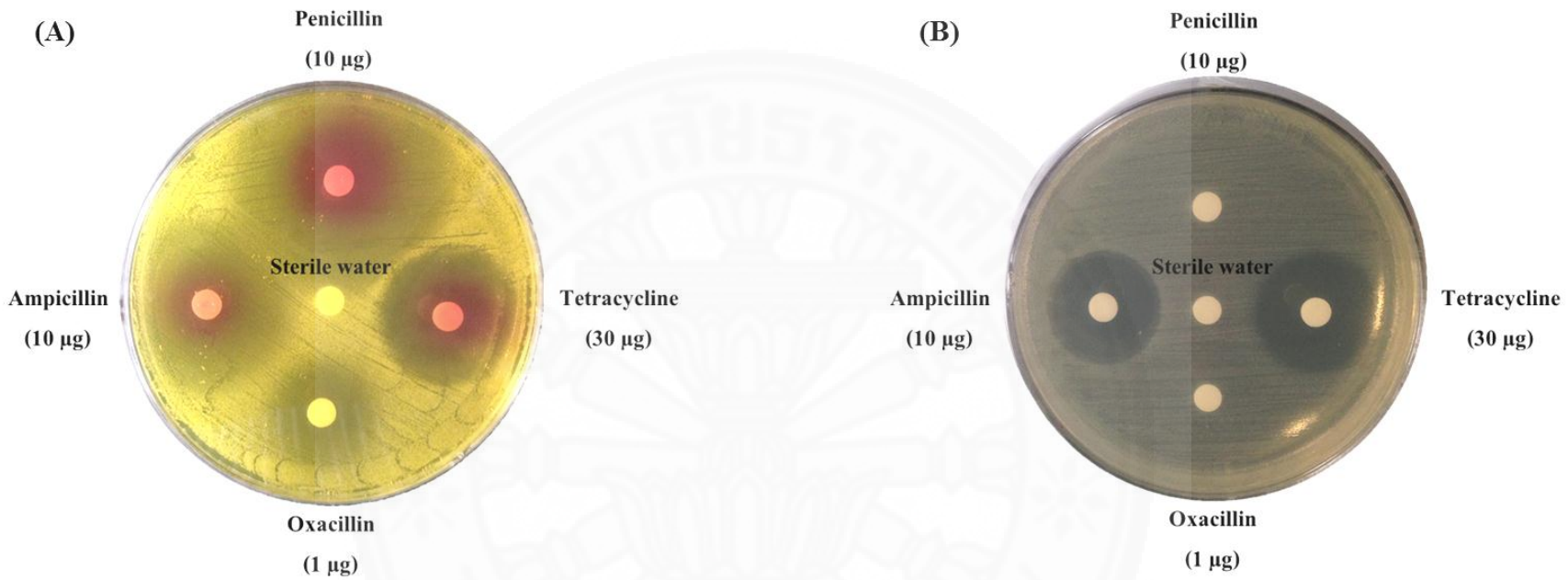


Figure 4.40 The inhibition zone of antibiotic drugs against (A) *S. aureus* ATCC 25923 and (B) *E. coli* ATCC 25922

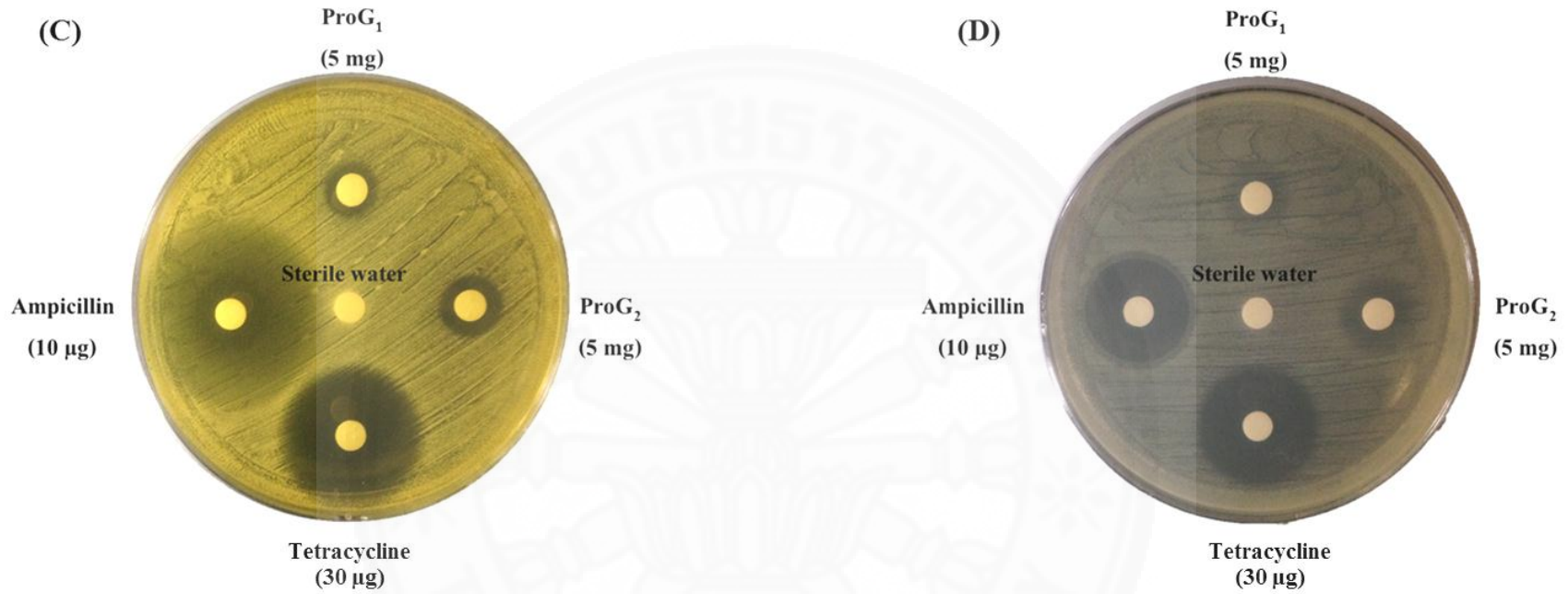


Figure 4.40 (cont.) The inhibition zone of $ProG_1$ and $ProG_2$ against (C) *S. aureus* ATCC 25923 and (D) *E. coli* ATCC 25922

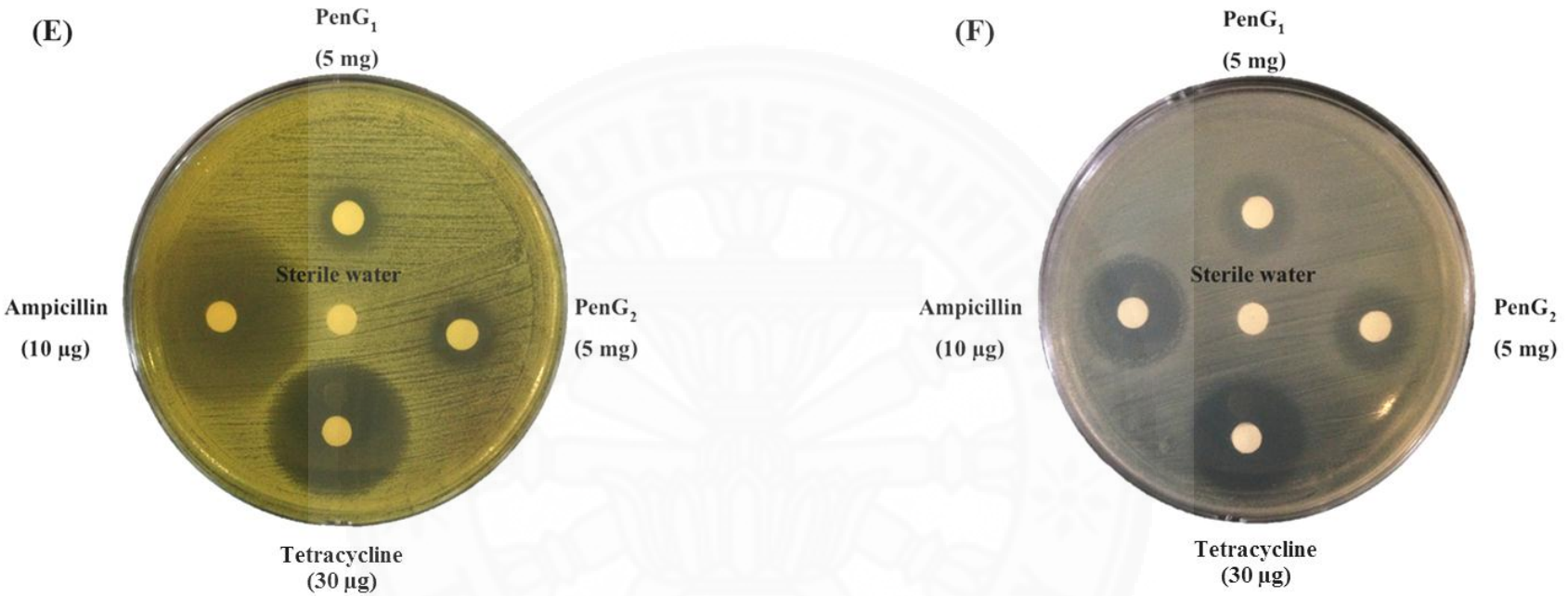


Figure 4.40 (cont.) The inhibition zone of $PenG_1$ and $PenG_2$ against (E) *S. aureus* ATCC 25923 and (F) *E. coli* ATCC 25922

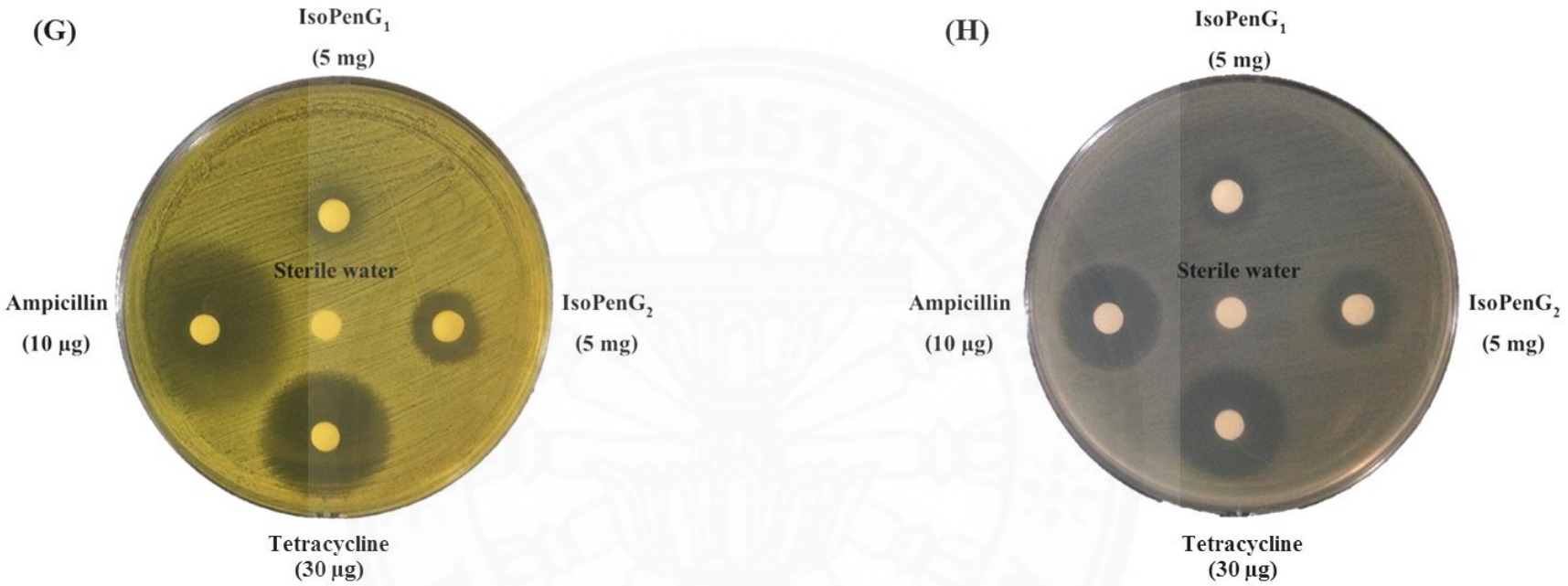


Figure 4.40 (cont.) The inhibition zone of $IsoPenG_1$ and $IsoPenG_2$ against (G) *S. aureus* ATCC 25923 and (H) *E. coli* ATCC 25922

Table 4.9 Antibacterial activity of standard antibiotic drugs and the synthesized alkyl glycosides

Microorganism	**Antibacterial activity (Zone of inhibition in mm of diameter[a])**									
	AMP[b] (10 µg)	**TET[b] (30 µg)**	**PEN[b] (10 µg)**	**OXA[b] (1 µg)**	**ProG$_1$ (5 mg)**	**ProG$_2$ (5 mg)**	**PenG$_1$ (5 mg)**	**PenG$_2$ (5 mg)**	**IsoPenG$_1$ (5 mg)**	**IsoPenG$_2$ (5 mg)**
S. aureus ATCC 25923	30 ± 0.2	26 ± 0.1	40 ± 0.2	24 ± 0.1	10 ± 0.1	12 ± 0.1	10 ± 0.1	12 ± 0.1	10 ± 0.1	12 ± 0.2
E. coli ATCC 25922	22 ± 0.1	24 ± 0.1	0	0	12 ± 0.2	14 ± 0.1	14 ± 0.1	16 ± 0.1	12 ± 0.2	15 ± 0.2

[a] Values of inhibitory zone in mm are the mean ± SD of three parallel measurements.

[b] The concentration of antibiotics (AMP, Ampicillin; TET, Tetracycline; PEN, Penicillin; OXA, Oxacillin) was used according to the standard concentration shown in Clinical and Laboratory Standards Institute (CLSI, 2014).

Table 4.10 Minimal inhibitory concentration (MIC) of ampicillin and the synthesized alkyl glycosides against microorganisms

Microorganism	**Minimal inhibitory concentration**[a]						
	Ampicillin (μg/mL)	**ProG$_1$ (mg/mL)**	**ProG$_2$ (mg/mL)**	**PenG$_1$ (mg/mL)**	**PenG$_2$ (mg/mL)**	**IsoPenG$_1$ (mg/mL)**	**IsoPenG$_2$ (mg/mL)**
S. aureus ATCC 25923	0.08 ± 0	3.75 ± 0	1.88 ± 0	3.75 ± 0	1.88 ± 0	3.75 ± 0	1.88 ± 0
E. coli ATCC 25922	0.98 ± 0	3.75 ± 0	1.88 ± 0	1.88 ± 0	0.94 ± 0	1.88 ± 0	1.88 ± 0

[a] All data are shown as mean ± SD derived from triplicate experiments.

Table 4.11 Minimal bactericidal concentration (MBC) of ampicillin and the synthesized alkyl glycosides against microorganisms

Microorganism	**Minimal bactericidal concentration**[a]						
	Ampicillin (μg/mL)	**ProG$_1$ (mg/mL)**	**ProG$_2$ (mg/mL)**	**PenG$_1$ (mg/mL)**	**PenG$_2$ (mg/mL)**	**IsoPenG$_1$ (mg/mL)**	**IsoPenG$_2$ (mg/mL)**
S. aureus ATCC 25923	0.08 ± 0	3.75 ± 0	3.75 ± 0	3.75 ± 0	1.88 ± 0	3.75 ± 0	3.75 ± 0
E. coli ATCC 25922	0.98 ± 0	3.75 ± 0	1.88 ± 0	1.88 ± 0	1.88 ± 0	3.75 ± 0	1.88 ± 0

[a] All data are shown as mean ± SD derived from triplicate experiments.

4.9.4 Solubilization of insoluble amylomaltase

The $PenG_2$, which exhibited the highest emulsification activity from Section 4.9.1, was chosen for the examination of bacterial insoluble protein solubility. The $PenG_2$ were added into sonication buffer and incubated with the *C. glutamicum* insoluble amylomaltase protein from *E. coli* BL21 (DE3) transformants as described in Section 3.16.4.2. The effect of $PenG_2$ on the solubility of insoluble amylomaltase was determined by 10% SDS-PAGE and amylomaltase activity, comparing with the commercial ionic and non-ionic surfactants. As shown in Figure 4.41, the amount of soluble amylomaltase (84 kDa) in soluble fraction increased when the sonication buffer composed of $PenG_2$ or other commercial surfactants. Although the reaction mixture of 1% (w/v) SDS gave the highest soluble amylomaltase protein, the specific activity of SDS-solubilized amylomaltase dramatically decreased. In contrast to SDS, the soluble amylomaltase from the action of 1% (w/v) $PenG_2$ showed the specific activity of 1.01 U/mL, which is similar to the 1.14 U/mL seen for 1% (w/v) Triton^{TM} X-100 (Table 4.12). In addition, the increasing of $PenG_2$ concentration resulted in the enhancing of the amount and activity of soluble amylomaltase (Figure 4.42 and Table 4.12). These results indicated that $PenG_2$ could be used in improving the solubility of insoluble protein and stabilizing of enzyme activity.

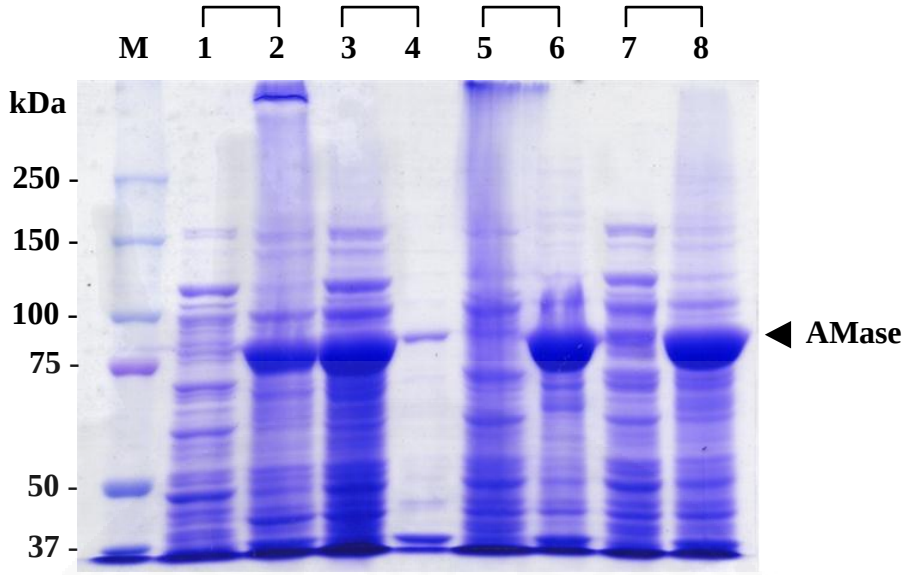


Figure 4.41 10% SDS-PAGE analysis of the improvement of aggregated amylomaltase solubility by the addition of several surfactants

Lane M: Protein marker (5 µL)

Lane 1-2: Soluble and insoluble fraction without the addition of surfactants (100 µg)

Lane 3-4: Soluble and insoluble fraction with 1% (w/v) SDS (100 µg)

Lane 5-6: Soluble and insoluble fraction with 1% (w/v) Triton™ X-100 (100 µg)

Lane 7-8: Soluble and insoluble fraction with 1% (w/v) $PenG_2$ (100 µg)

The arrow indicates a molecular weight of amylomaltase (AMase) (84 kDa).

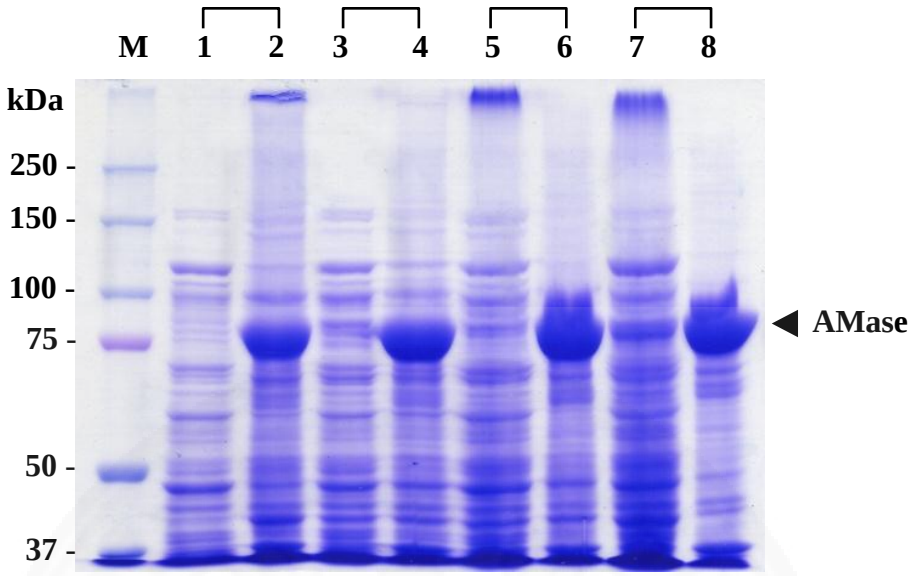


Figure 4.42 10% SDS-PAGE analysis of the effect of $PenG_2$ concentration on aggregated amylomaltase solubility

Lane M: Protein marker (5 µL)

Lane 1-2: Soluble and insoluble fraction without the addition of surfactants (100 µg)

Lane 3-4: Soluble and insoluble fraction with 1% (w/v) $PenG_2$ (100 µg)

Lane 5-6: Soluble and insoluble fraction with 3% (w/v) $PenG_2$ (100 µg)

Lane 7-8: Soluble and insoluble fraction with 5% (w/v) $PenG_2$ (100 µg)

The arrow indicates a molecular weight of amylomaltase (AMase) (84 kDa).

Table 4.12 Specific activity of amylomaltase from soluble and insoluble fractions with and without surfactant supplements

Supplement	Specific activity[a] (U/mg protein)	
	Soluble fraction	**Insoluble fraction**
Tris-HCl buffer pH 7.4	0.21 ± 0.04	0
1% (w/v) SDS	0.33 ± 0.02	0
1% (w/v) Triton^{TM} X-100	1.14 ± 0.02	0
1% (w/v) $PenG_2$	1.01 ± 0.01	0
3% (w/v) $PenG_2$	1.34 ± 0.03	0
5% (w/v) $PenG_2$	1.56 ± 0.02	0

[a] Calculated from starch transglycosylation activity and protein concentration.
All values are averaged from three replicate experiments.

CHAPTER 5
DISCUSSION AND CONCLUSIONS

5.1 Purification of CGTase from the pBC recombinant cell

There are several methods have been used to purify CGTase. For example, DEAE Sephadex A-50 followed by DEAE Sepharose CL-6B was used to purify *B. firmus* CGTase (Yim et al., 1997). Resource Q ion-exchange column, Phenyl-Superose column and α-CD affinity column were purification steps for hyperthermophilic archaeon *Thermococcus* sp. CGTase (Tachibana et al., 1999). For purification of *Bacillus* sp. 1070 CGTase, one-step affinity purification method using β-CD-Sepharose 4B column was used (Volkova, Lopatin, & Varlamov, 2000). Ammonium sulfate precipitation at 70% saturation combined with Sepharose 6B column was used to purify alkalophilic *Bacillus* sp. TS1-1 CGTase (Rahman, Illias, Hassan, Mahmood, & Rashid, 2006). In this study, CGTase which was produced from the pBC recombinant cell was an extracellular enzyme. So, crude CGTase was harvested after the removal of cells by centrifugation. The first common step of purification was starch adsorption. Based on this method, CGTase was specifically absorbed to starch substrate due to substrate-enzyme affinity binding, while the other non-binding proteins were separated by centrifugation. CGTase did not hydrolyze starch substrate because this purification step was performed under unsuitable temperature (4 °C) for enzyme catalysis. The absorbed CGTase was then eluted from starch by adding a buffer with maltose solution. Maltose which had the higher affinity for binding with CGTase than starch was bound with CGTase instead of starch. Therefore, CGTase-maltose that presented in soluble fraction was easily separated from starch by centrifugation. The CGTase-maltose solution was dialyzed against TB1 buffer to remove maltose from enzyme and the enzyme activity was then determined by dextrinizing assay. After the starch adsorption step, the CGTase had a specific dextrinizing activity of 1.08×10^4 units/mg protein, 71% yield and 6-fold purification (Table 4.1). The purity of purified CGTase was checked by SDS-PAGE analysis. As a result, the CGTase from starch adsorption still showed the smeared

bands of proteins that had nearby size with CGTase (Figure 4.3, Lane 2). So, the second step of purification was performed on DEAE-Toyopearl 650M column chromatography, an anion-exchange column chromatography with positive charge methacrylate resin. In this purification step, the pH of buffer that solubilized CGTase was 8.5 while the pI value of CGTase was 4.9 (Kaskangam, 1998). Therefore, the amino acids at the surface of CGTase were changed to the negative charge and bound with column. The proteins were eluted from the column by sodium chloride gradient solution and separated according to their charges. Sodium chloride reduced the ionic strength between proteins and resins. The weakest anionic protein was eluted first and followed by strong anionic protein. The fractions of 2 mL were collected and determined their protein and dextrinizing activity profiles by measuring the absorbance at 280 and 600 nm, respectively. From Figure 4.1, the purification profile of CGTase showed that the unwanted proteins were separated from the CGTase peak. In summary, after the two steps of purification, the purified CGTase was pure for using in a further experiment, giving only one band on SDS-PAGE analysis and had a specific dextrinizing activity of 1.30×10^4 units/mg protein, 43% yield and 7-fold purification (Figure 4.3, Lane 3 and Table 4.1).

5.2 Characterization of CGTase

5.2.1 Molecular weight determination

The purified CGTase was determined by 7.5% SDS-PAGE analysis and its relative molecular weight was calculated from a standard curve of protein markers. As shown in Figure 4.4, the molecular weight of CGTase from the pBC recombinant cell was estimated to be 72 kDa. The approximately sizes of CGTases in the range of 70-75 kDa were found in various *Bacillus* species (Tomita, Kaneda, Kawamura, & Nakanishi, 1993; Sian et al., 2005; Costa, del Canto, Ferrarotti, & de Juiménez Bonino, 2009; Atanasova, Kitayska, Bojadjieva, Yankov, & Tonkova, 2011). However, a larger size of CGTase at 110 kDa has been reported from *B. agaradhaerens* (Martins & Hatti-Kaul, 2002).

5.2.2 Effect of temperature on transglycosylation activity and stability

The optimum temperature for transglycosylation of CGTase from the pBC recombinant cell was observed at 50 °C (Figure 4.5). This optimum temperature was closed to *Klebsiella pneumoniae* AS-22 (optimum temperature at 45 °C) (Gawande & Patkar, 2001), *Bacillus* sp. C26 and *Amphibacillus* sp. NPST-10 (optimum temperature at 50 °C) CGTases (Cheirsilp, Kitcha, & Maneerat, 2010; Ibrahim, Al-Salamah, El-Tayeb, El-Badawi, & Antranikian, 2012). For temperature stability, after the pBC CGTase was preincubated at temperatures of 25, 40, 50 and 60 °C for 120 minutes, 93-100% activity of the CGTase was retained at temperatures of 25, 40 and 50 °C. At 60 °C, CGTase from the pBC recombinant cell in this study became denatured form and lost its function (Figure 4.6). Approximately 20% activity was remained after preincubation at 60 °C for 120 minutes. CGTase from thermophilic bacteria were different such as those from *B. stearothermophilus* ET1 (optimum temperature 80 °C and stable up to 90 °C) (Chung et al., 1998), *Thermoanaerobacterium thermosulfurigenes* EM1 (optimum temperature 85 °C and stable up to 90 °C) (Wind et al., 1995) and *Thermococcus* sp. B-1001 (optimum temperature 90 °C and stable up to 100 °C) (Tachibana et al., 1999).

5.2.3 Effect of pH on transglycosylation activity and stability

The effect of pH on the enzyme activity and stability was studied in the pHs ranging from 3.0 to 11.0. As a result, the extreme pH values were not suitable for the enzyme to perform transglycosylation activity. The enzyme exhibited the highest activity at pH 6.0 (Figure 4.7), which was in accord with some CGTases from *Bacillus* sp. G1 (Sian et al., 2005), *B. stearothermophilus* (Ahn, Hwang, & Kim, 1990) and *B. stearothermophilus* ET1 (Chung et al., 1998). However, the different optimum pHs were reported in alkaliphilic bacteria such as *B. agaradhaerens* (pH 9.0) (Martins & Hatti-Kaul, 2002) and *Brevibacterium* sp. No. 9605 (pH 10.0) (Mori, Hirose, Oya, & Kitahata, 1994). The determination of pH stability of CGTase showed that it was stable in the range of pH 5.0-11.0 and pH 5.0-10.0 at 4 and 50 °C in 24-hour preincubation, respectively (Figure 4.8). In comparison, the pH stability profile of CGTase in this study had a wider range than that of *K. pneumoniae* AS-22 (pH 6.0-9.0) (Gawande & Patkar, 2001), *B. firmus* (pH 5.5-9.0) (Sohn et al., 1997)

and *Bacillus* sp. G1 (pH 7.0-9.0) (Sian et al., 2005) CGTases. These results implied that CGTase from the pBC recombinant cell was an alkalophilic enzyme.

5.2.4 Donor specificity

The donor specificity of the enzyme was evaluated in the coupling reaction using α-CD, β-CD and γ-CD as glycosyl donors with cellobiose acceptor. The occurred transglycosylation products were determined by glucose oxidase assay and the TLC analysis. As a result, CGTase could transfer α-1,4-linked glucosyl residues from all of the CDs to a cellobiose acceptor, which was a β-1,4-glucosidic residue. Although these three substrates were effective as glucosyl donors for transglycosylation reaction of this CGTase, the highest coupling activity and the largest transglycosylation yield were observed when the β-CD donor was used (Figures 4.9 and 4.10). This appropriate glycosyl donor (β-CD) has also been reported in the previous study of CGTase from *Paenibacillus* sp. RB01 for the synthesis of epicatechin-α-glycosides using epicatechin acceptor (Aramsangtienchai, Chavasiri, Ito, & Pongsawasdi, 2011). However, the investigation of many researchers have reported the use of other substrates such as α-CD and starch as the best appropriate donor for transglycosylation reaction of *B. stearothermophilus* and *B. macerans* CGTases using L-ascorbic acid and β-arbutin as acceptors, respectively (Aga, Yoneyama, Sakai, & Yamamoto, 1991; Sugimoto, Nishimura, Nomura, Sugimoto, & Kuriki, 2003). These data indicated that the donor specificity of CGTase depended on the unique property of each CGTase that was used in the catalytic reaction to form a new covalent bond with the acceptor of interest.

5.3 Synthesis of alkyl glycosides

5.3.1 Effect of alcohol structure and concentration on transglycosylation reaction of CGTase

The use of an organic solvent such as alcohol in the enzymatic reaction mainly led to the extinction of enzyme function. However, in this study, alcohols were used as glycosyl acceptor in the transglycosylation reaction under the catalysis of CGTase. Therefore, the effect of alcohols on CGTase activity for the synthesis of alkyl glycosides was first investigated by the determination of remaining

coupling activity (phenolphthalein assay) and TLC analysis after 24-hour transglycosylation reaction. The reactions were separated into two groups (single phase and two phase reactions) due to the solubility of alcohol acceptors in water. The longer chain alcohol was low soluble in water than short chain alcohol. From Figure 4.11, in single phase reaction, the alkyl chain length and concentration of alcohol had a significant effect on the coupling activity of CGTase. The longer alkyl chain and the higher concentration of alcohol resulted in the lower coupling activity. The effect of alcohol on the activity of other enzymes has already been observed by other researchers. For example, in the synthesis of alkyl glucoside catalyzed by β-glucosidase from Thai rosewood, the enzyme was more inactivated by the long alkyl chain lengths and high concentrations of alcohols (Lirdprapamongkol & Svasti, 2000). The activity and stability of invertase from *S. cerevisiae* were also found to be lowered and totally disappeared when the concentration of alcohol was added to 60% (v/v) of the reaction mixture (Rodríguez, Gómez, Gónzalez, Barzana, & López-Munguía, 1997). Many studies have suggested that there are many causes of these behaviors such as the negative influence of the reduction of water activity, enzyme transition-state destabilization and enzyme conformational modifications, which resulted in the loss of enzyme activity including product yield in alcoholic solutions (Itoh & Kamiyama, 1995; Rodríguez et al., 1997; Ito, Ebe, Shibasaki, Fukuda, & Kondo, 2007; Wang et al., 2012).

However, the different result was observed in the two phase reaction. The increasing of alkyl length and alcohol concentration did not make the reduction of coupling activity. This result could be a consequence of the limitation of enzyme-substrate interaction. In a single phase reaction in which all substances were in the same phase, the effect of enzyme-substrate interaction between phases was very strong. On the other hand, in a two phase reaction, the enzyme was in a different phase and could not have completely contacted to alcohol substrate. Consequently, its activity was still high and undamaged from the high concentration of alcohol when compared with the single phase reaction (Vulfson, Patel, Beecher, Andrews, & Law; 1990). In addition, when the activities of reactions with 1-pentanol and isopentanol were compared, it was found that the structure of alcohol acceptor also had an important factor on the CGTase activity. The higher activity was observed when using

1-pentanol as the acceptor. This result indicated that the appropriate acceptor specificity of the transglycosylation reaction of CGTase was found to be normal primary alcohol with linear carbon chain. The tendency of acceptor specificity in the enzymatic synthesis of alkyl glycosides in this study was similar to those of other alkyl glycosides in the previous studies. The branched primary alcohol, secondary alcohol and tertiary alcohol were shown to be poor glycosyl acceptors in the transglycosylation reactions of *F. oxysporum* and Thai rosewood β-glucosidases (Makropoulou et al., 1998; Lirdprapamongkol & Svasti, 2000), *A. niger* α-rhamnosidase (Martearena, Blanco, & Ellenrieder, 2003) and *Paenibacillus* sp. RB01 CGTase (Chotipanang et al., 2011;).

The effect of alcohols on alkyl glycoside synthesis was also determined by subjecting the reaction products to TLC analysis. This chromatography was a basic separation method which composes of silica gel as the polar stationary phase. After the sample had been applied on TLC plate, the mixture of solvents was used as the non-polar mobile phase. The polar compounds such as oligosaccharides were strongly absorbed on silica gel resulted in the less R_f value, while the expected alkyl glycoside products with the non-polar property of the alkyl chains were migrated to a higher position (high R_f value). The reaction products on TLC plate were visualized by dipping with a sulfuric solution and heating at high temperature. The organic products on TLC plate were turned into a black spot of carbon due to degradation of an organic compound by sulfuric acid. Then, the concentration of alkyl glycosides was measured using a scanning densitometer with the Quantity One® 1-D analysis program. The results showed that the relationship between the alcohol concentration and alkyl glycoside yield was found to be a bell-shaped curve (Figure 4.12). The product yield increased with the increasing concentration of alcohol but at alcohol concentrations exceeding 30% (w/v) methanol, 20% (w/v) ethanol, 10% (v/v) 1-propanol, 5% (w/v) 1-butanol, 10% (w/v) 1-pentanol and 20% (w/v) isopentanol, alkyl glycoside yield decreased. This pattern could explain that at the initial increasing of alcohol concentration, the alcohol as an acceptor substrate was converted into more products due to the higher turnover rate of the enzyme. However, higher alcohol concentrations might deactivate enzyme, resulting in lower product yield. Moreover, the more product yield obtained from the reaction

with 1-pentanol than isopentanol confirmed that CGTase preferred the linear primary alcohol to the iso-primary alcohol in transglycosylation reaction.

5.3.2 Transglycosylation from β-CD to alcohols

The reactions with appropriate concentration of alcohols were then analyzed on TLC plate. From Figure 4.13, after 24 hours transglycosylation reaction of CGTase, each alcohol acceptor gave the different numbers of product spots with higher R_f value than oligosaccharides due to their polarity which correlated with the number of glucose and the length of the alkyl chain. These spots were expected to be the derivatives of alkyl glycosides which were synthesized by CGTase-catalyzed transglycosylation reaction from β-CD to the alcohol acceptors. In addition to alkyl glycosides, the hydrolysis products such as glucose and other oligosaccharides which resulted from the transfer of β-CD to water were formed in a lower R_f value. So, it is possible that other products with longer carbohydrate chain were also produced in reaction mixtures with each alcohol acceptor, but they may have been obscured by the hydrolysis products. The high hydrolysis products in reactions indicated that water was the best acceptor for the enzyme. In comparison to the other bacterial enzymes involved in the synthesis of alkyl glycosides, *A. oryzae* β-galactosidase, *A. Niger* β-mannosidase and *L. mesenteroides* dextransucrase gave only one alkyl glycoside product with one unit of glucose (Ooi, Hashimoto, Mitsuo, & Satoh, 1985; Itoh & Kamiyama, 1995; Kim et al., 2009). CGTase in this study yielded at least 1-3 products depending on alcohol acceptors. Therefore, it can be concluded that recombinant CGTase from *B. circulans* A11 was a unique enzyme that acted differently from other enzymes in the transglycosylation of oligosaccharides from β-CD to alcohols for the synthesis of alkyl glycosides. From the previous study, Chotipanang et al. (2011) reported for the first time that CGTase could transfer oligosaccharides from β-CD to methanol giving methyl glycosides with 1-3 glucose units. However, there have been no reports on the use of longer and branched alcohols as an acceptor for transglycosylation reaction of CGTase. Moreover, the properties of alkyl glycosides which were synthesized from longer primary linear and branched alcohol acceptors have never been studied before. Thus, 1-propanol, 1-pentanol and isopentanol were chosen as acceptors for further experiment.

5.3.3 Optimal conditions for the synthesis of alkyl glycosides

Prior to the optimization of transglycosylation reaction, the reaction mixture for the synthesis of alkyl glycosides consisted of 0.64% (w/v) β-CD, various alcohols (10% (w/v) 1-propanol and 1-pentanol, and 20% (w/v) isopentanol) and 75 U/mL CGTase in 20 mM phosphate buffer at pH 6.0. The reaction was performed at 50 °C for 24 hours and then analyzed on TLC plate. The obtained alkyl glycoside yields were quantified by scanning densitometer compared to the spot intensity of standard MG_1. As a result, three alkyl glycoside products were observed in the reactions using 1-propanol, 1-pentanol and isopentanol acceptors with yields of 10.6, 21.8 and 21.0% (w/v) of total products, respectively. The amount of three products from each alcohol was different because CGTase also exhibited glucoamylase-like activity, which hydrolyzed glucose residues from the non-reducing end of alkyl maltooligosaccharide.

To achieve the highest yield of propyl-, pentyl- and isopentyl-glycosides, several parameters involved in the synthesis of these alkyl glycosides were sequentially and independently optimized by changing various conditions of transglycosylation reaction. These parameters were the alcohol and β-CD concentrations, enzyme units, incubation times, temperatures and pHs. The optimal conditions for the synthesis of alkyl glycosides were judged from the reaction which gave the maximal product yield. From Figures 4.14-4.16, after the optimization of transglycosylation reaction was completed, the yields of propyl-, pentyl- and isopentyl- glycosides comprised up to 37, 48 and 42% (w/w) of total products, which were an increase of greater than 3.5, 2.2 and 2.0-folds compared to the yield before optimization, respectively. This increase in product yield was found in all alkyl glycosides, including alkyl glucoside (AG_1), alkyl maltoside (AG_2) and alkyl maltotrioside (AG_3) products. These results indicated that the yield of product could be controlled by adjusting the substrate and enzyme concentrations together with other conditions (temperature, pH and incubation time). In general, different bacterial enzymes catalyzing systems produced propyl- and pentyl- glucoside product have been reported. For example, the incubation of *L. mesenteroides* dextransucrase with sucrose and 1-propanol at 28 °C and pH 5.2 could catalyze the synthesis of propyl glucoside with yields of 38% (w/v) (Kim et al., 2009). Wang et al. (2012) reported

that the use of reverse hydrolysis activity of almond meal β-glucosidase resulted in 13.7% (w/v) of pentyl glucoside. However, these glycosides consisted of one glucose residue. Thus, the propyl- and pentyl- glycoside yields obtained in this study were quite good compared to that from the other enzymes. For isopentyl glycoside synthesis, this study was the first report on the use of isopentanol as an acceptor in the transglycosylation reaction to produce isopentyl glycosides.

5.3.4 Large scale preparation and purification of alkyl glycosides

To prepare larger amounts of glycoside products for characterization, the reaction mixtures for the synthesis of propyl-, pentyl- and isopentyl- glycosides were scaled up to 250 mL and performed under the optimal conditions. Each alkyl glycoside product was isolated by PLC, a glass plate coated with thick silica gel. Similar to TLC, this simple method could isolate the expected alkyl glycoside products at different R_f values in a large volume based on their polarity differences. Only the mono- and di- glycosyl derivatives of alkyl glycosides were scraped from PLC while the tri- glycosyl derivative could not be isolated because of its very low yield. Alkyl glycoside products were extracted from silica gel by methanol and their purities were checked by TLC. As a result, each glycoside product of propyl-, pentyl- and isopentyl- glycosides was completely isolated and gave only one spot on TLC (Figure 4.17). The purified alkyl glycoside products in methanol were continuously evaporated to a dry powder by a centrifugal evaporator at 45 °C. By the different purification method, HPLC technique with various types of the column has been used for purification of alkyl glycosides. For example, Makropoulou et al. (1998) reported the purification of methyl-, ethyl-, propyl-, isopropyl-, butyl-, isobutyl- and 2-butyl- glycosides by HPLC on the NH_2-Spherisorb column (4.6×250 mm). This column contained a silica-based aminopropyl resin which was used to form polar interaction with sugar. Elution was performed with a mixture of acetonitrile-water (75:25 (v/v) ratio). Products in each fraction were detected by both a refractometer and TLC analysis. HPLC with Aminex HPX-87H column (7.8×300 mm) which was used for analyzing carbohydrates has also been used to purify methyl-, ethyl-, propyl-, butyl- and pentyl- glycosides. These glycosides were eluted with 5 mmol per litre of H_2SO_4 and detected using a refractive index detector (Wang et al., 2012).

5.4 Characterization of alkyl glycosides

5.4.1 Structure of alkyl glycosides

The structure of synthesized alkyl glycoside products was preliminarily analyzed in terms of the number of glucosyl units and its configuration. Each mixture of two purified alkyl glycoside products was digested with glucoamylase and α-glucosidase. Glucoamylase (α-1,4-D-glucan glucohydrolase, E.C. 3.2.1.3) is an exohydrolase enzyme which catalyzes the hydrolysis of terminal α-1,4-D-glucan from non-reducing ends of starch and other related oligo- and poly-saccharides to release β-glucose (Chiba, 1997). From Figure 4.18 (Lanes 4, 8 and 12), the disappearance of G_2-derivative spots and the increasing of G_1-derivatives and glucose spots indicated that glucoamylase hydrolyzed glycosidic linkage between glucose units of the G_2-derivatives. By contrast, the linkage between the alkyl chains and the first glucosyl unit could not be digested by glucoamylase. So, G_2-derivative spots that disappeared after treatment with glucoamylase were assumed to be alkyl glycosides with disaccharide units conjugated by the α-1,4-glycosidic bond. α-Glucosidase was then used to check the configuration of the alkyl glycoside products. α-Glucosidase (α-D-glucan glucohydrolase, E.C. 3.2.1.20) releases the α-D-glucose from the non-reducing terminal of both homogeneous and heterogeneous substrates (Chiba, 1997). From Figure 4.18 (Lanes 5, 9 and 13), all purified products were converted into glucose by the action of α-glucosidase. This result reflected that the first glycosyl unit linked to alkyl chain of all alkyl glycoside products was an α-configuration.

Using mass spectrometry, the positive ion ESI-mass spectrum showed the pseudomolecular ion peaks of three synthesized products from each alcohol acceptor which corresponded to the molecular masses of propyl-, pentyl- and isopentyl- glycosides having glucose, maltose and maltotriose attached to the alkyl groups of alcohols (Figures 4.20-4.22). Two main products of each alkyl glycoside were confirmed their structures by ^{1}H- and ^{13}C-NMR techniques (Tables 4.6-4.7). As a result, the products of $ProG_{1\ and\ 2}$, $PenG_{1\ and\ 2}$ and $IsoPenG_{1\ and\ 2}$ were alkyl-α-D-glucopyranoside and alkyl-α-D-maltopyranoside with propyl-, pentyl- and isopentyl- hydrophobic tails, respectively (Figure 4.37). To our knowledge, this study

was the first report on the use of MS and NMR analyses to solve the complete structures of these alkyl glycosides. These alkyl glycoside structures which were produced from recombinant pBC CGTase had the different number of glucosyl units, type of linkage and configuration as those previously reported. For example, the propyl- and pentyl- glucosides with only one glucose unit were formed in β-linkage by the action of Thai rosewood and almond meal β-glucosidases (Lirdprapamongkol & Svasti, 2000; Wang et al., 2012). Kim et al. (2009) and Bousquet, Willemot, Monsan, and Boures (1998) also reported the use of *L. mesentoroides* α-dextransucrase and *T. duponti* α-transglucosidase in the production of propyl- and pentyl- α-glycosides, respectively, but these products contained only one attached unit of glucose. From these reports, it was evidenced that the structure of alkyl glycosides as well as the number of glucosyl unit attached were dependent on type and source of enzyme. In this study, α-CGTase is specific for an α-1,4-linkage transfer, and the configuration of the catalyzed product from this enzyme was found to be an α-form (Nakamura, Haga, & Yamane, 1994).

5.4.2 Biological properties of alkyl glycosides

The properties of alkyl glycosides were investigated to evaluate its usefulness.

5.4.2.1 Emulsification property

The purified alkyl glycoside products were characterized for their emulsification properties using n-hexadecane as a substrate, compared to commercial non-ionic surfactants such as MG_1 and Triton^{TM} X-100. These properties were discussed in terms of emulsification activity and stability and previously used to judge the surfactant properties of several surfactants, such as methyl- and ethyl-glycosides (Kim et al., 2009), bio-surfactants produced by *Candida lipolytica*, *Klebsiella* sp. Y6-1 and *Yarrowia lipolytica* (Cirigliano & Carman, 1984; Lee et al., 2008; Souza, Salgueiro, & Albuquerque, 2012). As a result, the purified alkyl glycosides could form and stabilize an oil-in-water emulsion during the time tested. The higher emulsification activity and stability were found in alkyl-α-D-glycosides with linear hydrophobic structure and longer chains of alkyl and carbohydrate groups (Figures 4.38-4.39). One possible explanation for this result was the increase in the solubility of the alkyl glycosides in the oil and water phases by the long alkyl and

carbohydrate chains, respectively. Moreover, many previous studies suggested that the linear alkyl chain facilitated the formation of an emulsion. The branched structure in alkyl chains of both alkyl-α-glycoside and alkyl-β-glycoside was found to interrupt the packing of micelle molecules resulting in the decreasing of surfactant efficiency (Nilsson, Söderman, & Johansson, 1998; Boyd et al., 2001). von Rybinski (1996) and Nainggolan, Radiman, Hamzah, and Hashim (2009) reported that the surfactant efficiency of alkyl glycosides depended on the alkyl and carbohydrate chain lengths as well as the configuration of linkage. The longer chains of both alkyl and carbohydrate groups with α-configuration exhibited better surfactant efficiency. Furthermore, the alkyl chain length is the major influence on emulsification activity and stability compared to the number of glucose groups in the alkyl glycosides (von Rybinski & Hill, 1998). These experiments confirmed that the emulsification property related to the unique physicochemical structure of alkyl glycosides.

5.4.2.2 Antibacterial property

The antibacterial property of the synthesized alkyl glycosides was tested against human normal flora bacteria: Gram-positive *S. aureus* ATCC 25923 and Gram-negative *E. coli* ATCC 29522. The method of disc diffusion, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), was applied. From Tables 4.9-4.11, all alkyl glycosides had the moderate antibacterial property against both tested bacterial strains. This property might be the result of the interaction of alkyl glycosides with the phospholipids, which are formed as lipid bilayer structure in bacterial cell membrane (Figure 5.1) (Wenk, Alt, Seelig, & Seelig, 1997; Nobmann, Bourke, Dunne, & Henehan, 2010; Lichtenberg, Ahyayauch, & Goñi, 2013). The presence of the hydrophobic alkyl chains in the alkyl glycoside structure caused the adsorption of these molecules to cellular membrane of the bacteria due to the similar property between these chains and the alkyl chains of phospholipids in the bacterial lipid bilayer. Lipids were grouped together because they had the same hydrophobic property based on their molecular structure. So, alkyl glycosides become integrated with the phospholipid bilayer of the cell membrane. This integration resulted in a disruption of the cell membrane which led to the leakage of the cell components, followed by the death of the bacteria. The mechanism of solubilization of a lipid bilayer by alkyl glycosides was shown in Figure 5.2.

Moreover, as a result, the antibacterial efficiency of alkyl glycosides was the most effective for inhibiting *E. coli* growth. This special feature could be explained by the observation that the lipopolysaccharides and outer phospholipid membrane of gram-negative bacterial cell wall were directly disrupted by alkyl glycosides, while the thick peptidoglycan of gram-positive bacteria could prevent the alkyl glycosides to reach the cytoplasmic membrane (Figure 5.1).

In this study, the $PenG_2$ was found to be the best antibacterial agent which exhibited the highest inhibition zone and the lowest MIC and MBC values. This result revealed that the difference in antibacterial efficiency of alkyl-α-glycosides depends on the length of hydrophilic and hydrophobic chains as well as the structure of alkyl chain. The longer alkyl chain length of alkyl glycosides that have a greater hydrophobic effect results in more closely to interact with the plasma membrane. The linear alkyl structure of alkyl glycosides promotes the lipid solubility and cell membrane penetration. In addition, the increasing of carbohydrate groups enhances both water solubility and surface activity which lead to the good interaction between environmental components and cell membrane throughout depressing their selective permeability (Lindstedt, Allenmark, Thompson, & Edebo, 1990; Nilsson et al., 1998; Boyd et al., 2001; Sułek, Ogorzałek, Wasilewski, Klimaszewska, 2013). Based on the mechanism of penetrating microbial cell membrane, other alkyl glycosides such as butyl glycoside, dodecyl mannoside and methyl-6-O-dodecanyl glycoside were also reported that they showed the broad spectrum antimicrobial functionalities against gram-positive and gram-negative bacteria and fungi (Matin et al., 2013; Matsumura et al., 1990; Nobmann et al., 2010; Chaveriat, Gosselin, Machut, & Martin, 2013). The non-ionic surfactants with germanium metal structure increased the antibacterial activity and lipid membrane penetration by blocking of metal binding sites on the enzymes of the microorganisms (Zaki & Tawfik, 2014). By contrast, the antibiotics, ampicillin inhibited peptidoglycan synthesis in the cell wall of gram-positive bacteria, while tetracycline bound at 30S subunit ribosome and blocked the protein synthesis of microorganisms.

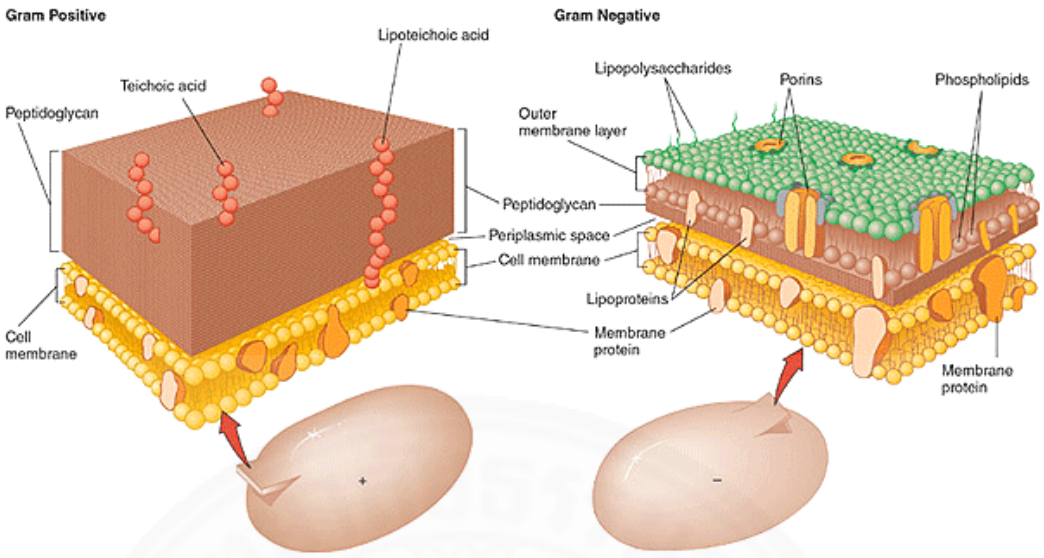


Figure 5.1 Structure of bacterial cell walls (copy from Zaki & Tawfik, 2014)

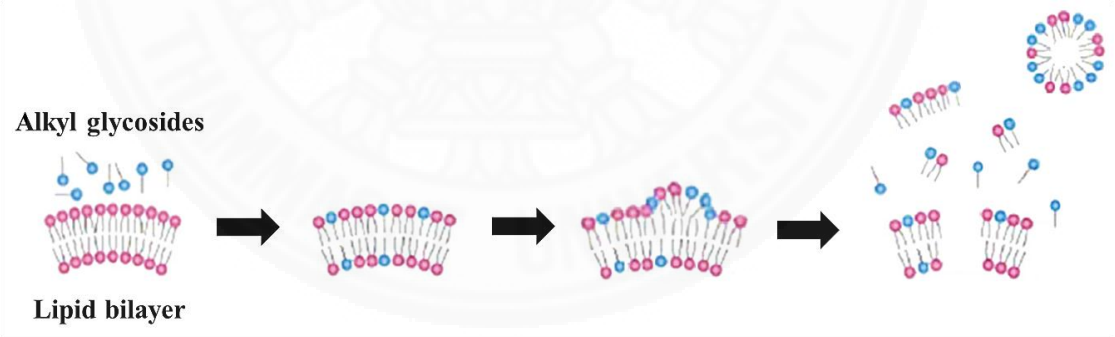


Figure 5.2 Mechanism of alkyl glycosides in the solubilization of lipid bilayer (copy and modified from Lichtenberg et al., 2013)

5.4.2.3 Insoluble proteins solubilizing property

In this experiment, the A406R-amylomaltase that had a high level of insoluble recombinant protein expression in a total cell lysate was used as a model. The ability of alkyl glycosides in the solubilization of insoluble proteins was demonstrated by the amount of soluble amylomaltase on SDS-PAGE and its specific transglycosylation activity. As a result, close to 100% of the total proteins was observed in a soluble fraction which was performed in lysis buffer with 1% (w/v) SDS (Figure 4.41, Lane 3). However, the specific activity of SDS-solubilized amylomaltase did not significantly increase (Table 4.12). These results indicated that SDS, a strong anionic surfactant, disrupts non-covalent bond of proteins and causes a denaturation of the native structure, coupled with a loss of biological activity of proteins. In contrast, the addition of 1% (w/v) $PenG_2$ gave the positive result of the amylomaltase solubilization with the activity of 1.01 U/mg (Figure 4.41, Lane 7 and Table 4.12). Moreover, the trend of the increasing $PenG_2$ concentration to increased amylomaltase solubility and activity was found to be concentration-dependent effect (Figure 4.42 and Table 4.12). These results indicated that $PenG_2$ could refold the aggregated amylomaltase to a bioactive soluble enzyme. There may be two possibilities to explain this application of $PenG_2$, either the disruption of insoluble protein structure or the assistance of protein folding. For the first possibility, $PenG_2$ was a non-ionic surfactant that inhibited protein-protein and protein-lipid bonding which were formed in the secondary structure of insoluble proteins, resulting in the increasing of protein solubility. Also, this alkyl glycoside had mild and non-denaturing-protein properties, it shows more ability to maintain the activity and stability of amylomaltase than SDS. Another possibility of $PenG_2$ application was the use as the preserving agent in maintaining enzyme activity and conformation by the addition in the lysis buffer. Recombinant proteins from different organisms which were expressed in *E. coli* might be in a partially folded form in the cytosol. When the cells were lysed in the buffer contained $PenG_2$, the alkyl chain of $PenG_2$ formed weak binding to the hydrophobic surface of the protein and acted as a chemical chaperone, leading to the correct folding of proteins. The proposed mechanism of PenG2 in the assistance of recombinant protein solubility was shown in Figure 5.3. For 1% (w/v) Triton^{TM} X-100, although it gave the highest recovery of

amylomaltase activity when compared to other surfactants at the same concentration (Figure 4.41, Lane 5 and Table 4.12), it had a low critical micelle concentration (CMC) value and high molecular weight. These properties made it difficult to remove entirely from enzyme solution (Varhač, Robinson, & Musatov, 2009). Many studies reported that the enzyme solution which contained this unremoved Triton™ X-100 had negative influences on structural analysis, enzyme purification and immobilization (Sánchez-Otero, Valerio-Alfaro, García-Galindo, & Oliart-Ros, 2008; Varhač et al., 2009).

Generally, several methods have been used to increase protein solubility or to diminish inclusion body formation. For example, fusion protein construction, cell-free expression, cold-shock system and removing of amino residues that affect solubility. These methods were complicated and spent more time and cost. Interestingly, the addition of $PenG_2$ during sonication method in this study was effective in refolding insoluble proteins avoiding to change gene constructions and expression systems. Furthermore, because the $PenG_2$ had the low molecular weight, it was removed easily from enzyme solution by either dialysis or gel filtration. In comparison with previous studies, there are a very large number of compounds have been used to solubilize protein. Parker and Song (1992) reported that many proteins and enzymes such as albumin, immunoglobulin, pepsin and alcohol dehydrogenase resisted SDS-denaturation and could be folded in the native secondary structure by interaction with SDS. Octyl glucoside, octyl mannoside, dodecyl maltoside and hexadecyl maltoside have been reported that they had an important roles in the formation, stabilization and refolding of many proteins (Kleinschmidt, Wiener, & Tamm, 1999; Stöckel, Döring, Malotka, Jähnig, & Dornmair, 1997; Jastrzebska et al., 2006). In addition to surfactants, Bandyopadhyay et al. (2012) showed that trehalose and trimethylamine N-oxide aided in the surface and core amino acid preventions of protein aggregation and the folding of mutated proteins. From these observations and the results in this study, they can be summarized by noting that the different proteins from various organisms required the specific agents to assist their folding, solubility and stability.

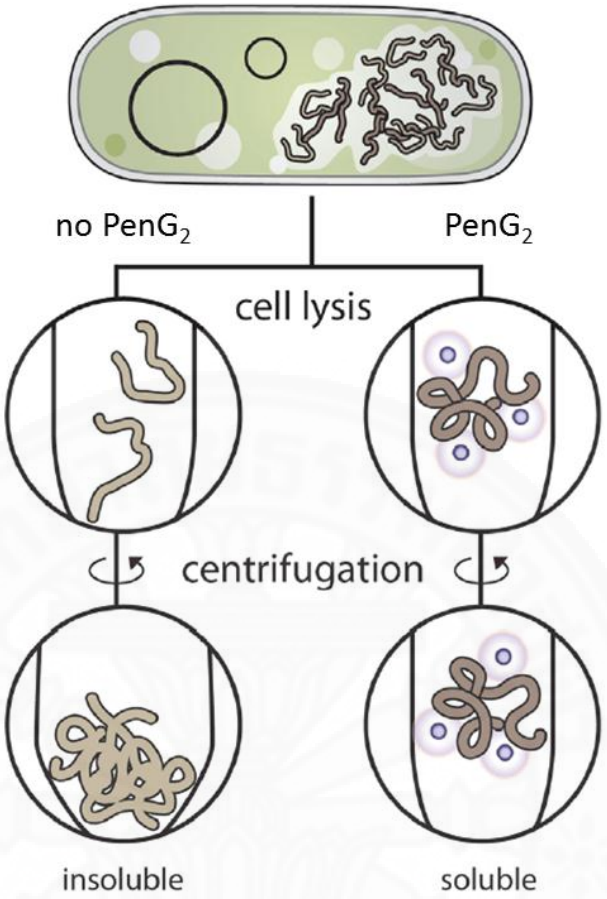


Figure 5.3 Proposed mechanism of $PenG_2$ in the assistance of recombinant protein solubility (copy from Leibly et al., 2012)

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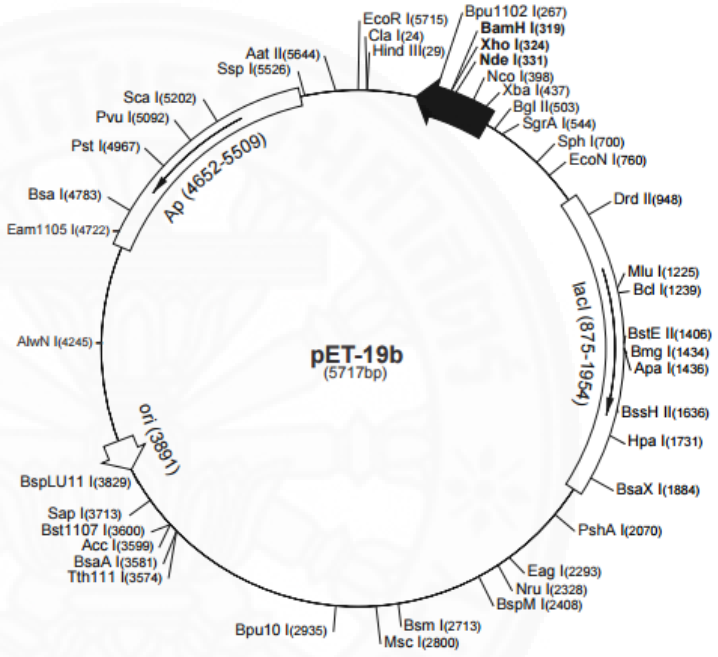
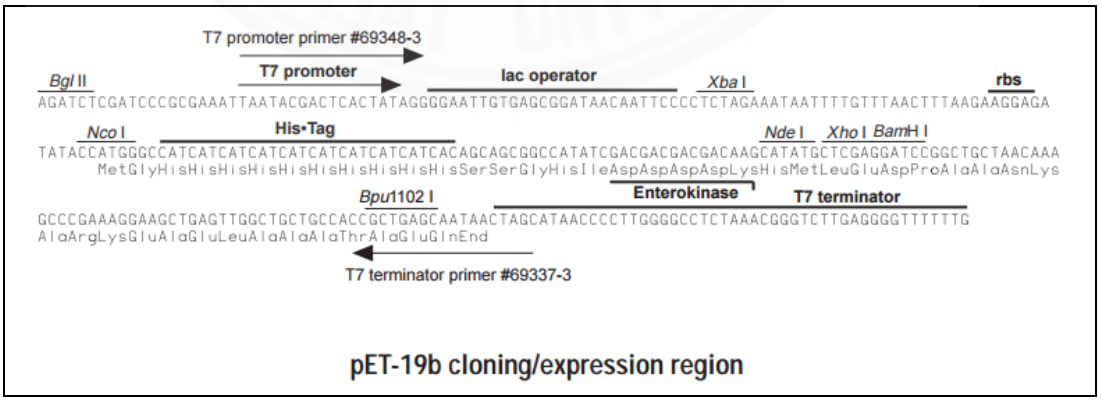
APPENDICES

APPENDIX A

RESTRICTION MAP OF PET-19B VECTOR

pET-19b sequence landmarks	
T7 promoter	472-488
T7 transcription start	471
His•Tag coding sequence	366-395
Multiple cloning sites	
(*Nde* I -*Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	875-1954
pBR322 origin	3891
bla coding sequence	4652-5509





pET-19b cloning/expression region

APPENDIX B
PREPARATION FOR CULTURE MEDIUM

LB broth

- Tryptone	1 g
- Yeast extract	0.5 g
- Sodium chloride (NaCl)	1 g

Added distilled water to 100 mL

LB agar

- Tryptone	1 g
- Yeast extract	0.5 g
- Sodium chloride (NaCl)	1 g
- Agar	1.5 g

Added distilled water to 100 mL

Mannitol salt agar

- Mannitol salt agar (Becton Dickinson & Company, U.S.A)	11.1 g

Added distilled water to 100 mL

APPENDIX C
PREPARATION FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

1. Stock reagents

2 M Tris-HCl, pH 8.8

- Tris base 24.2 g

Adjusted pH to 8.8 with 1 M HCl and added distilled water to 100 mL

1.5 M Tris-HCl, pH 8.8

- Tris base 18.17 g

Adjusted pH to 8.8 with 1 M HCl and added distilled water to 100 mL

1 M Tris-HCl, pH 6.8

- Tris base 12.1 g

Adjusted pH to 6.8 with 1 M HCl and added distilled water to 100 mL

0.5 M Tris-HCl, pH 6.8

- Tris base 6.06 g

Adjusted pH to 6.8 with 1 M HCl and added distilled water to 100 mL

10% (w/v) SDS

- Sodium dodecyl sulfate 10 g

Added distilled water to 100 mL

50% (v/v) Glycerol

- 100% Glycerol 50 mL

Added distilled water to 50 mL

1% (w/v) Bromophenol blue

- Bromophenol blue 0.1 g

Added distilled water to 10 mL and filtrated to remove the aggregated dye

10% (w/v) Ammonium persulfate

- Ammonium persulfate ($(NH_4)_2S_2O_8$) 0.5 g

Added distilled water to 5 mL

Solution A

- 30% Acrylamide/Bis solution, 29:1 (Bio-Rad, U.S.A.)

Solution B

- 2 M Tris-HCl, pH 8.8	75 mL
- 10% (w/v) SDS	4 mL
- Distilled water	21 mL

Solution C

- 1 M Tris-HCl, pH 6.8	50 mL
- 10% (w/v) SDS	4 mL
- Distilled water	46 mL

2. Working solutions (Native-PAGE)

7.5% Separating gel

- Solution A	1.41 mL
- 1.5 M Tris-HCl, pH 8.8	2.50 mL
- Distilled water	3.49 mL
- 10% (w/v) Ammonium persulfate	100 µL
- TEMED	10 µL

5.0% Stacking gel

- Solution A	0.32 mL
- 0.5 M Tris-HCl, pH 6.8	0.50 mL
- Distilled water	1.70 mL
- 10% (w/v) Ammonium persulfate	25 µL
- TEMED	3 µL

5x Sample buffer

- 1 M Tris-HCl, pH 6.8	3.1 mL
- 50% (v/v) Glycerol	5.0 mL
- 1% (w/v) Bromophenol blue	0.5 mL
- Distilled water	1.4 mL

10x Electrophoresis buffer

- Tris base	30 g
- Glycine	144 g

Added distilled water to 1 litre

3. Working solutions (SDS-PAGE)

10.0% Separating gel

- Solution A	2.50 mL
- Solution B	2.50 mL
- Distilled water	2.39 mL
- 10% (w/v) Ammonium persulfate	100 µL
- TEMED	10 µL

7.5% Seperating gel

- Solution A	1.41 mL
- Solution B	2.50 mL
- Distilled water	3.49 mL
- 10% (w/v) Ammonium persulfate	100 µL
- TEMED	10 µL

5.0% Stacking gel

- Solution A	0.32 mL
- Solution C	0.50 mL
- Distilled water	1.70 mL
- 10% (w/v) Ammonium persulfate	25 µL
- TEMED	3 µL

5x Sample buffer

- 1 M Tris-HCl, pH 6.8	0.6 mL
- 50% (v/v) Glycerol	5.0 mL
- 1% (w/v) Bromophenol blue	1.0 mL
- 2-Mercaptoethanol	0.5 mL
- 10% (w/v) SDS	2.0 mL
- Distelled water	0.9 mL

10x Electrophoresis buffer

- Tris base	30 g
- Glycine	144 g
- SDS	10 g

Added distilled water to 1 litre

APPENDIX D

PREPARATION FOR BUFFER AND SOLUTION

0.2 M Acetate buffer, pH 3.0-6.0

- Sodium acetate (CH_3COONa) 1.64 g

Adjusted pH to 3.0-6.0 by 0.2 M acetic acid and added distilled water to 100 mL

0.2 M Phosphate buffer, pH 5.0

- Potassium dihydrogen phosphate (KH_2PO_4) 1.35 g
- di-Potassium hydrogen phosphate (K_2HPO_4) 0.14 g

Added distilled water to 100 mL

0.2 M Phosphate buffer, pH 6.0

- Potassium dihydrogen phosphate (KH_2PO_4) 2.27 g
- di-Potassium hydrogen phosphate (K_2HPO_4) 0.58 g

Added distilled water to 100 mL

0.2 M Phosphate buffer, pH 7.0

- Potassium dihydrogen phosphate (KH_2PO_4) 0.91 g
- di-Potassium hydrogen phosphate (K_2HPO_4) 2.32 g

Added distilled water to 100 mL

0.2 M Tris-HCl buffer, pH 7.0-9.0

- Tris base 2.42 g

Adjusted pH to 7.0-9.0 by 1 M HCl and added distilled water to 100 mL

0.2 M Glyine-NaOH buffer, pH 9.0-11.0

- Glycine 1.50 g

Adjusted pH to 9.0-11.0 by 1 M NaOH and added distilled water to 100 mL

TB1 buffer (10 mM Tris-HCl, pH 8.5 with 10 mM $CaCl_2$)

- Tris base 1.21 g
- Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) 1.47 g

Adjusted pH to 8.5 by 1 M HCl and added distilled water to 1 litre

Iodine solution (stock solution) (0.2% (w/v) I_2 in 2.0% (w/v) KI)

- Iodine (I_2) 2.0 g
- Potassium iodide (KI) 0.2 g

Added distilled water to 100 mL

Iodine solution (working solution) (0.02% (w/v) I_2 in 0.2% (w/v) KI)

- Iodine solution (stock solution) 10 mL
- Distilled water 90 mL

Phenolphthalein solution (stock solution)

4 mM Phenolpthalein in absolute ethanol

- Phenolpthalein 127.3 mg

Added absolute ethanol to 100 mL

125 mM Sodium carbonate solution

- Sodium carbonate (Na_2CO_3) 1.33 g

Added distilled water to 100 mL

Phenolphthalein solution (working solution)

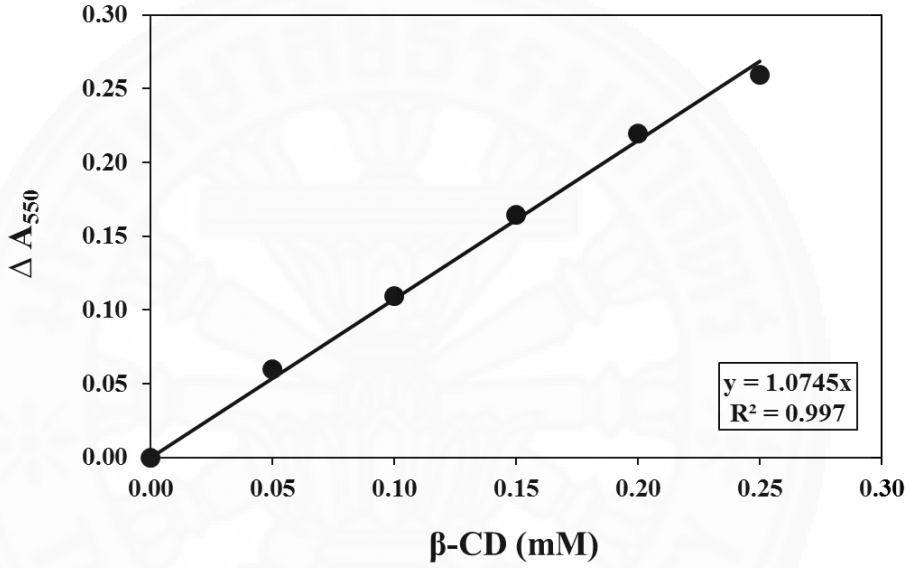
- 4 mM Phenolphthalein in absolute ethanol 1 mL
- 125 mM Sodium carbonate solution 100 mL
- Absolute ethanol 4 mL

Buffer for emulsification activity (0.5 mM Tris-HCl, pH 8.0 with 0.05 M $MgSO_4$)

- Magnesium sulfate ($MgSO_4$) 0.6 g
- 10 mM Tris-HCl buffer 5.0 mL

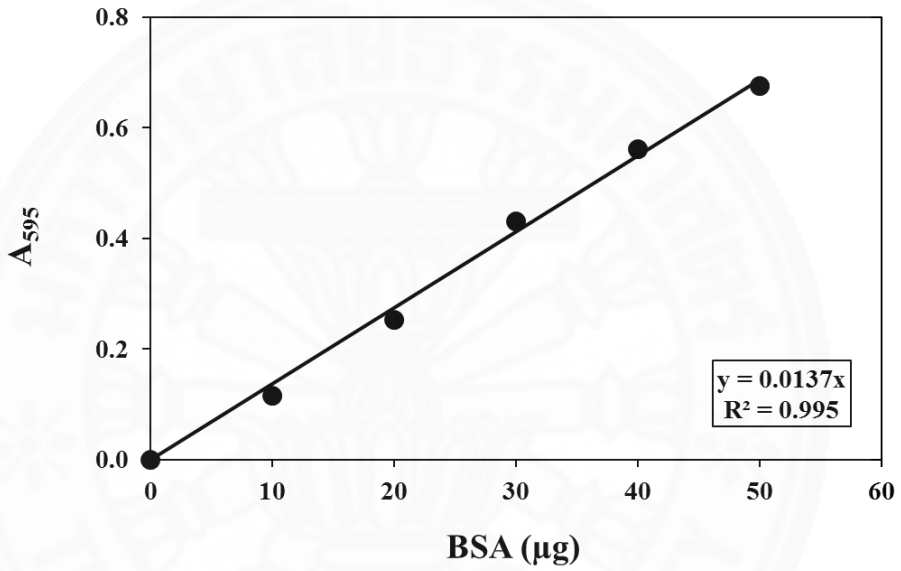
Adjusted pH to 8.0 by HCl and added distilled water to 100 mL

APPENDIX E
STANDARD CURVE FOR β-CD DETERMINATION BY PHENOLPHTHALEIN METHOD



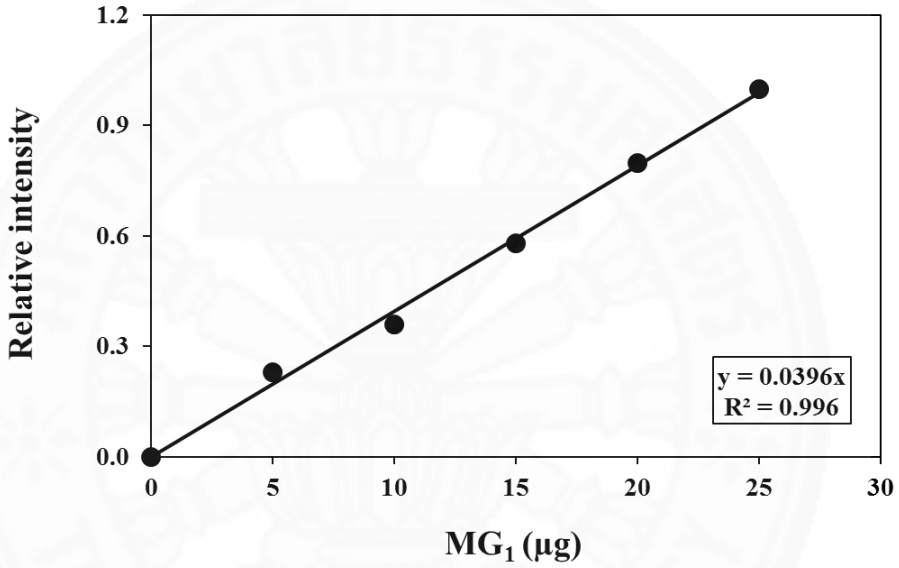
APPENDIX F

STANDARD CURVE FOR PROTEIN DETERMINATION BY BRADFORD'S METHOD



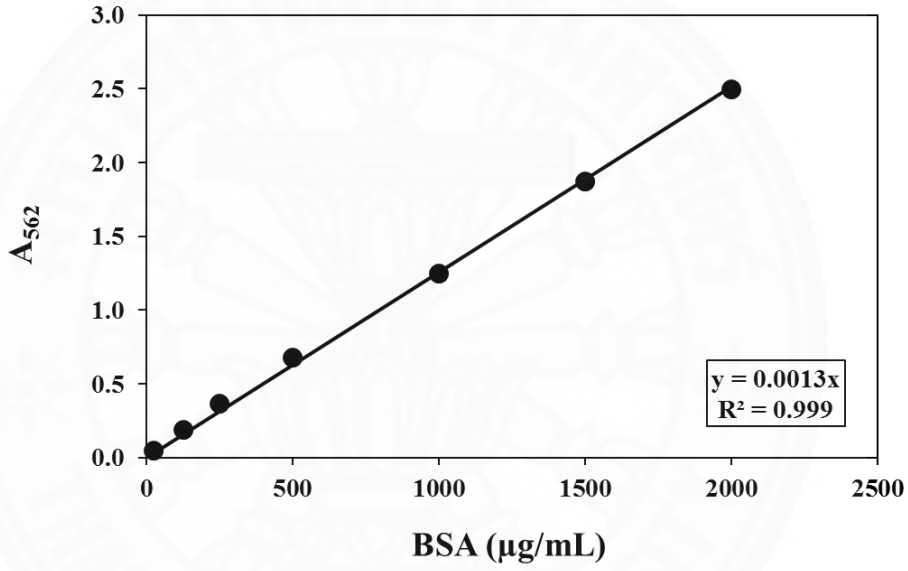
APPENDIX G

STANDARD CURVE FOR MG_1 CONCENTRATION BY DETERMINATION OF SPOT INTENSITY ON TLC PLATE



APPENDIX H

STANDARD CURVE FOR PROTEIN DETERMINATION BY PIERCE™ BCA PROTEIN ASSAY KIT



BIOGRAPHY

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Scholarship	2014: The Ph.D. Research Assistant Scholarship of Thammasat University

Publications

Charoensapyanan, R., Ito, K., Rudeekulthamrong, P., & Kaulpiboon, J. (2016). Enzymatic synthesis of propyl-α-glycosides and their application as emulsifying and antibacterial agents. *Biotechnol Bioprocess Eng 21*(3), 389-401.

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