ปฏิกิริยาแทรนส์ไกลโคซิเลชันของไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรส จาก *Paenibacillus* sp. RB01 สำหรับการสังเคราะห์แอลคิลไกลโคไซด์สายโซ่ขนาดกลาง

นางสาวเกษิณี เกตุเลขา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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TRANSGLYCOSYLATION REACTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01 FOR THE SYNTHESIS OF MEDIUM-CHAIN ALKYL GLYCOSIDES

Miss Kasinee Katelakha

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
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Thesis Title	TRANSGLYCOSYLATION REACTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01 FOR THE SYNTHESIS OF MEDIUM-CHAIN ALKYL GLYCOSIDES
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เกษิณี เกตุเลขา : ปฏิกิริยาแทรนส์ไกลโคซิเลชันของไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรส จาก *Paenibacillus* sp. RB01 สำหรับการสังเคราะห์แอลคิลไกลโคไซด์สายโซ่ขนาดกลาง . (TRANSGLYCOSYLATION REACTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01 FOR THE SYNTHESIS OF MEDIUM–CHAIN ALKYL GLYCOSIDES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. มัญชุมาส เพราะสุนทร , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, 150 หน้า.

แอลคิลไกลโคไซด์เป็นสารชะล้างชนิดไม่มีประจุซึ่งสามารถสังเคราะห์ได้โดยปฏิกิริยาการโยกย้ายหมู่ไกลโคซิลจาก บีตา– ไซโคลเดกซ์ทริน ไปยังแอลกอฮอล์ตัวรับ โดยใช้เอนไซม์ ไซโคลเดกซ์–ทรินไกลโคซิลแทรนส์เฟอเรสจาก *Paenibacillus* sp. RB01 เป็นตัวเร่ง ทำการศึกษาผลของแอลกอฮอล์ต่อเอนไซม์ด้วยวิธีฟีนอล์ฟทาลีน พบว่าแอลกอฮอล์ตัวรับมีผลต่อเอนไซม์ ปฏิกิริยาในระบบสองเฟสแม้ว่าจะมีผลต่อเอนไซม์น้อยกว่าแต่ให้ผลิตภัณฑ์ไกลโคไซด์รวมน้อยกว่า ในที่นี้ในกลุ่มของแอลกอฮอล์สายโซ่ขนาดกลาง พบว่าจะได้ผลิตภัณฑ์ไกลโคไซด์มากที่สุด เมื่อใช้ไอโซบิวทานอล เป็นตัวรับหมู่ไกลโคซิล ทั้งนี้พบว่าบีตา– ไซโคลเดกซ์ทรินเป็นตัวให้ที่ดีที่สุด ผล การบ่มผลิตภัณฑ์ที่สังเคราะห์ได้ด้วย เอนไซม์กลูโคแอมิเลส และ เอนไซม์แอลฟากลูโคซิเดส สามารถสรุปเบื้องต้นได้ว่า ผลิตภัณฑ์ที่ได้มีทั้ง isobutyl monoglucoside และ isobutyl (poly)glucoside ที่เชื่อมด้วยพันธะแอลฟา 1, 4 ภาวะที่เหมาะสมในการสังเคราะห์คือ การบ่มปฏิกิริยาโดยใช้ไอโซบิวทานอล 5% (v/v) กับ บีตา–ไซโคลเดกซ์ทริน เข้มข้น 1.2 % (w/v) ใน 0.5 โมลาร์ อะซิเตท บัฟเฟอร์ พีเอช 6.0 ด้วยเอนไซม์ 450 ยูนิต/มล. เป็นเวลา 48 ชั่วโมง โดยพบผลิตภัณฑ์หลัก 3 ชนิด ที่ Rt ~7, 8, และ 9 นาทีโดยวิธี HPLC หลังจากนั้นแยกผลิตภัณฑ์ด้วยวิธี PLC เพื่อวิเคราะห์น้ำหนักของผลิตภัณฑ์หลักสองชนิด พบว่าผลิตภัณฑ์ ที่ Rf = 0. 74 (Rt ~9 นาที) และ Rf 0.56 = (Rt~8 นาที) มีน้ำหนัก 259.1 และ 421.2 g/mol ตามลำดับ จึงสรุปได้ว่าผลิตภัณฑ์ดังกล่าวคือ isobutyl–α–monoglucoside และ isobutyl–α–polyglucoside ตามลำดับ ต่อมาเพิ่มปริมาณการผลิต และทำการแยกด้วย Amberlite IRA–900 และทดสอบสมบัติการเป็นสารชะล้างของผลิตภัณฑ์โดยใช้ n–hexadecane เป็นสารตั้งต้นในการเกิดอีมัลชัน พบว่า isobutyl–α–monoglucoside มีอิมัลซิฟิเคชัน แอกทิวิตี เท่ากับ 11.4 % ขณะที่ isobutyl–α–maltoside มีค่าเท่ากับ 41.1 % ของ triton X– 100 และผลิตภัณฑ์ทั้งสองชนิดมีค่า emulsification stability มากกว่า 80 %

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : MEDIUM-CHAIN ALCOHOL / ALKYL GLYCOSIDE / CYCLODEXTRIN GLYCOSYLTRANSFERASE / *Paenibacillus* sp. / TRANSGLYCOSYLATION
KASINEE KATELAKHA : TRANSGLYCOSYLATION REACTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01 FOR THE SYNTHESIS OF MEDIUM–CHAIN ALKYL GLYCOSIDES. ADVISOR : ASST. PROF. MANCHUMAS PROUSOONTORN, Ph.D., CO–ADVISOR : PROF. PIAMSOOK PONGSAWASDI, Ph.D., 150 pp.

Alkyl glycosides belong to the group of non–ionic surfactant. They were synthesized by transglycosylation reaction from β–cyclodextrin donor to alcohol acceptors using cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp. RB01 as a catalyst. The effect of alcohol acceptors on enzyme was determined by phenolphthalein method. The results showed that alcohol had effect on enzyme to some extent. The reaction performed as two phase system had lower influence to enzyme. However, the yield of transglycosylation products was also found to be lower. In the group of medium chain alcohol acceptor, isobutanol gave the highest yield while β–CD was reported as an appropriate donor. The results obtained after treatment the products with glucoamylase and α–glucosidase could be preliminary summarized that the products were both isobutyl monoglucoside and isobutyl (poly)glucoside with α 1, 4–linkage. The suitable condition was to incubate 5% (v/v) of isobutanol with 1.2 % (w/v) β–CD in 0.5 M acetate buffer pH 6.0 with enzyme 450 units/mL for 48 hours. The three main products were observed at Rt~7, 8 and 9 min by HPLC analysis. PLC was employed to isolate the expected products at indicated Rf values to confirm by mass relationship. The molecular weight of product at Rf = 0.74 (Rt~9 min) and Rf = 0.56 (Rt~8 min) were 259.1 and 421.2 g/mol, respectively. Thus, they were isobutyl–α–monoglucoside and isobutyl–α–maltoside, respectively. Reaction was then prepared in larger scale and purified by Amberlite IRA–900 column chromatography. The two purified products were checked for the emulsification properties using n–hexadecane forming–emulsion. The results revealed that isobutyl–α–monoglucoside had emulsification activity of 11.4% while isobutyl–α–maltoside had 41.1% of triton X–100. The emulsification stability of both products was more than 80 %.

Department : Biochemistry Student's Signature .....
Field of Study : Biochemistry and Molecular Biology Advisor's Signature .....
Academic Year : 2012 Co–advisor's Signature .....

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A	Absorbance
BSA	bovine serum albumin
CDs	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
cm	centimeter
°C	degree Celsius
ESI-TOF-MS	Electrospray Ionization-Time of Flight Mass Spectrometry
et al.	Et. Alii (latin), and others
g	gram
h	hour(s)
HPLC	High Performance Liquid Chromatography
kDa	kiloDalton
K_d	dissociation constant
L	Liter
µg	Microgram
µL	Microliter
µmole	Micromole
M	Molar
min	minute(s)
mA	Milliampere
mg	Milligram
mL	Milliliter
mM	Millimolar

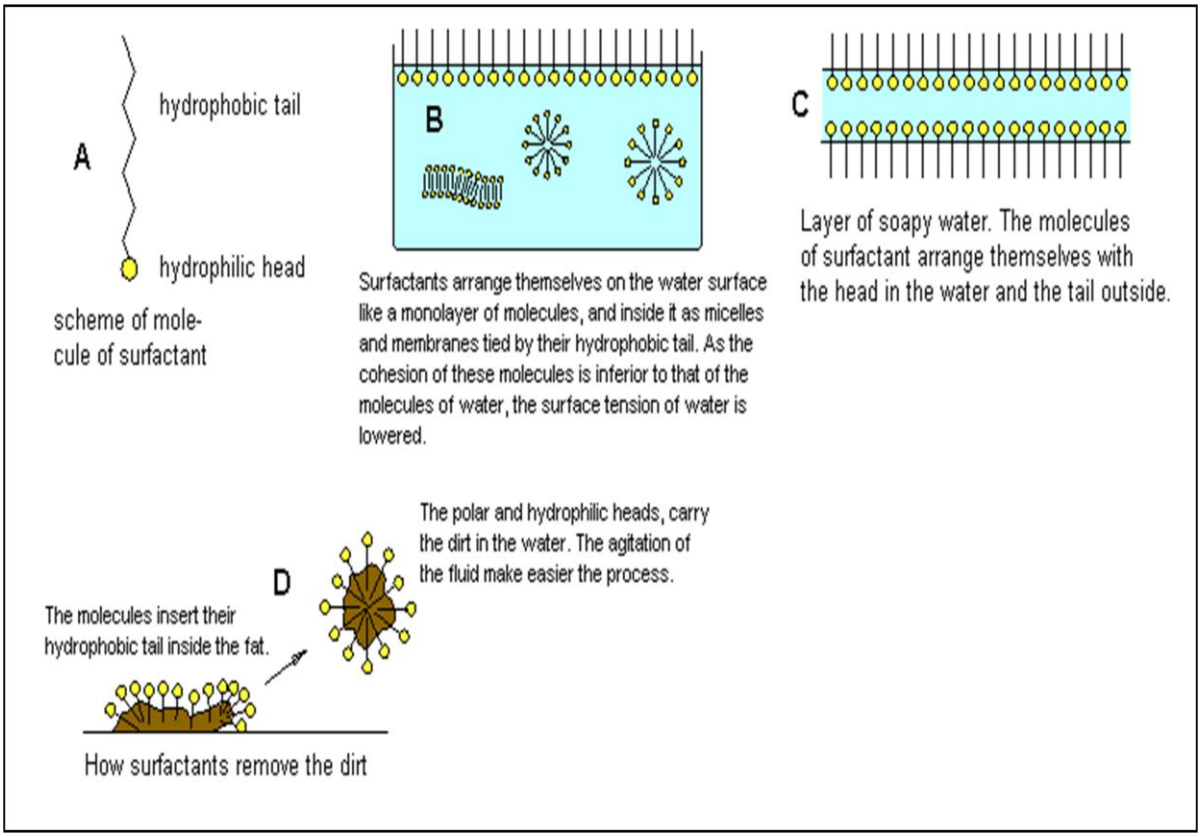
MS	Mass Spectrometry
PAGE	Polyacrylamide gel electrophoresis
PLC	Preparatrive thin layer chromatography
rpm	revolution per minute
Rf	Relative mobility
Rt	Relation time
SDS	Sodium dodecyl sulfate
TLC	Thin Layer Chromatography
U	Unit(s)
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Surfactants

Surfactants are compounds that lower the surface tension of a liquid, the interfacial tension between two liquids, or that between a liquid and a solid (Mishra et al., 1993). Their properties are known as emulsifiers which have been used in various applications including in household products (Fountain et al., 1991). Surfactant composed of hydrophilic and hydrophobic moieties, therefore they are well defined as amphipathic compounds (McCarty et al., 1994). Like other emulsifiers, hydrophobic moiety enclosed the contaminated lipid while hydrophilic moiety exposed to water molecule and form structure called micelle. These properties increase the solubility of sparingly soluble compound in water (Mall et al, 1996). Classification of surfactant was done by different methods and one of them is the determination of the net charge on the hydrophilic moiety of surfactant. Surfactants can be classified into 4 types which are anionic, cationic, non–ionic and amphipathic surfactants dependent on their net charge. Surfactant molecules in water are shown in Figure 1.



(source : http://www.funsci.com/fun3_en/exper2/exper2.htm)

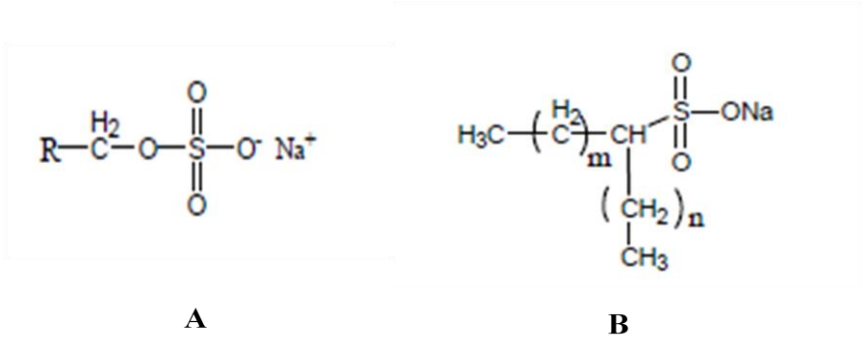
Figure 1 Surfactant molecules in water; **(A)** scheme of molecular structure of surfactant (which has a hydrophilic head and a hydrophobic tail), **(B)** surfactant forming monomolecular layer, bilayer and micelle, **(C)** formation of soapy membrane and **(D)** how surfactants remove the dirt.

1.1.1 Anionic surfactants

Anionic surfactants are one sub–class of surfactants. Applications of anionic and cationic surfactants differ from nonionic surfactants. Anionic surfactants are usually used in detergent industries (Zhang et al., 2009). They are inexpensive but sensitive to hard water containing calcium or magnesium due to the fact that they could be formed as salt of anionic surfactant with these divalent ions (Wanless and Ducker, 1997). Thus, their applications have been limited in hard water. However, incorporation with nonionic surfactants could improve its water hardness tolerant property (Homendra and Devi, 2004). Anionic surfactants had been found to cause facial skin tightness due to its lipid removal ability (Kawai and Imokaw, 1984). Sulfonates ($–SO^{-}_{3}$) and sulfates ($–O–SO^{-}_{3}$) are considered to be well–known for anionic surfactants. Anionic surfactants are able to form an ion pair with methylene blue (Jurado et al., 2006) and acridine orange (Adak et al., 2005). Thus, their properties can usually be determined by spectrophotometric method. The structures of anionic surfactants are shown in the Figure 2.

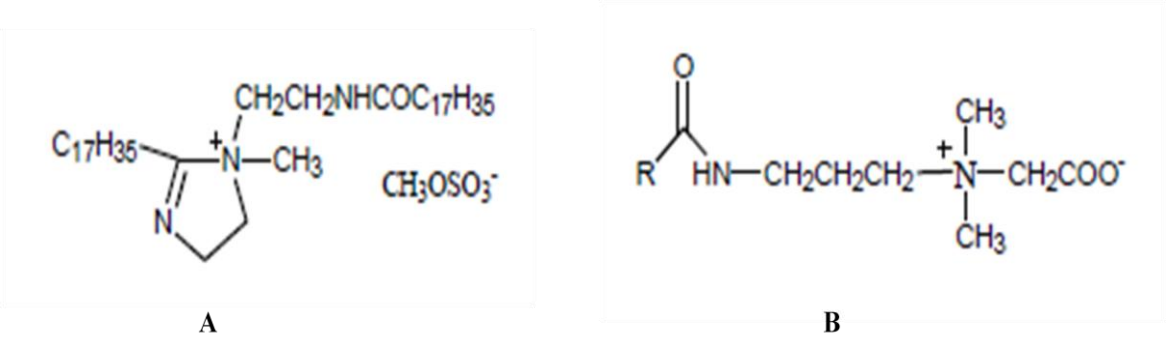
1.1.2 Cationic surfactants

Cationic surfactants could form strong adsorption layer with negative charge of natural colloid (Shukla and Tyagi, 2006). With the positive charge of hydrophilic moiety, cationic surfactants are extensively used as conditioning agents also in a wide variety of fabric, hair conditioner and softener (Giolando et al., 1995). They also showed good biodegradable which were less toxic for aquatic organism, thus they have been used as germicidal agents (Yamane et al., 2008). Most of the quaternary ammonium cationic surfactants have good degradable properties which related to hydrophobic group on their



A B

Figure 2 Anionic surfactants; (**A**) sulfonate surfactant and (**B**) sulfate surfactant (Matthew, 2008).



A B

Figure 3 Cationic surfactants; (**A**) quaternary ammonium surfactant and (**B**) alkyl betaines surfactant (Matthew, 2008).

molecular structure (Qin et al., 2005). The mixture of cationic and anionic surfactants leads to the formation of pseudo–nonionic surfactant which is formed by water insoluble complex between cation and anion in the solution. Forming of pseudo–

nonionic surfactant will reduce the CMC (critical micelle concentration) value to lower than those values obtained from either cationic or anionic surfactant (Borrego et al., 1999). CMC is temperature dependent (Noudeh et al., 2007). Long hydrophobic alkyl chain length on the compound provided the favorable properties, lower CMC and high foaming power (Badache et al., 2012). The structures of quaternary ammonium and alkyl betaines surfactants are shown in Figure 3.

1.1.3 Nonionic surfactants

Unlike, anionic and cationic surfactants, nonionic surfactant are usually known as sugar–surfactants. The structure is dependent on the combination of the hydrophilic and hydrophobic moiety which are derived from carbohydrate group and fatty acid, respectively (Boyat et al., 2000). Because of its sugar head group is natural compound thus, it is likely to be low toxic (Razafindralambo et al., 2012). Therefore, much interest has been led to use renewable raw materials in place of petrochemical substances as starting materials. Nonionic surfactants show broad applications in many industries and household use. They have low sensitivity to hard water and pH. This could be explained by the fact that they do not possess any electrical charge on their molecules (Holmberg et al., 2004). Moreover, they have efficiently been used as co–surfactant with anionic surfactant classes which have benefits to solubilize the magnesium or calcium salt of anionic surfactants. The driving force for the adsorption and CMC value was dependent on the alkyl chain length and was not affected by the type of the sugar head group (Varga et al., 2012). Ether carboxylic acid is one of nonionic surfactant that shows well wetting and foaming stability which could not be hydrolyzed by alkalis or acids. Therefore, they have high potential in acidic toilet

bowl cleaners and for mild cosmetic personal care products (Louis, 1999). The structures of non–ionic surfactants are shown in Figure 4.

1.1.4 Amphoteric (zwitterionic) surfactants

Amphoteric surfactants are a group of surfactants that carry both anionic and cationic charges on their molecules. Thus, they can function either as anionic, cationic, or non–ionic surfactant based upon the pH of solution. The positive charges are almost from the ammonium whereas the negative charges can be more variable (carboxylate, sulphate, sulphonate). Amphoteric surfactants may have been known as β–alanine, betaine, imidazoline or α–amino acid surfactants. Among this group, β–alanine derivative showed excellent active surfactant properties with mild effect to human skin and microorganism (Hidaka et al., 2003). Nevertheless, Vonlanthen et al. (2011) revealed the effect of betaine amphoteric surfactant (CAPB) on the marine organism *U. lactuca.* In addition, glycine–amphoteric surfactant exhibited good bactericidal efficiency to denture plaque (Hashiguchi et al., 2009). The example structure of amino acid (sodium glycinate) surfactants is shown in Figure 5.

1.2 Bio–surfactants

Biosurfactants are defined as surface–active compounds which are synthesized from microorganisms. Among the microorganism–producing surfactants, bacteria in the genus *Pseudomonas* were established to be the producer of the rhamnolipid–type

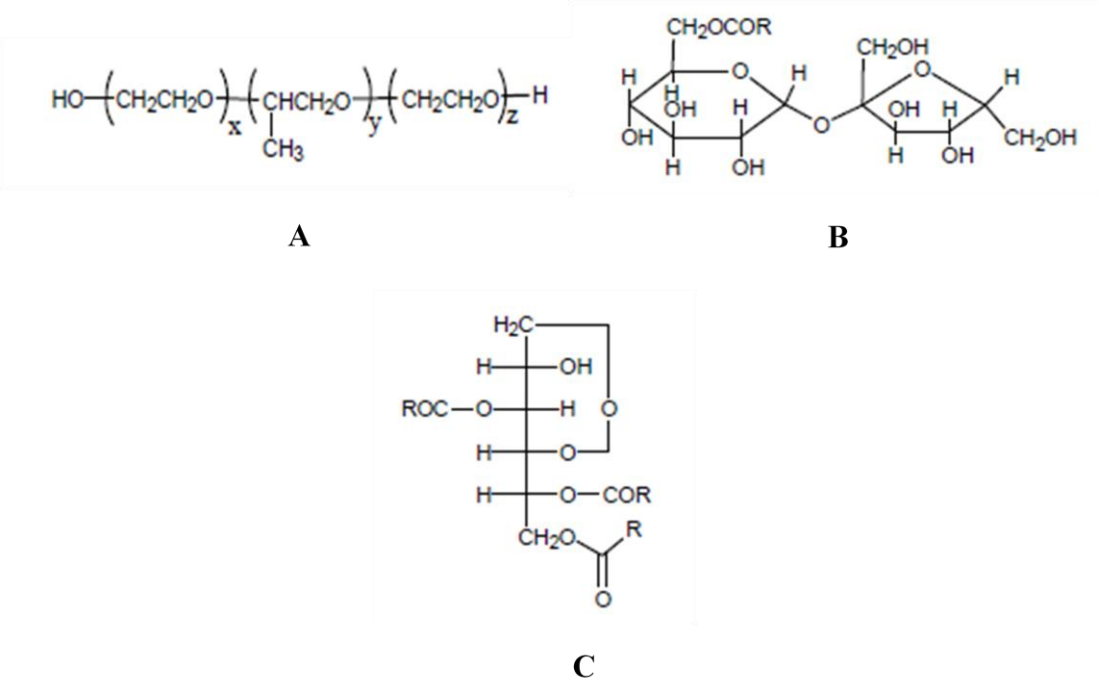


Figure 4 Non–ionic surfactants; (**A**) ethoxilate ether surfactant, (**B**) alkyl carbohydrate ester and (**C**) sorbitan trioester (Matthew, 2008).

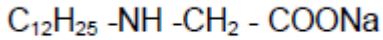


Figure 5 Amphoteric surfactants; (Matthew, 2008).

biosurfactants and glycolipid surfactants, which are known to be capable of using broad during cultivation. Reduction of the production cost can be achieved by finding alternative carbon and energy sources for the cultivation of microorganisms (Raza et al., 2007). In addition, *Klebsiella* sp. Y6–1 isolated from waste soybean oil gave crude bio–surfactant which could be extracted from culture supernatant. Also this extracted–bio surfactant showed better emulsification properties than chemical–synthesis surfactants (Lee et al., 2008). While, growing *Nocardia* sp.L–417 in the presence of n–hexadecane as substrates a carbon source, two surfactants were produced with different emulsifying properties (Kim et al., 2000). Moreover, the bio–surfactant could also be isolated from marine organisms, *Nocardiopsis* sp. B4 (Khopade et al., 2012). The list of microorganisms producing bio–surfactant is presented in the Table1.

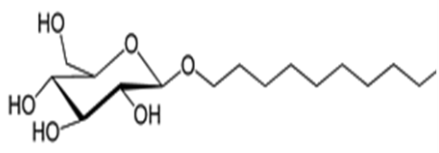
1.3 Alkyl glucosides (alkyl polyglycosides)

Alkyl glucosides or alkyl glycosides and alkyl polyglycosides belong to subclass of nonionic surfactants. Alkyl glycosides are the acetal products from the reaction of glucose source and long carbon chain primary alcohols or alcohol mixtures (Varvil et al., 2009). The hydrophilic carbohydrate derived moiety could ionize but no charge presented on their molecules, thus they are classified as non–ionic surfactant. The structures of alkyl glucoside and alkyl poly glycoside are shown in Figure 6. Since, they are prepared from naturally occurring and renewable resources, they are cheap and non–toxic. They have therefore, been extensively studied, especially, to reduce the production cost. Alkyl glycosides also have been shown to have good

Table 1 Bio–surfactant producing organisms (modified from Saharan et al., 2011).

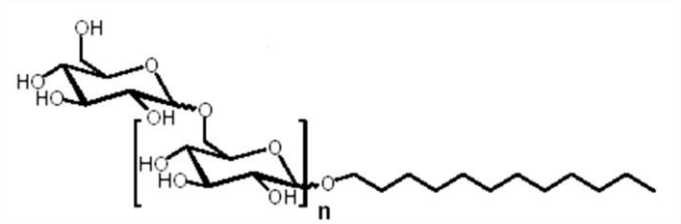
Biosurfactant	Microorganism(s)	Current economic importance
Cellobiose lipids	*Ustilago maydis*	Antifungal Compounds
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas chlororaphis,Serratia Rubidea*	Bioremediation, Antimicrobial and biocontrol properties
Carbohydrate–lipid	*P.fluorescens, Debaryomyces polmorphus*	Bio–emulsifiers
Whole cell	*Cyanobacteria*	Bio–flocculent
Diglycosyl diglycerides	*Lactobacillus fermentum*	Bio–remediation
Subtilisin	*B. subtilis*	Antimicrobial Properties
Aminoacids lipids	*Bacillus* sp.	Antimicrobial Properties

A



source:http://swiftcraftymonkey.blogspot.com/2010/04/surfactants-alkyl-polyglucosides-or.html

B



source: http://www.cyberlipid.org/glycolip/glyl0062.htm

Figure 6 Structure of alkyl glycosides; (**A**) alkyl (mono)glucoside and (**B**) alkyl (poly) glucoside.

surfactant properties where water solubility is high and they are also biodegradable. Therefore, it is well–known that alkyl glycosides are ecologically safe (Matsumura et al., 1990). The solubility is dependent on the content of hydrophilic to hydrophobic of their whole molecules. This group of surfactant is especially used in personal care cleansers and they are also considered to be mild to the skin (Savic et al., 2011).

1.4 The glycosylation reactions for the synthesis of alkyl glycosides

The glycosylation reactions for the synthesis of alkyl glycosides require special activation methods as shown in the Figure 7. The production procedures are divided into chemical procedures and enzyme or microbial procedures (von Rybinski and Hill, 1998).

1.4.1 Chemical procedures

1.4.1.1 Acid activation

The chemical glycosylation using acid–catalyzed reaction of glucoses with alcohols had been reported by Emil Fischer (1893). Scheme of fischer glycosylation (fischer glycosidation) for the synthesis of alkyl glycoside from hemiacetalic glucose is shown in Figure 8. The polyfunctionality of carbohydrates cause the formation of

randomly linked glucoside anomer. The product mixtures were 2:1 of α:β–anomers (Capon, 1969).

1.4.1.2 Base activation

The anomeric oxygen of sugars can also be activated by bases, which is known as anomeric–*O*–alkylation (Figure 9). Upon treatment a hemiacetalic sugar with a base, the generated anomeric oxide can be alkylated leading directly and irreversibly to a glycoside (Schmidt, 1986).

1.4.1.3 Activation by leaving group

Synthesis of alkyl glycosides by activation of halide leaving group was purposed by Koenigs and Knorr (1901). The Koenigs–Knorr reaction is generally used for the glycosylation of aliphatic alcohols (Wulff and Röhle, 1974). Figure 10 showed the scheme of the Koenigs–Knorr reaction for the synthesis of alkyl glycosides using glycosyl halide as a glycosyl donor.

In conclusions, the chemical approach for the synthesis of alkyl glycosides employed chemical catalysts to enhance rate of the reactions. In any case, large amount of catalyst must be used (50−100% (w/w) compared to the amount of oligosaccharide substrate). It is also taken by using high temperature to promote the reaction (Katsuraya et al., 1995). The limitation of this method is also the products obtained were mixture of anomers. The product selectivity can be improved by protection/deprotection of the reactive function groups using organic solvent in the presence of Lewis acid catalysts (Katsuraya et al., 1999) or using butyl glycoside

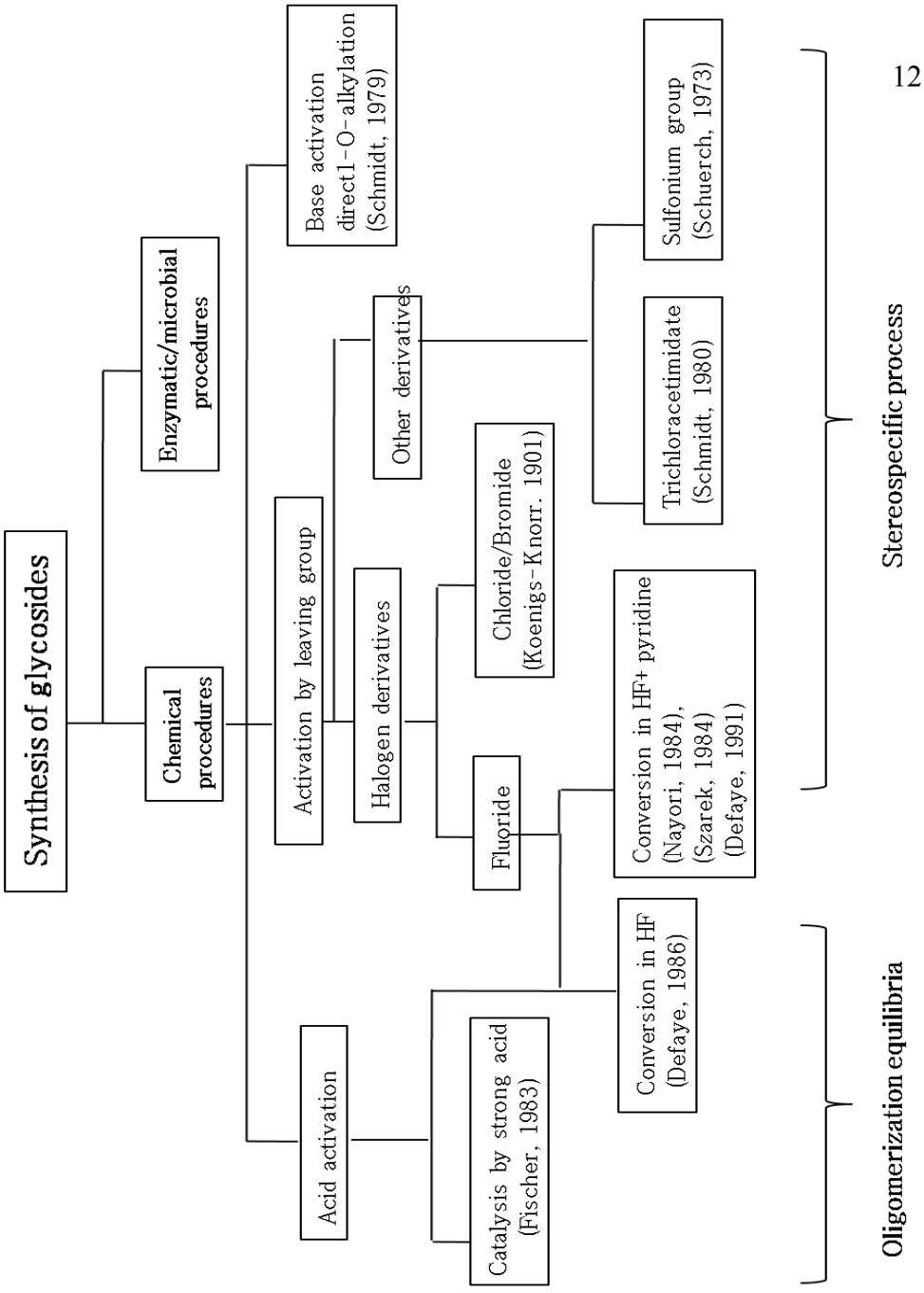
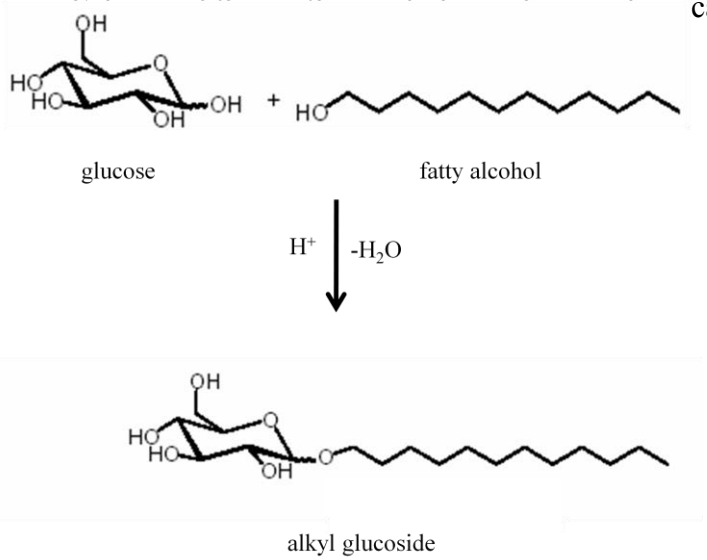


Figure 7 [illegible] cal and biological meth



source: http://www.cyberlipid.org/glycolip/glyl0062.htm

Figure 8 Fischer glycosylation for the synthesis of alkyl glycosides.

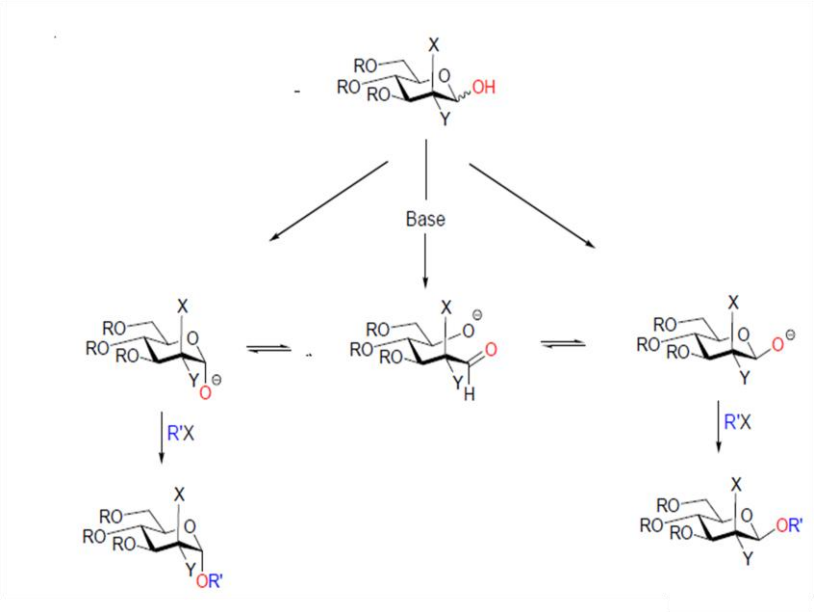


Figure 9 The anomeric–*O*–alkylation; R'; alkyl group, X; halide group (Schmidt, 1986).

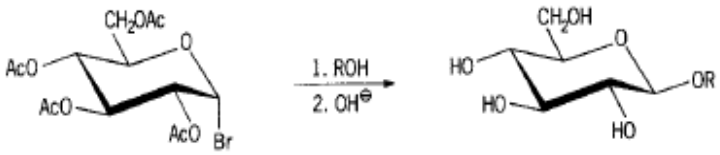


Figure 10 Synthesis of alkyl glycosides according to the Koenigs–Knorr reaction (Koenigs and Knorr, 1901).

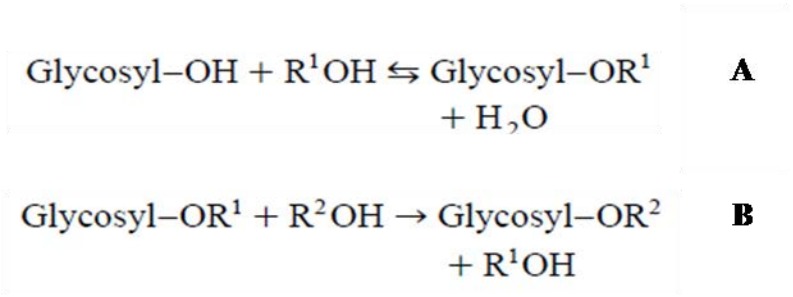


Figure 11 Scheme of β–glucosidases catalyze reactions for the synthesis of alkyl glycosides; (**A**) reverse hydrolysis and (**B**) transglycosylation reaction.

itself as starting material in the presence of Hbeta zeolite as catalyst at 120 °C (Camblor et al., 1997). Moreover, the product mixture needs many steps of purification.

In order to reduce waste generation, use of safer solvents, reduce energy consumption, the method is replaced by enzymatic procedures.

1.4.2 Enzymatic procedures

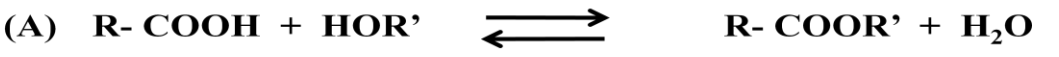
Enzymatic procedure for the synthesis of alkyl glycosides is an attractive method which has been developed to solve the environmental problems. This method is more advantageous over the chemical method because their conditions used are milder and also lower waste is produced.

Enzymes are well known as biological catalysts derived from living organisms. They catalyze their substrates to products by lowering the activation energy of the reactions similar with those of chemical catalysts. Thus, they can increase the rate of the reaction. One of the most important characteristics of enzymes that recognize them important and advantage over chemical catalysts is that they are selective and specific to their substrates. Therefore, alkyl glycoside products obtained from this method will have high selectivity. Although enzymatic method has been found to become an attractive synthesizing route for alkyl glycoside, it has been discovered only to a minor extent. Among the enzyme catalyst, glucosidase and lipase can usually be carried out for the production of alkyl glycosides.

a) β–glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of glycosidic bond under physiological condition. However, the alkyl glycosides synthesizing miscible solvent and thus under the kinetic control, the properties of enzyme has large influence on the yield of alkyl glycoside obtained (Ljunger et al., 1994). The synthesis of alkyl glycoside by β–glucosidase can also be taken by transglycosylation reaction. The scheme of the β–glucosidase catalyzed reaction is shown in Figure 11. Synthesizing by the glycosidase enzymes includes intermediate step of glycosyl–enzyme complex formation (Sinnott, 1990). Thus, the synthesis of alkyl glycosides is generally hampered by the low solubility of carbohydrates in organic media also the

yield is often lowered by the hydrolysis activity. In addition, glycosidases showed lack activity in organic media (Laroute and Willemot, 1992). To use β–glucosidase in non–aqueous media, several methods have thus been reported, for example, the synthesis under the system of controlled water activity (Vulfson et al., 1990) or the use of an immobilized enzyme (Ljunger et al., 1994). Moreover, production in the system of reverse Aerosol–OT micelles in octane (pseudohomogeneous media) to improve the contract area of enzyme and its hydrophobic substrate had been reported by Kouptsova et al., (2001).

b) Lipases (EC 3.1.1.3) catalyze ester synthesis or reverse hydrolysis reaction and tranesterification which occurs in the low water content condition or in organic solvent (Figure 12). The production yield from the reverse hydrolysis reaction is strongly affected by type of solvent, water content, solubility of substrates and stability of enzyme in organic solvent (Degn and Zimmermann 2001). Mutua and Akoh (1993) used lipase from *Candida* sp. to synthesize alkyl glycosides in organic media using methyl–α–D–glucopyranoside, methyl–β–D–glucopyranoside and octyl–β–D–glucopyranoside as starting materials.



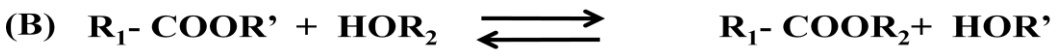


Figure 12 Lipases catalyzed reactions; (**A**) esterification or reverse hydrolysis and (**B**) tranesterification reaction.

1.5 Surfactant properties

Surfactants display general properties such as, CMC, emulsification and foaming properties. Moreover, Rhamnolipid biosurfactant from *P. aeruginosa* was reported previously that it had antiproliferative activity toward cancer cell line (Thanomsub et al., 2007).

1.5.1 Critical micelle concentration (CMC)

One of the most physical characteristic of surfactant is the critical micelles concentration. CMC is defined as the concentration of surfactants above which micelles are formed. From Figure 13, the amphiphilic structure of surfactants, the micelles formation was dependent upon the hydrophilic head group and long hydrophobic tail. CMC of nonionic surfactant is usually lower than that of ionic surfactant. This property is known as temperature dependence. Many methods have been employed for the determination of the CMC of surfactants by following the position of the breaking point in concentration dependencies on the physical or chemical properties of surfactants. CMC of ionic surfactant (anionic and cationic) is more conveniently measured by following the breaking point of the electro conductivity which is the function dependence on the surfactant concentration in the solution (Kallay et al., 1994). The simplified method for the determination of the CMC value of nonionic surfactant is to measure the maximum absorption spectrum changed by complexing with iodine (Chang et al., 1988).

1.5.2 Emulsification activity and emulsification stability

Emulsification consists of dispersing one immiscible fluid into another (typically, although not uniquely, oil and water) via creation and stabilization of an interface (Payet and Terentjev, 2008). Emulsification activity is the capability of surfactant to form emulsion with oil under the given conditions (Cheng et al., 2008). The turbidity of colloidal systems depends on the size and concentration of scatterers, and thus can be used to measure these parameters (Mandel et al., 1985). Turbidity measurements are rapid and simple to carry out and require relatively small quantities of sample. In addition, the light intensity can be measured using ultraviolet–visible (UV–vis) spectrophotometers, which are widely available in many research laboratories. Turbidity measurements may therefore offer a convenient method of monitoring solubilization of emulsion droplets by surfactant solutions (Julian and Dungan, 1995).

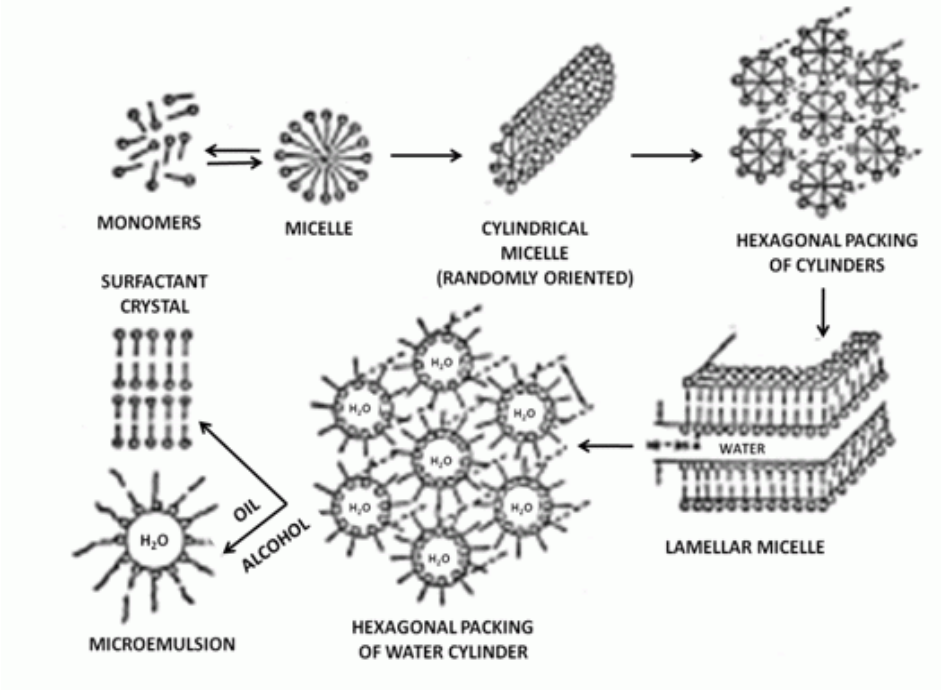


Figure 13 Illustrating the formation of various structures in surfactant solution with increasing surfactant concentration (Shah et al., 1977).

It has been reported that emulsions are not stable in thermodynamic stable sense due to the high free energy of the interface between the two phases (Pearce and Kinsella, 1978, and Cameron et al., 1991). The emulsification stability is to study how slowly separation of emulsion into oil and water phases (Cheng et al., 2008). Emulsion stability can be determined in term of height of cream layer as a percentage of the initial height of the emulsion by heated centrifugation (Yasumatsu et al., 1972). The emulsification stability was also expressed in term of disassociation constant obtained from following the turbidity of the emulsion (Cirigliano and Carman, 1985).

1.6 Cyclodextrin glycosyltransferase (CGTase)

1.6.1 General information of CGTase

Cyclodextrin glycosyltransferase or cyclodextrin glucanotransferase, (CGTase, E.C.2.1.4.19) is classified as a starch degrading enzyme. CGTase belongs to glycosyl hydrolase (GH 13) or α–amylase family (Henrissat, 1991). This family generally hydrolyzes the internal α 1→4 bonds between glucose residues of starch via α–retaining double displacement mechanism (Sinnott, 1990 and Uitdehaag et al., 2000). Scheme of the α–retaining mechanism is shown in Figure 14.

CGTase is ~75 kDa enzyme that consists of five domains, labeled A–E (Jespersen et al., 1991) (Figure 15). Domain A is the catalytic $(\alpha/\beta)_8$ or TIM barrel domain which CGTase has in common with other α–amylase family members (Janecek et al., 1997). Domain B is the substrate binding domain. A β–sheet structure which is specialized in binding to raw starch granules is labeled as domain C and E (Penninga et al.1996). The function of domain D remains unknown (Kelly et al., 2009). CGTase is an extracellular enzyme and the bacteria producing CGTase subsequently use these cyclodextrins as a carbon source for growth (Bender, 1993). CGTase is widely distributed in bacteria commonly in *Bacilli* genus (Nogrady et al., 1995) including *Bacillus macerans* (Lane and Pirt, 1971). *Bacillus circulans* (Maréchal et al., 1976) and *Thermoanaerobacter* sp. P4 (Avci and Dönmez, 2012). However, CGTase has also been successfully extracted from *Thermococcus*–a single archaeon (Tachibana et al., 1999).

1.6.2 Mechanism of CGTase

CGTase catalyzes four different transglycosylation reactions: cyclization, disproportionation, coupling and hydrolysis reaction. CGTase binds its starch substrate across multiple sugar binding subsites (labeled–7 to +2) (Strokopytov et al., 1996). Cyclization reaction is an intramolecular transglycosylation reaction used to produce cyclic oligosaccharide in which CGTase cleaves an α–(1, 4) linkage of starch chain and concomitantly linking the non–reducing and reducing end (Figure 16 A). This cyclic oligosaccharide is known as cyclodextrin (CDs). Disproportionation reaction is a major reaction in which the linear malto–oligosaccharide is cleaved and transferred to another linear acceptor substrate (Figure 16 B). CGTase also catalyzes an opening of CDs ring and transfer the linear oligosaccharide obtained to the acceptor substrates (coupling reaction) (Figure 16 C). When water is used as an acceptor instead of carbohydrate acceptor, the result is the hydrolysis of amylase or linearization of CDs (Figure 16 D) (van der Veen et al., 2000).

CDs produced from CGTase consisted of glucose varied from 6–8 units namely α, β and γ CD, respectively (Figure 17 A). They are linked by α 1→4 glycosidic bonds. CDs are a doughnut–shaped structure with hydrophilic outer surface from the steric positioning of hydroxyl groups and hydrophobic cavity which capable to form an inclusion complex with a wide variety of guest molecules, solid, liquid, or gaseous molecules (Figure 17 B) (Villiers, 1891). The central cavity of CDs is lined by the skeletal carbons and ethereal oxygens of the glucose residues which gives a lipophilic character (Figure 17 C) (Loftsson et al., 2005). The outstanding characteristic of CDs is their capability to form solid inclusion complex with wide variety of solid, liquid or gaseous compounds by molecular complexation (Villiers, 1891). This can lead to the change of the chemical or physiological properties of guest

molecules. In addition, CDs and their derivatives are applicable to be used in analytical chemistry, agriculture, pharmaceutical field, and food industries (Singh et al., 2002). The important characteristics of CDs are summarized in Table 2.

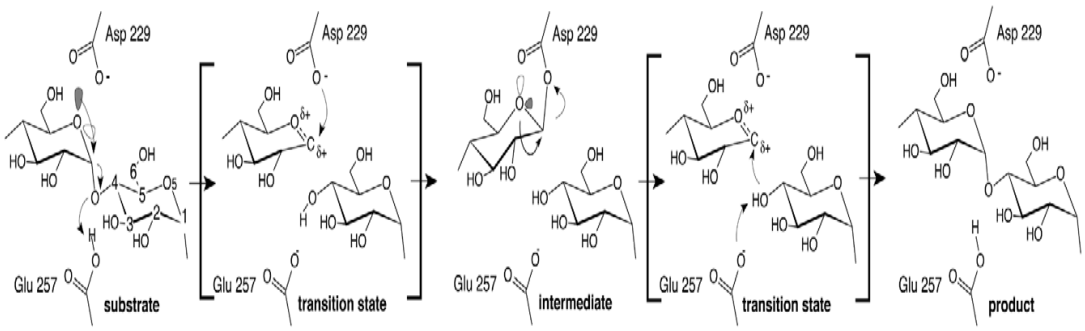


Figure 14 Scheme of the α–retaining mechanism as used by the α–amylase family (Uitdehaag et al., 2002).

a.

b.

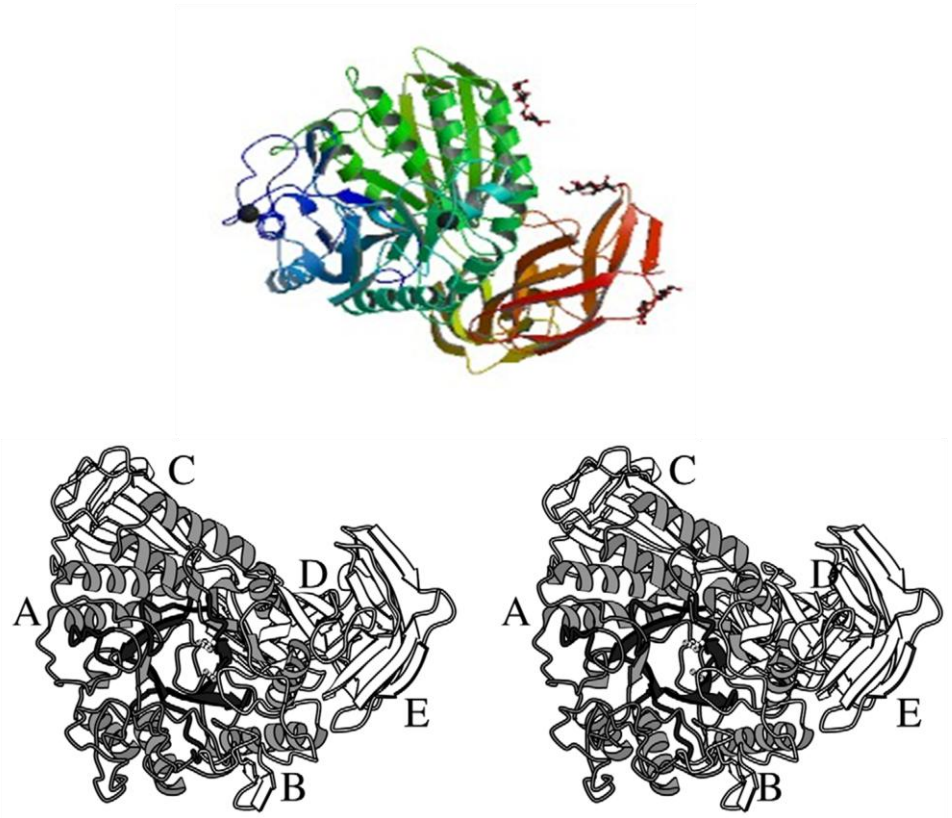


Figure 15 (**a)** X–ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose–dependent crystal form (Lawson et al., 1994).

(b) Stereo–view of the structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose–dependent crystal form (Uitdehaag et al., 2002).

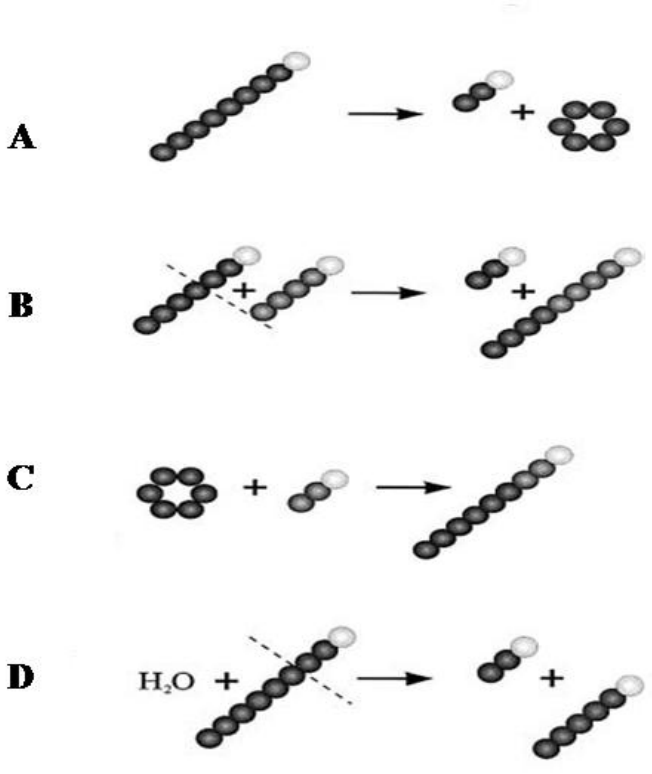


Figure 16 Schematic representation of the four reactions catalyzed by CGTase ; (**A**) hydrolysis reaction, **(B)** dispropertionation activity **(C**) cyclization activity and **(D)** coupling activity (van der Veen et al., 2000).

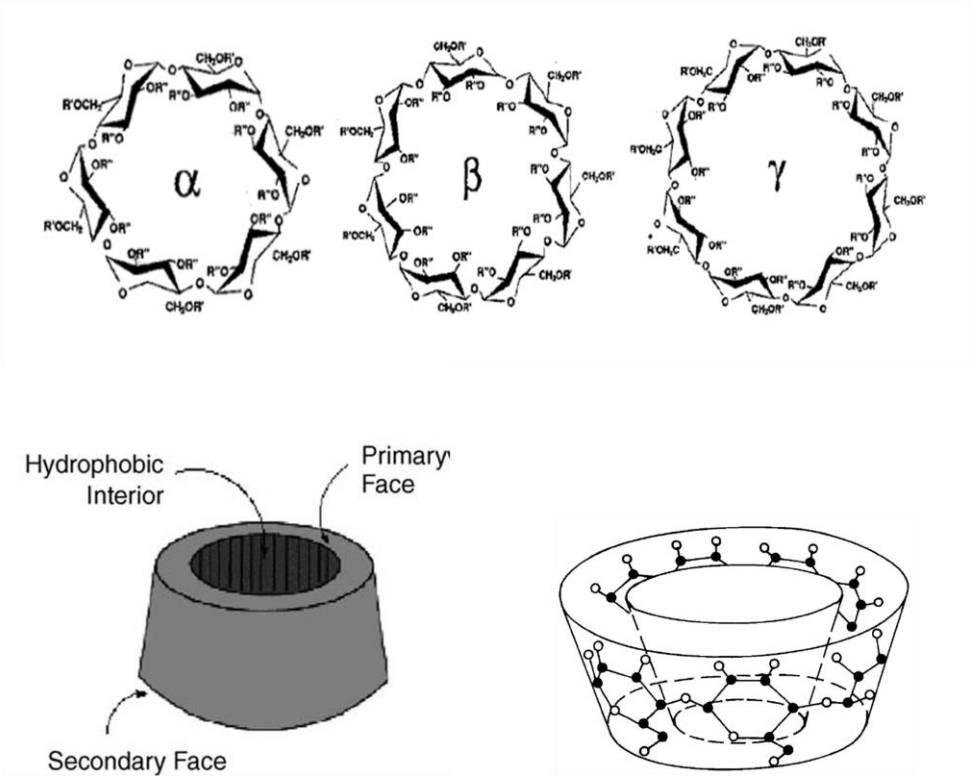


Figure 17 Chemical structure of cyclodextrins;

(**A**) α, β, and γ–cyclodextrin consisting of 6, 7 or 8 glucose units (Tachibana, 1999).

(**B**) Schematic drawing of the cyclodextrin cylinder (Del Valle, 2003).

(**C**) The conical shape of the β–cyclodextrin molecule (Loftsson et al., 2005).

Properties	α–CD	β–CD	γ–CD

Table 2 Characteristics of α–, β–, and γ–CDs (Del Valle, 2004).

Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25◦C (%, w/v)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7–5.3	6.0–6.5	7.5–8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	174	262	427

1.6.3 Transglycosylation activity of CGTase

Like other enzymes in α–amylase family, using intermolecular–transglycosylation reaction, this enzyme was applied for the synthesis of the new

glycoside products with new physical or chemical properties. The transglycosylation activity of CGTase is utilized for industrial application in saccharide or glycoside products such as the synthesis of maltooligosyl–fructose (coupling sugar). Kitahata (2000) successfully synthesized coupling sugar using intermolecular transglycosylation activity of CGTase from the mixture of starch hydrolyzates and sucrose as an acceptor. CGTase immobilized on Eupergit C was able to use starch as a donor and sucrose as an acceptor for the synthesis of maltooligosyl–fructose, resulting in an excellent yield of 92% (Martín et al., 2004). In addition, the industrial processes for the synthesis of coupling sugar using CGTase from *Bacillus macerans* catalyzed reaction have been patented (Petersin, 1992). The sweetness of coupling sugar is ~50 of that of sucrose with low cariogenicity property. This is widely employed in the manufacture of chocolates and cookies (Ikeda, 2007).

Using disproportionation activity of a highly thermostable CGTase from *Thermoanaerobacter* sp, the malto–oligosaccharide (MOS) was produced from starch donor substrate and glucose as an acceptor (Martín et al., 2000). MOS are usually produced from commercially debranching enzymes such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) combined with hydrolysis by various α–amylases. This product constitutes an important class of oligosaccharides in food industry (Crittenden and Playne, 1996).

There are many groups of substances which are able to be used as a nucleophilic acceptor for CGTase to produce the useful glucosides. Examples are as follows:

a) Suzuki and Suzuki (1991) synthesized 4^{G}–α–D–glucopyranosyl rutin by transglycosylation with CGTase from *stearothermophilus* using

dextrin as a glycosyl donor in 50% methanol. The solubility of synthesized product in water was much higher than that of rutin.

b) Prousoontorn and Pantatan (2007) immobilized CGTase from *Paenibacillus* sp. A11 on alumina. The immobilized enzyme was further used in a batch production of 2–O–α–glucopyranosyl–L–ascorbic acid from ascorbic acid and β–cyclodextrin substrate.

c) Shimoda et al. (2008) produced oligosaccharides of genistein and quercetin. Genistein and quercetin were glycosylated by cultured with cells yielding the corresponding glucosides derivatives. Then these glucosides were further glycosylated to the corresponding β–maltosides and β–maltotriosides by CGTase from *Bacillus macerans.* The glucoside products showed potent inhibitory effects on histamine released from rat peritoneal mast cells.

d) Aramsangtienchai et al. (2011) synthesized (−)−epicatechin glucosides using (−)–epicatechin (EC) as an acceptor and β–CD donor through the transglycosylation reaction catalyzed by CGTase from *Paenibacillus* sp. RB01. The water solubility and stability against UV irradiation of transglycosylated products were significantly higher than that of EC.

From the literatures, it has recently been concluded that different natures of substrates can be used as acceptors for transglycosylation reaction catalyzed by CGTase. Therefore, to synthesize alkyl glycosides with the prospective of an alternative surfactant, CGTase seems to be a very interesting enzyme. The maltodextrin glycosides were synthesized from methyl–α, methyl–β, phenyl–α and phenyl–β–D–glucoside as acceptor and α–CD as donor with the action of CGTase

from *Bacillus macerans* (Yoon and Robyt, 2006). To achieve longer chain alky glycosides, Svensson and co–workers (2009) used dodecyl–β–D–maltooctaoside (DDM) as an acceptor and α–CD as a donor to yield dodecyl–β–D–maltoside (DDMO) by activity of CGTase from *Bacillus macerans* (Figure 18). In 2011, Chotipanang and co–workers successfully used CGTase from *Paenibacillus* sp. RB01 for the production of alkyl glycosides from several alcohol acceptors (methanol, ethanol, propanol and butanol) and β–CD donor.

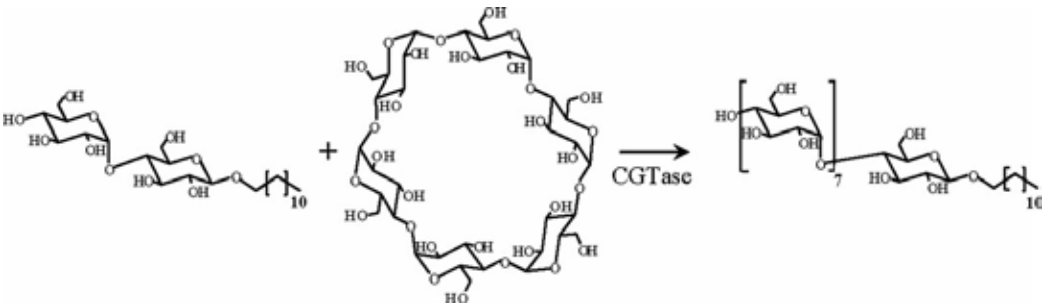


Figure 18 CGTase catalyzed coupling reaction between α–CD and DDM synthesizing DDMO (Svensson et al., 2009).

The starch and cyclodextrin research unit at the Department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on CGTase isolated from thermotolerant *Paenibacillus* sp. RB01. This strain had previously been isolated from hot spring soil area in Rachaburi, Thailand. CGTase from this strain was first partially purified and characterized by Tesana (2001). In 2002, this enzyme was completely purified and characterized by Yenpech (Yenpech, 2002). The optimum temperature and pH were at 40° C and at 7–9 for dextrinizing activity (Yenpech,

2002). CGTase from this stain had previously been used to catalyze transglycosylation reaction to various natures of acceptor (Aramsangtienchai et al., 2011, Thanadolsatein et al., 2010). However in 2011 Chotipanang et al (2011) has successfully been reported that CGTase was able to catalyze the synthesis of alkyl glycoside using short chain alcohols as an acceptor. Due to the fact that alcohol had an effect on the stability and enzyme activity, the production of alkyl glycoside were thus, usually short chain alkyl glycoside. For more potential use in the surfactant applications, the requirement was a search for longer chain alkyl glycosides. The development of using CGTase in the synthesis of beneficial alkyl glycoside from activated donor to alcohol acceptor has recently drawn much attention of the researchers. Therefore, the aim of this research was to synthesize medium chain alkyl glycosides using CGTase catalyzes the transfer of glucosyl unit from donor to alcohols. The effect of alcohols on the coupling activity of enzyme was also determined. Finally, the large scale production of the glycoside products was performed so as to apply this method for industrial purpose. Since alkyl glycosides could be applied in surfactant industry as nonionic surfactant which exhibits antimicrobial activity (Matsumura et al., 1990). Longer alkyl glycosides chain showed that they can be used in membrane solubilization (Rosevear et al., 1980), and crystallization of membrane proteins (Priví, 2007). Thus, the longer chain of alkyl glycosides synthesized by CGTase can also be further used as emulsifier. It has been reported that CGTase was able to synthesize oligosaccharide conjugated to corresponding acceptors, therefore, a novel glycosylated products from CGTase was alkyl (poly) glycosides from low cost substrate. This, however cannot be obtained from α–or β–glucosidases.

The objectives of this research were:

I) To study the transglycosylation ability of *Paenibacillus* sp.RB01 CGTase from the glycosyl donor to medium chain alcohols.

II) To determine the effect of alcohol acceptors on the coupling activity of enzyme.

III) To determine the optimal condition for the production of medium chain alkyl glycoside.

IV) To purify and evaluate its use by testing the emulsification activity and emulsification stability.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: MLS–3020, Sanyo electric Co., Ltd., Japan

Autopipette: Nichipet EX, Nichiryo, Japan

Balance: AB204–S, Mettler Toledo, Switzerland

Balance: PB303–S, Mettler Toledo, Switzerland

Cellulose tubular membrane (MWCO 12,000–14,000): CelluSep T4, Membrane Filtration Products, Inc., USA

Centrifuge, refrigerated: Avanti™ J–30I, Beckman Instrument Inc., USA

Centrivap: Centrivap Concentrator, Labconco Corporation, USA

Electrophoresis unit: Mini–PROTEAN® 3 Electrophoresis, Bio–Rad, USA

Filter paper (125ID No.1): Whatman®, England

Bruker 100 MHz spectrometer, Bruker Daltonics Inc., USA (^{13}C NMR)

Fraction collector: Model Frac–920, Amersham Biosciences, Sweden

Freeze–dryer: Labconco corporation, USA

Gel document: Syngene, England

High Performance Liquid Chromatography unit: Shimadzu, Japan

- Auto injector: SIL–10AD
- Column oven: CTO–10AD
- Degasser: DGU–14A
- Pump: LC–10AD

Refractive index detector: RID–10A

System controller: SCL–10A

Incubator: Gallenkamp, England

Incubator shaker: Gallenkamp, England

Laminar flow: Bio Clean Bench, Sanyo, Japan

Magnetic stirrer and heater: Model 512P–2, Barnstead/ Thermolyne Corporation, USA

Mass spectrometer model Micro TOF, Bruker Daltonics, Germany.

Membrane filter, ultrafiltration: Vivaflow 50 with 10,000 MWCO (polyethersulfone), Generon Ltd., England

Membrane filter: Nylon, pore size 0.45 μm, National Scientific Company, USA

Microwave oven: Edition–I, Daewoo, Korea

Oven: Contherm, New Zealand

Peristaltic pump: Pump P–1, Pharmacia Biotech, Sweden

pH meter: SevenEasy, Mettler Toledo, Switzerland

PLC plates: Silica gel 60 F_{254} (1 mm), Merck, Germany

Power supply: PowerPac Basic™, Bio–Rad, USA

TLC plates: Silica gel 60 F_{254}, Merck, Germany

UV–VIS Spectrophotometer: DU®650 Spectrophotometer, Backman Instrument Inc., USA

VIS Spectrophotometer: 6400 Spectrophotometer Jenway, LABQUIP, England

Vortex: Model K–550–GE, Scientific Industries, USA

Water bath: Memmert, Germany

YMC HPLC column: YMC co., Ltd, Japan

2.2 Chemicals

40% w/v Acrylamide monomer solution containing 5% (w/v) N,N'–methylenebis–acrylamide: Amersham Biosciences, Sweden

Agar: Scharlau, Spain

α–Glucosidase (maltase) from *Saccharomyces cerevisiae* 5.8 U/mg: Fluka, Switzerland

Amberlite® IRA–900 (Chloride form), Aldrich Chemistry, USA

25% Ammonia solution: BDH, England

Ammonium persulfate: USB Coporation, USA

Amyloglucosidase (glucoamylase) from *Aspergillus niger* 73.8 U/mg: Fluka, Switzerland

Beef extract: Biomark Laboratories, India

β–Cyclodextrin: Sigma, USA

Bovine serum albumin: Sigma, USA

Bromophenol blue: Merck, Germany

1–Butanol: Carlo Erba Reagents, Italy

2–Butanol: BDH, England

Calcium chloride: Scharlau, Spain

Conc. Sulfuric acid: J.T.Baker, Thailand

Coomassie blue G–250: Sigma, USA

Coomassie blue R–250: Sigma, USA

Cornstarch: Unilever, Thailand

Cyclohexanol: Merck, Germany

di–Potassium hydrogen orthophosphate: Univar, Australia

Ethanol, absolute: Merck, Germany

Ethyl acetate: LAB–SCAN, Thailand

Ethylenediamine tetraacetic acid (EDTA): Univar, Austraria

Flomax®: National Starch, USA

γ–Cyclodextrin: Sigma, USA

Glacial acetic acid: Mallinckrodt Chemicals, Thailand

Glucose: Univar, Austraria

Glycerol: Merck, Germany

Glycine: Sigma, USA

1–Heptanol: BDH, England

2–Hexanol: Fluka, Switzerland

Hydrochloric acid: Carlo Eaba Reagents, Italy

Iodine: Merck, Germany

Isobutanol: BDH, England

Isopentanol: BDH, England

Magnesium sulfate heptahydrate: Scharlau, Spain

Maltose: Laboratorios CONDA, Spain

Maltotriose: Fluka, Switzerland

2–Mercaptoethanol: Fluka, Switzerland

Methanol: Merck, Germany

Methyl–α–D–glucopyranoside: Sigma, USA

1–Octanol: Merck, Germany

85% Orthophosphoric acid: BDH, England

1–Pentanol: BDH, England

Peptone from casein: Scharlau, Spain

Phenol: Fisher Scientific, England

Phenolphthalein: M&B Laboratory Chemicals, England

Potassium dihydrogen phosphate: Univar, Austraria

Potassium iodide: Merck, Germany

1–Propanol: Carlo Eaba Reagents, Italy

2–Propanol: Carlo Eaba Reagents, Italy

Sodium acetate: BDH, England

Sodium carbonate: Univar, Australia

Sodium chloride: Carlo Eaba Reagents, Italy

Sodium dodecyl sulfate: Sigma, USA

Soluble starch: Scharlau, Spain

Standard molecular weight marker protein: GE Healthcare, England

TEMED (N,N,N',N'–tetramethylene–ethylenediamine): Fluka, Switzerland

tert–Butanol: BDH, England

Tris (hydroxymethyl) aminomethane: Research Organics Inc., USA

Yeast extract: Scharlau, Spain

2.3 Bacteria

Bacterial CGTase was produced from *Paenibacillus* sp. RB01 which was isolated from hot spring soil in Ratchaburi province, Thailand (Tesana, 2001).

2.4 Cultivation media for the production of CGTase

2.4.1 Medium I

Paenibacillus sp. RB01 was grown in medium I. Medium I was prepared by the addition of 1.0% (w/v) soluble starch, 0.5% (w/v) beef extract, 1.0% (w/v) peptone from meet, 0.2% (w/v) NaCl, and 0.2% (w/v) yeast extract. The pH of media was adjusted to 7.2 with 1 N NaOH solution. The solid medium I was prepared by adding of 1.5% (w/v) agar before subjected to sterilization at 121°C for 15 minutes using an autoclave.

2.4.2 Horikoshi's medium

Horikoshi's medium is well defined as production media for extracellular CGTase. The media was prepared according to previous reported (Rutchtorn, 1993) which was slightly modified from Horikoshi (1971). The medium formula composed of 1.0% (w/v) of cassava starch (flomax®), 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) and $MgSO_4 \cdot 7H_2O$. Medium was sterilized as previously described. Na_2CO_3 at 0.75% (w/v) was sterilized separately and added to Horikoshi's medium. The pH of the medium was 10.1–10.2.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

Paenibacillus sp. RB01 was streaked on Medium I plate and incubated at 37°C for 18 hours. One loop was inoculated into liquid Medium I and grown until A_{660} reached 0.3–0.5.

2.5.2 Enzyme production

A 1.0% (v/v) of starter inoculum was transferred into 300 mL of Horikoshi's medium in a 1,000 mL Erlenmeyer flask. The cultivation was performed at 40°C with continuous shaking at 250 rpm in shaking incubator for 72 hours. Crude CGTase was obtained after removal of cells by centrifugation at 5,000 rpm for 30 minutes at 4°C. Supernatant of crude CGTase was kept at 4°C for further purification.

2.6 Partial purification of CGTase

Crude CGTase from previous step was partially purified by starch adsorption method (Kato and Horikoshi, 1984) which was slightly modified by Laloknam (1997).

Corn starch (commercial grade) was oven dried at 120 ºC for 30 minutes and cooled to room temperature before use. Dried starch was gradually sprinkled into stirring supernatant of crude CGTase to make a final concentration of 5% (w/v) at 4 ºC. The supernatant was left to stir overnight. Subsequently, the starch cake was collected by centrifugation at 8,000 rpm for 30 minutes at 4 ºC. The starch cake obtained from this step was subjected to wash twice with TB1 buffer (10 mM tris–HCl pH 8.5 containing 10 mM $CaCl_2$). CGTase was then eluted from the starch cake with 62.5 mL of 0.2 M maltose in TB1 for starting broth of 1 L. CGTase was eluted again with the same process. The mixture was stirred for at least 30 minutes before

the remained starch was removed by centrifugation at 8,000 rpm for 30 minutes at 4 ºC. The solution of CGTase was concentrated with viva flow (10,000 MWCO) and dialysis was performed to remove maltose with three changes of distilled water at 4°C.

2. 7 Enzyme analysis

2.7.1 Non–denaturing polyacrylamide gel elctrophoresis (Native–PAGE)

Starch degrading activity of partial purified enzyme from starch adsorption was determined from the migration of protein (enzyme) on non–denaturing gel. Separating gel was 7.5 % (w/v) acrylamide while the stacking gel used here was 5.0 % (w/v) acrylamide. The purified protein and crude extract were mixed with sample buffer before loaded into the wells. Tris–glycine buffer pH 8.0 was used as electrode buffer. The system was preceded by setting the constant current of 12 mA per slap using a Mini–Gel electrophoresis unit from cathode towards anode. The electrophoresis was allowed to perform at room temperature. When the dye front reached the bottom of the gel, the gel was cut in to 2 parts for the determination by protein staining and activity staining.

2.7.2 Coomassie blue staining (Protein staining)

A gel from Native–PAGE was subjected to protein staining by the addition of 0.1% (w/v) of Coomassie brilliant blue R–250 in 45% (v/v) methanol and 10% (v/v)

acetic acid) and incubated for 30–60 minutes. Subsequently, the gel was destained with several changes of destaining solution (10% (v/v) methanol and 10% (v/v) acetic acid).

2.7.3 Dextrinizing activity staining

For dextrinizing activity staining, the native gel was soaked with 2.0 % (w/v) soluble starch in 0.2 M phosphate buffer pH 6.0, at 40 °C for 10 minutes. The gel was rinsed several times with distilled water to remove the remaining starch on gel. After that 10 mL of I_2 staining reagent (0.2 g% (w/v) I_2 in 2.0% (w/v) KI) was added and incubated at 40°C for color development. The starch degrading activity was observed by the clear zone on the blue background on the gel.

2.7.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

The denaturing gel electrophoresis was carried out on 7.5 % (w/v) separating gel and 5.0% (w/v) of stacking gel. Electrophoresis buffer was Tris–glycine pH 8.0, containing 0.1 % SDS. Protein samples were treated with 5x sample buffer (1% (w/v) bromophenol blue and 50% (v/v) glycerol in 1 M Tris–HCl pH 6.8) and boiled for 5 minutes before loading onto the gel. The system was operated at 12 mA of constant current per slap using a Mini–Gel electrophoresis unit from cathode towards anode. After electrophoresis, the gel was stained with Coomassie brilliant blue for visualization of the separated protein. The approximate protein bands fractionated on the gel were determined by comparing the travel distance with known molecular weight protein markers. The molecular weight marker proteins were carbonic

anhydrase (30 kDa), ovalbumin (45 kDa) albumin (66 kDa) and phosphorylase b (97 kDa).

2.8 Determination of the enzyme activity

2.8.1 Dextrinizing activity

The measurement of starch hydrolytic activity of CGTase was performed by a method which was slightly modified from Fuwa (1954). The soluble starch (0.2 g %) was prepared in 0.2 M phosphate buffer pH 6.0 prior to be used as a substrate for CGTase. Enzyme sample (0.05 mL) was incubated with 0.15 mL of starch substrate at 40°C for 10 minutes. The reaction was stopped by the addition of 2 mL of 0.2 M HCl. The iodine reagent (0.02% (w/v) I_2 in 0.2% (w/v) KI) 0.25 mL was added to the sample reaction. The reaction was adjusted to a final volume of 5 mL with distilled water and the absorbance at 600 nm was measured. Control reaction was performed by adding HCl to the reaction mixture before adding the enzyme. One unit of enzyme was defined as the amount of enzyme which reduced 10% of the blue color of the starch–iodine complex per minute under the described conditions.

2.8.2 Coupling activity

This method measures the disappearance of β–CD in the reaction mixture by the phenolphthalein solution which was slightly modified from Goel and Nene (1995). The inclusion complex formation of phenolphthalein in the hydrophobic cavity of cyclodextrin results in the decrease of the absorbance at 550 nm. Two–hundred and fifty microliters of standard β–CD or sample reaction (CGTase pre–incubated with β–CD and alcohol acceptors) was incubated with 750 µL of phenolphthalein solution for

15 minutes. The absorbance at 550 nm was then read. Relative coupling activity was calculated by conversion of ΔA_{550} to μmoles β–CD which can be quantitated from the β–CD–phenolphthalein calibration curve (see Appendix C). The disappearance of β–CD in the reaction mixture was calculated from the difference between the amount of β–CD substrate at 0 and 24 hour incubation with CGTase and acceptors (see Eq 1.). The activity of enzyme in buffer was set as 100 percent.

$$\begin{matrix}\text{μmol} \\ \text{(β-CD disappeated)}\end{matrix} = \left(\frac{\text{A550 (sample)} - \text{A550 (control)}}{\text{Slope from the calibration curve}} \times 0.25 \right) \qquad \text{Eq 1.}$$

2.9 Protein determination

Bradford's method (1976) has been known as an analytical procedure for the determination of the protein concentration in the solution under acidic condition where the red–brown form of dye is converted to bluer form due to its ability to form inclusion complex with amino acid of protein sample. Enzyme sample (100 μL) was mixed with 1.0 mL of Coomassie blue reagent. The reaction was incubated for 10 minutes at room temperature. Absorbance was measured at 595 nm. The calibration curve of standard bovine serum albumin was constructed (see Appendix D).

One liter of Coomassie blue reagent contains 100 mg of Coomassie blue G–250, 50 mL of absolute ethanol, 100 mL of 85% H_3PO_4 and distilled water. The reagent was filtered with Whatman® No.1 filter paper and kept in the dark bottle before use.

2.10 Transglycosylation reactions

The capability of the CGTase to catalyze the transferring of glycosyl unit from donor to alcohol acceptors was examined by varying the alcohol type and the concentration using β–CD as a glycosyl donor. The alcohols used here were 10–50% (v/v) of methanol, ethanol, 1–propanol, and 2–propanol. C_4, alcohol acceptors (1–butanol, isobutanol, 2–butanol and *tert*–butanol), C_5 alcohols (1–pentanol, 2–pentanol and C_6 alcohol used here were 2–hexanol and cyclohexanol) were used at the concentration of 2–30 % (v/v). Long chain alcohol acceptors were 1–heptanol and 1–octanol and were used at the concentration of 0.8–30% (v/v). The reaction mixture contained final concentration of 0.6% (w/v) β–CD and various alcohol concentrations as described above. They were incubated with 200 dextrinizing units/mL of CGTase at 40°C for 24 hours with continuous shaking in 50 mM acetate buffer pH 6.0. The influence of alcohol on the coupling activity of CGTase was examined by phenolphthalein method as previously described in section 2.8.2. The remaining coupling activity was calculated by followed Eq 1 compared with the reaction incubated in buffer. The suitable acceptor was selected from relative intensity which was measured by GeneTools program. Glucose spot (15 µg) was set as standard value.

2.11 Donor specificity

Appropriate glycosyl donors for the transglycosylation reaction catalyzed by CGTase were determined by varying the carbohydrate sources. Several donors used here were β–CD, soluble starch both from potato and cassava–a commercial named flomax® and raw starch. All glycosyl donors at the final concentration of 0.6% (w/v)

and selected alcohols acceptor (from previous step section 2.10) were incubated with 200 U/mL CGTase in 50 mM acetate buffer pH 6.0 at 40°C for 24 hours with continuous shaking. Samples were analyzed with TLC. To determine the amount of transglycosylated products, the intensity of transglycosylated product was calculated from the calibration curve of methyl–α–glucoside (Appendix E).

2.12 Characterization of reaction products using glucoamylase and α–glucosidase

The reaction mixture of CGTase catalyzed transglycosylation reaction was incubated with two amylolytic enzymes; glucoamylase and α–glucosidase. Glucoamylase is an exo–acting enzyme which hydrolyzes glycosidic linkage between glucose residues from the non–reducing end of starch and related substrate. While α–glucosidase is known to catalyze the hydrolysis of 1→4 linked alpha glucose residues from non–reducing terminal and the alpha linkage between glucose and alcohol acceptor. This was to preliminary check the configuration between alcohol and glycosyl units and whether the product obtained were glycoside derivatives. The transglycosylation reaction was performed according to section 2.10. After that the alcohol in the reaction was evaporated under vacuum at 40 °C before it was treated with glucoamylase. Glucoamylase at the final activity of 30 units/mL in 50 mM acetate buffer pH 6.0 was added and incubated at 40°C for 1 hour. After that, glucoamylase was inactivated by boiling for 10 minutes. To investigate the linkage between alcohol acceptor and glucosyl residues, the reaction mixture was incubated with α–glucosidase (final activity of 30 unit/mL) for 3 hours under the same

conditions as glucoamylase treatment which was described above. The products after treatment with two amylolytic enzymes were then analyzed by TLC and HPLC.

2.13 Determination of alkyl glycoside products

2.13.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was used to analyze the transglycosylation products by applying 20 μL of reaction samples on Merck silica gel 60 F_{254} aluminum sheet (20 cm in height). Mobile phase used was ethyl acetate: acetic acid: water (3:1:1 by volume). Spots were visualized by spraying with conc. sulfuric acid: methanol (1:2) followed by heating at 110°C for 15 minutes. The intensity of glycoside products on TLC were measured by scanning TLC chromatograms with GeneTools program of SYNGENE. Glucose spot (15 μg) was set as standard value.

2.13.2 High performance liquid chromatography (HPLC)

Alkyl glycoside products were analyzed by HPLC using YMC–pack ODS–AM 303 column (4.6 mm diam, 250 mm ht, YMC, Kyoto). HPLC was performed using a Kontron 420 pump system with a refractive index detector (RID). Polar mobile phase was a mixture of 40% methanol and ultrapure water containing 0.2 % (v/v) acetic acid. Alcohol in the reaction mixture was evaporated prior subjected to the HPLC analysis and the absorbed alkyl glycosides were eluted at the flow rate of 0.5 mL/min with the controlled temperature of 50 °C. The amount of the transglycosylated products were judged from total peak area compared with the standard curve of methyl–α–D–monoglucoside (Appendix F).

2.13.3 Mass spectrometry (MS)

The reaction mixture was concentrated by CentriVap Concentrator® before subjected to the molecular mass measurement by mass spectrometry. High resolution mass spectra (HRMS) were obtained using time–of–flight (TOF) which was measured on a mass spectrometer model Micro TOF, Bruker Daltonics, Germany. The compounds were ionized by electrospray ionization on the positive–ion mode. MS was performed at Chulabhorn Research Institute (CRI).

2.14 Optimization of alkyl glycoside production

The synthesis of alkyl glycosides using CGTase catalyzed transglycosylation reaction was performed under the optimum pH and temperature of CGTase. The optimum temperature and pH of CGTase from *Paenibacillus* sp. RB01 for transglycosylation reaction had been successfully reported by Chotipanang (2010). The optimum pH and temperature for transglycosylation by CGTase were at pH 6.0 and at 40°C, respectively.

2.14.1 Optimization of isobutanol concentration

Optimization for a novel product was started by varying isobutanol concentration. Isobutanol concentration ranging from 0–30 % (v/v) and 0.6% (w/v) β–CD were incubated with CGTase (200 U/mL) at 40 °C in 50 mM acetate buffer, pH 6.0 for 24 hours with continuous shaking, to find an appropriate acceptor

concentration. Reaction was stopped by boiling for 5 minutes and evaporated at 40 °C for 3 hours to remove alcohol remaining in the reaction mixture. Subsequently, the final volume was adjusted to 0.5 mL with distilled water. The reaction products were then analyzed by HPLC. Yield was calculated as mole of total products relative to mole of acceptor presence in reaction mixture (see Eq.2).

$$\% \text{ yield} = \left(\frac{\text{The amount of total product (mole)}}{\text{The amount of acceptor (mole)}} \times 100 \right) \quad \text{Eq 2.}$$

2.14.2 Optimization of β–cyclodextrin concentration

β–CD was selected as an appropriate donor for the synthesis of alkyl glycoside by transglycosylation reaction. The amount of β–CD substrate was varied. The final concentration of β–CD ranging from 0–1.8% (w/v) in acetate buffer pH 6.0 and mixed with optimum concentration of isobutanol from section 2.14.1 and 200 U/mL CGTase in 50 mM acetate buffer pH 6.0. The reaction mixture was incubated at 40°C for 24 hours with continuous shaking. The reaction products were analyzed by HPLC.

2.14.3 Optimization of enzyme concentration

To find the optimum concentration of CGTase, isobutanol acceptor concentration (section 2.14.1) and β–CD substrate concentration (section 2.14.2) which were selected from previous investigation were incubated with varying amount of CGTase (0–500 units/mL of CGTase) in 50 mM acetate buffer pH 6.0 at 40 °C for

24 hours with continuous shaking. The appropriate enzyme concentration was determined by HPLC.

2.14.4 Optimization of incubation time

Reaction mixture consisted of an appropriate concentration of isobutanol was mixed with appropriate β–CD concentration under optimum pH (pH 6.0) and temperature (40°C) of CGTase for transglycosylation reaction. Optimum CGTase concentration from previous section was used and the reaction was incubated from 0 hours to 7 days with continuous shaking. The reaction was stopped by boiling for 5 minute. The suitable time for the production of alkyl glycosides was chosen from HPLC analysis.

2.15 Large scale production of isobutyl glycoside

Large scale production of isobutyl glycoside was performed under the optimum conditions obtained from previous section (section 2.14). In the initial experiments, transglycosylation reaction was performed in the reaction mixture of 0.25 mL. To produce large amount of products, the total volume was raised to 12.5 mL. After the incubation was complete, the reaction mixture was concentrated by evaporation. The resulting reaction mixture was adjusted to 1 mL with 0.1 M NaOH. The products of CGTase catalyzed reaction from this section were purified by column chromatography before characterization for the emulsification activity.

2.16 Purification of alkyl glycosides

2.16.1 Preparative thin–layer chromatography (PLC)

Like paper chromatography, preparative thin–layer chromatography is a chromatographic technique which is able to analyze the products dependent on their partitioning ability. Mobile system used here was the same as previously described in TLC analysis (section 2.13.1). Reaction mixture from previous step was concentrated with rotary evaporator at 40 °C before applied to the preparative silica plate. The PLC was allowed to run with the same mobile system until the solvent front reached to the top. The preparative plate was dried. One lane of the reaction mixture on the plate was then carefully cut to detect the glycoside products using methanol sulfuric developing solution (section 2.13.1). Each product from PLC which had approximately the same Rf values as those confirmed by methanol sulfuric test were scraped separately. Each glycoside product was extracted from adsorbent (silica) by dissolving with methanol for 30 minutes at room temperature, then centrifuged at 6000 rpm at 25 °C for 1 hour. Methanol was further removed by evaporation using rotary evaporator at 40 °C. Purified alkyl glycoside was confirmed by mass spectroscopy.

2.16.2 Strong anionic column chromatography

Enzymatic synthesis of isobutyl glycoside which was achieved by CGTase catalyzed intermolecular transglycosylation reaction under the optimum condition, was purified by strong anion exchange chromatography. Anionic exchanger Amberlite® IRA–900 which was well swelled with 0.1 M NaOH was packed into 2.3 × 22 cm column. Column was allowed to equilibrate with 0.1 M NaOH to substitute the functional group with OH^-. Reaction mixture (0.5 mL), after concentrated with rotary evaporator, was loaded into the column. Alkyl glycoside products were eluted from resin with ultrapure water with the flow rate of 0.5 mL/min at room temperature.

The 2–mL fractions were collected to further determine for sugar consumption by phenol–sulfuric test (section 2.16.3).

2.16.3 Phenol–Sulfuric acid assay (Rao and Pattabiraman 1989)

A 5% (w/v) of phenol solution was prepared and kept at 4 °C before use. Samples (0.1 mL) were treated with 0.5 mL of cool phenol solution for 15 minutes then conc. sulfuric acid solution was added. Samples were placed at room temperature for 10 minutes before the absorbance at 480 nm was measured. Purification profile was constructed and fractions containing glycosyl units were collected for further characterization by TLC and HPLC.

2.17 Characterization of the alkyl glycoside products

2.17.1 Emulsification activity (Kim et al., 2009)

The final concentration (0.5 mg/mL) of purified isobutyl–monoglucoside and isobutyl–maltoside was prepared by diluting with 0.5 mM Tris–HCl buffer containing 0.05 M $MgSO_4$ (pH 8). The 4.4 mL solution was mixed with 0.1 mL of n–hexadecane substrate to form oil–in water emulsion by vigorous mixing on vortex for 2 minutes. The emulsion was obtained after the reaction was left to stand for 10 minutes. The turbidity was determined by spectrophotometer at 540 nm. Commercial non–ionic surfactant, Triton X–100®, isobutanol and standard methyl–α–monoglucoside were also studied.

2.17.2 Emulsification stability (Kim et al., 2009)

Emulsion was performed as described in 2.17.1. 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 was calculated from slope obtained from plotting the time versus log $OD_{540.}$

CHAPTER III

RESULTS

3.1 Partial purification of CGTase from *Paenibacillus* sp. RB01 using starch adsorption

After *Paenibacillus* sp. RB01 was activated in the medium I for 18 hours at 37 °C, it was then cultivated in Horikoshi's medium at 40°C with continuous shaking at 250 rpm for 72 hours for the production of an extracellular CGTase. The supernatant containing crude CGTase was obtained after the removal of cells by centrifugation at 5,000 rpm. Partial purification of CGTase using starch adsorption method was carried out, as previously described in Material and Methods section 2.4, to remove other proteins. Partially purified CGTase obtained from starch adsorption was concentrated with viva flow and subjected to dialysis against 3 changes of distilled water at 4 °C. Enzyme obtained was assayed for dextrinizing activity by following the absorbance at 600 nm of starch–iodine complex. Control reaction was also carried out where the reaction was stopped before the addition of an enzyme solution.

The purification table is shown in Table3. Enzyme had purification fold of 120 with 50.6% yield. Specific activity of the enzyme was 4800 units/mg protein which was calculated and expressed in terms of dextrinizing activity per mg protein. Purity of enzyme after purification through starch adsorption was then checked with electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed to check the size of obtained protein. The gel was stained with Coomassie blue for demonstration of protein bands. The SDS gel is shown in Figure

19. This result revealed that after the crude enzyme was purified through starch adsorption method, the number of protein bands observed on the gel decreased, suggesting that the enzyme was successfully purified using only starch adsorption technique. The molecular mass of CGTase was estimated to be 70 kDa by this SDS–PAGE by comparison the band with those of known molecular weight of standard proteins.

Native polyacrylamide gel electrophoresis (Native–PAGE) was carried out for co–confirmation with SDS–PAGE that the protein obtained was CGTase by following the starch–degrading property. This was observed from staining the native gel with iodine solution (0.2% iodine solution in 2.0% of potassium iodide). After the addition of soluble starch substrate (2.0%, w/v) onto the gel, the degradation of starch substrate by the action of CGTase showed distinct clear zones which corresponded to the mobility of the active bands (Figure 20 A). The 3 active bands from the activity staining were coincided with those from protein staining (Figure 20 B). This result implied that the partial purified enzyme had 3 isoforms with different conformation and net charge.

Table3 Purification table of CGTase from *Paenibacillus* sp. RB01 by starch adsorption method.

Purification step	Volume (mL)	Total activity* ($Ux10^3$)	Total protein (mg)	Specific activity* (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1,050	39.9	991	40.3	1	100
Starch adsorption	69	20.2	4.20	4800	120	50.6

*Dextrinizing activity

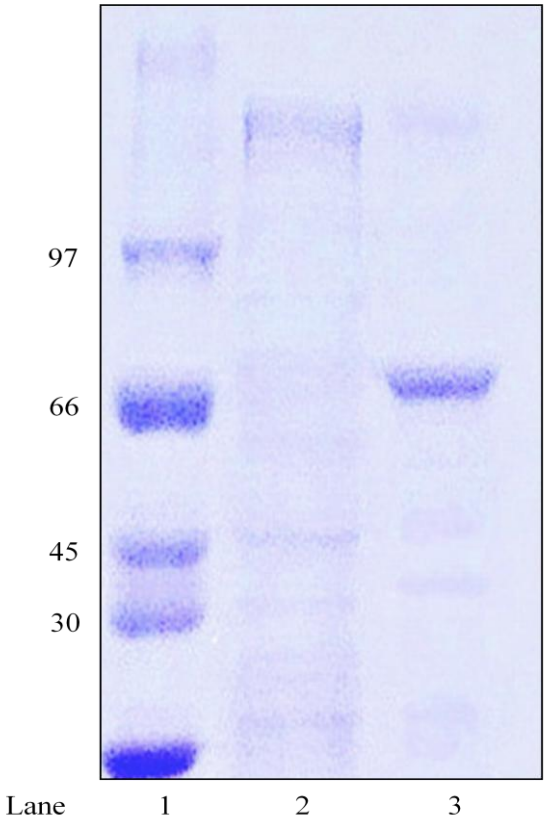


Figure 19 **SDS–PAGE of CGTase from *Paenibacillus* sp. RB01 before and after purification.**

Lane 1: Low molecular weight protein marker

Phosphorylase b (97 kDa)

Albumin (66kDa)

Ovalbumin (45 kDa)

Carbonic anhydrase (30 kDa)

Lane 2: Crude enzyme (50 μg)

Lane 3: Partial purified enzyme from starch adsorption (10 μg)

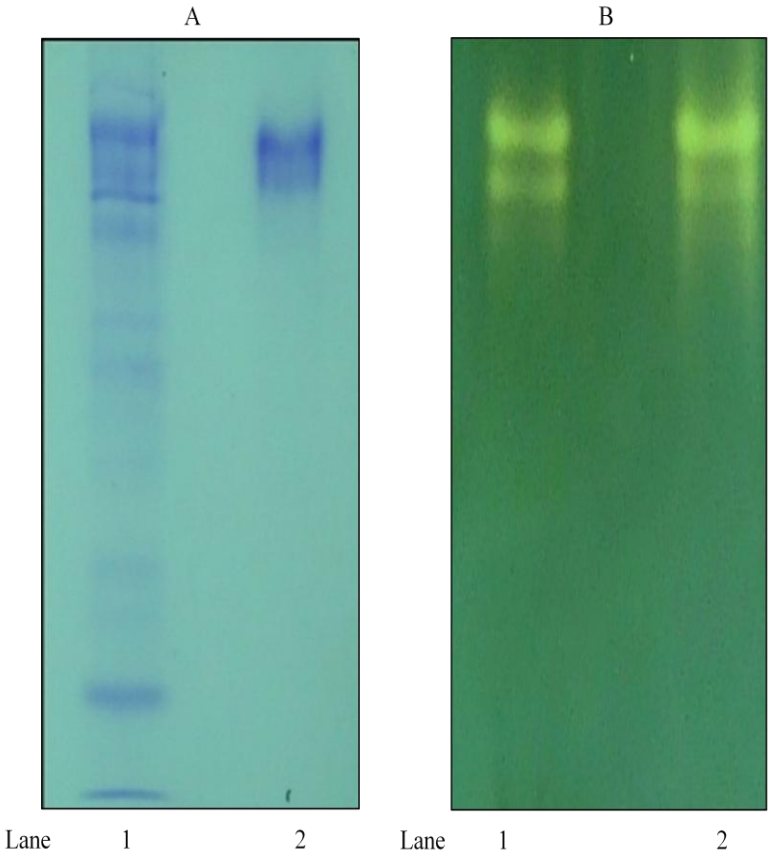


Figure 20 **Native–PAGE of CGTase from *Paenibacillus* sp. RB01 before and after purification.**

(A) Coomassie blue staining

Lane 1: Crude enzyme (50 μg)

Lane 2: Partial purified enzyme from starch adsorption (20 μg)

(B) Dextrinizing activity staining

Lane 1: Crude enzyme (0.02 U)

Lane 2: Partial purified enzyme from starch adsorption (0.02 U)

3.2 Effect of alcohols on the coupling activity of CGTase

The use of organic solvents in the enzymatic reaction is limited by the fact that many organic solvents are well known to cause enzyme inactivation. However, the purpose of this study was to synthesize alkyl glycosides using alcohols as an acceptor under the catalysis of CGTase via intermolecular transglycosylation reaction. Therefore, the effect of alcohols on enzyme activity under the condition used for the synthesis of the desired products was first investigated. The reaction media containing 0.6% (w/v) of β–CD substrate was incubated with CGTase and various alcohols under optimized condition of CGTase catalyzed transglycosylation reaction. Short chain alcohol acceptors used were methanol, ethanol, 1–propanol and 2–propanol at the concentration of 10–50% (v/v). Medium chain alcohols used were 1–butanol, 2–butanol, isobutanol, *tert*–butanol, 1–pentanol, isopentanol, 2–hexanol and cyclohexanol at the concentration of 5–30% (v/v) and long chain alcohols (heptanol and octanol) at the concentration of 2–30% (v/v) were used.

The effect of alcohols on the enzyme activity was studied by varying the alcohols concentration. Short chain still had good water solubility when alcohol concentration as high as 50 % (v/v) was used and thus, the reactions had single phase (homogeneous). Medium chain alcohols (except *tert*–butanol), however, had relatively lower solubility where C_4, C_5 and C_6 alcohols were soluble at concentration as high as 8, 4 and 2 % (v/v), respectively. For a group of long chain alcohols, heptanol was still soluble at the concentration of 0.8% (v/v) while octanol was insoluble at all concentration used. According to the solubility of these alcohol acceptors, the reaction mixtures containing low water soluble alcohols were separated into two phases. Thus, the reactions were performed with continuous shaking. The

transglycosylation reactions were carried out under the optimum conditions of the enzyme which were to use 50 mM of sodium acetate buffer pH 6.0 and the temperature of 40ºC with continuous shaking for 24 hours. The reactions were stopped by boiling at 100 ºC for 10 minutes. To study the effect of alcohols on the transglycosylation reaction catalyzed by CGTase, an aliquot of 10 µl was withdrawn for the determination of the disappearance of β–CD after the action of enzyme using phenolphthalein method (coupling activity). Control experiments were also carried out where β–CD and alcohol were incubated with CGTase at 0 minute. The inclusion complex of phenolphthalein in the hydrophobic cavity of β–CD resulted in the decrease of an absorbance at 550 nm. Coupling activity after treatment with alcohols was calculated and expressed in terms of relative coupling activity (see Equation 1 section 2.8). Coupling activity of CGTase after incubation with β–CD substrate without alcohol for 24 hours was set as 100%. All samples were done in duplicate.

For short chain alcohol (methanol, ethanol, 1–propanol and 2–propanol), they were still soluble under the range of concentration studied, thus, a single phase was obtained. As can be seen in Figure 21 (A), the coupling activity of CGTase was dramatically decreased when the concentration of alcohol increased. The remaining activity of CGTase, after treatment with alcohol was highest in methanol, followed by ethanol, 1–propanol ≈ 2–propanol, respectively. For medium chain alcohols, due to the lower water solubility, the reactions were one and two phase system, depending on the type and concentration of alcohol used. According to Figure 21 (B), the influence of these medium chain alcohols, which still resulted in one phase system, on CGTase decreased with increasing alcohol concentration. The enzyme still retained activity for more than 40% in the reaction mixture containing butanol isobutanol,

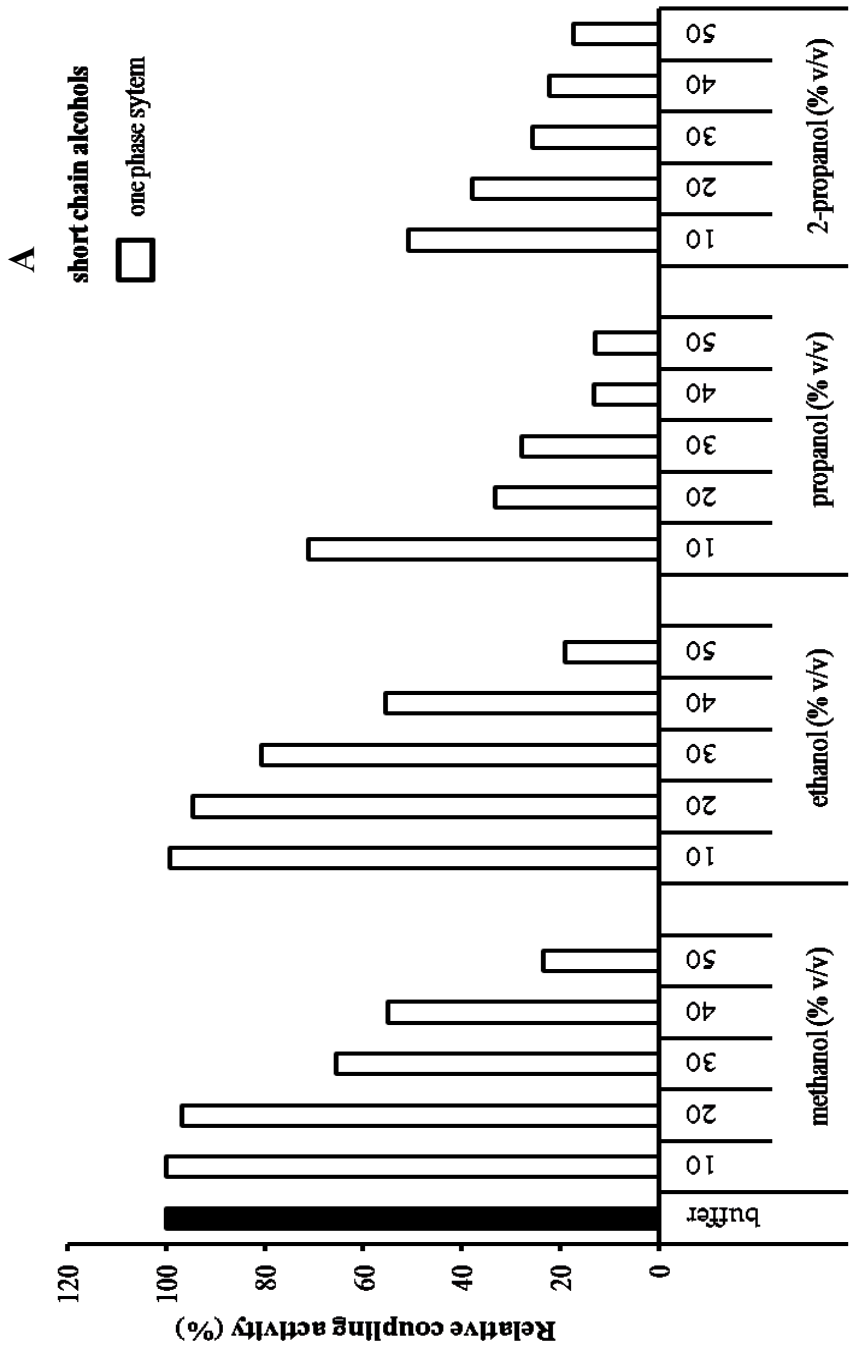


Figure 21 **Relative coupling activity of CGTase in alcohol solvent mixtures; (A) incubated with short chain alcohols, (B) incubated with medium chain alcohols and (C) incubated with long chain alcohols.**

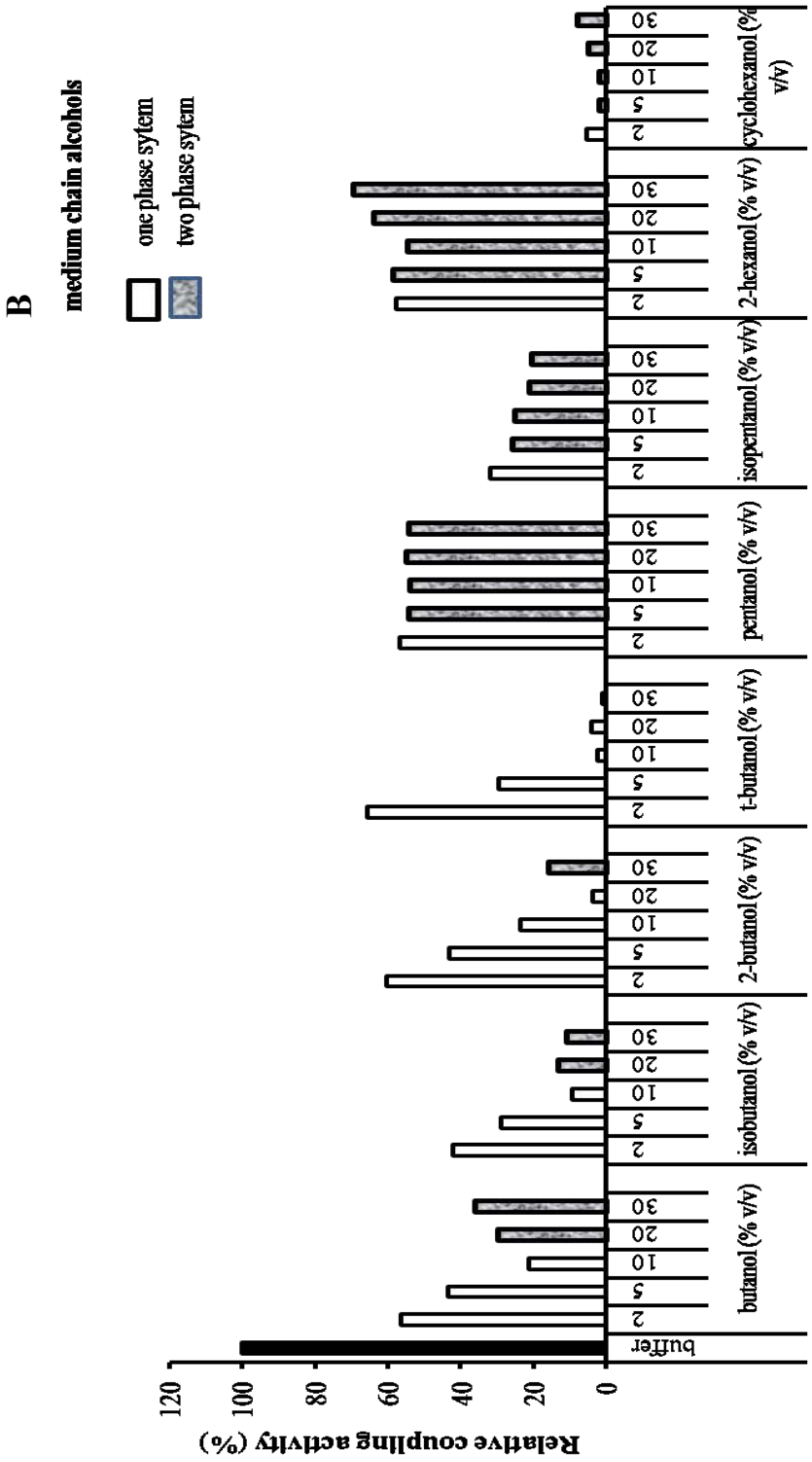


Figure 21 **Relative coupling activity of CGTase in alcohol solvent mixtures; (A) incubated with short chain alcohols, (B)incubated with medium chain alcohols and (C) incubated with long chain alcohols.** Continue,

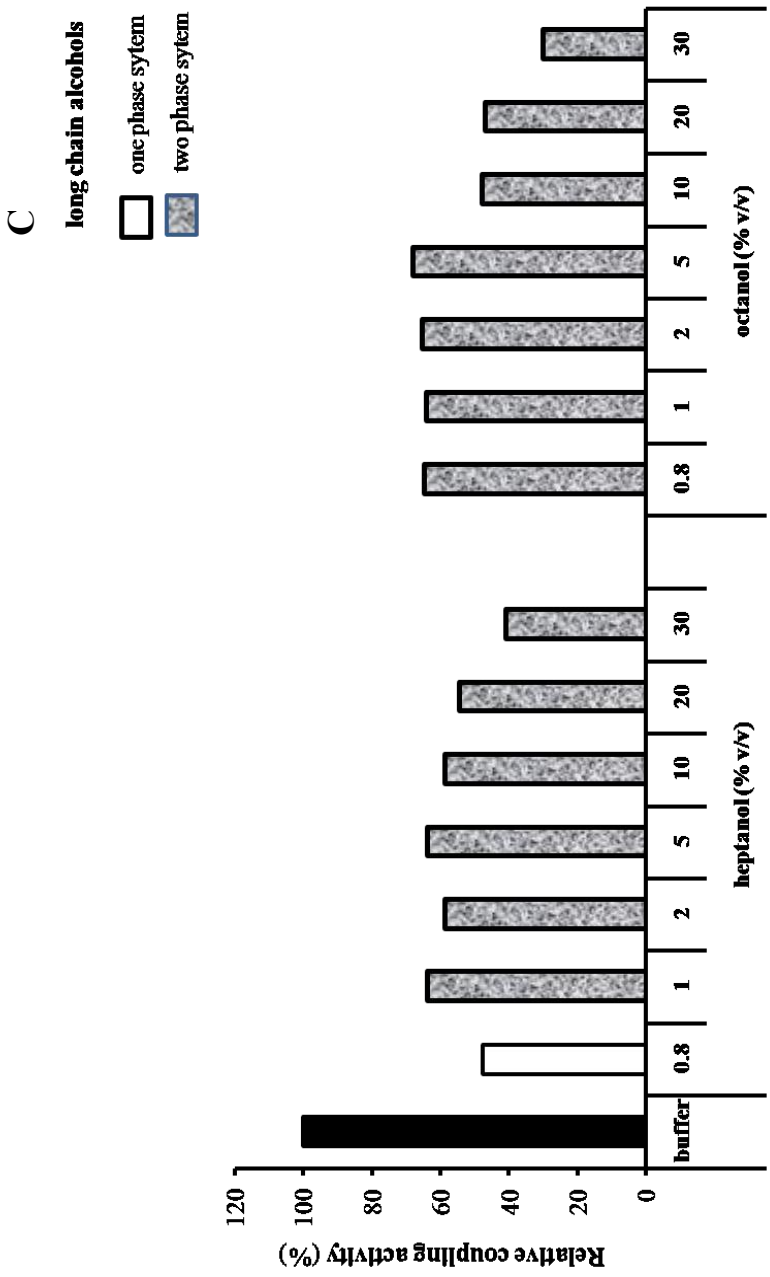


Figure 21 **Relative coupling activity of CGTase in alcohol solvent mixtures; (A) incubated with short chain alcohols, (B) incubated with medium chain alcohols and (C) incubated with long chain alcohols.**

Continue,

2–butanol and *tert*–butanol when the concentration of 2–5 (%, v/v) was used. However, the activity remained after treatment with C_5, C_6 (2–hexanol) (Figure 27 B), C_7 and C_8 alcohols (Figure 27 C) were still high and the activity did not decrease much when increasing their concentration to as high as 30%. These reactions were found to be in two phase system. In addition, alcohol with branch chain structure (isobutanol and isopentanol) had more effect on their corresponding primary alcohol with the same chain length. These results revealed that the structure of alcohol had significant effect on CGTase activity. In the case of cyclohexanol, the influence on CGTase activity studied by the phenolphthalein test was found to be stronger than other alcohol acceptors. The results can be described by the fact that the cyclic structure of cyclohexanol made it enable to form an inclusion complex with hydrophobic cavity of CDs. This is possible to compete with phenolphthalein molecule to form an inclusion complex with β–CD cavity. (Yu et al., 2004).

In conclusion, the results showed that alcohols had an effect on the CGTase's coupling activity. In one phase reaction system, the effect of alcohol on CGTase activity was dependent on the structure and the concentration of alcohol acceptors. The remaining activity of CGTase after incubation with methanol and ethanol were more than 50 % at the alcohol concentration of 10–30 % (v/v). Longer alcohol chain length had stronger effect on the enzyme than short chain alcohols in one phase system. Increasing alcohol concentration led the reaction media into two–phase system although the activity of enzyme did not dramatically decrease. Unlike the single phase reaction system, it was found that the effect of alcohol concentration on the enzyme activity was more pronounced when increasing alcohol concentration. Solubility of alcohol acceptors used was different depending on the length and

structure of alcohols. Long chain alcohol was low soluble in water than short chain alcohol. While secondary and tertiary structures were more soluble than primary straight chain of the same alkyl chain length. Therefore, effect of alcohol on coupling activity of CGTase did not only depend upon its concentration but also whether the reaction catalyzed by CGTase was in one or two phase system. This was due to the fact that CGTase could not react with its substrate uniformly in two–phase reaction system. To select an appropriate acceptor, the reaction products of various alcohols were further determined the alkyl glycoside yield by comparison of the products intensity on TLC.

3.3 TLC analysis of transglycosylation products

The alkyl glycoside products obtained from the transglycosylation reaction catalyzed by CGTase were determined with thin layer chromatography (TLC). The migration of substance on the TLC (stationary phase) was dependent on characteristic of the substance, stationary phase and mobile phase. Reaction was aliquot and applied to 20x20 cm of silica plate. Non–polar mobile system was prepared using ethyl acetate : acetic acid : water (3:1:1 by volume). The mobile system was left to equilibrate before use. The spots were observed by spraying with methanol : sulfuric acid (2:1 by volume). Subsequently, TLC plate was heated at 110 ºC for 15 minutes. The expected transglycosylation products should have less polarity than standard sugars (glucose, maltose and maltotriose) because of the attachment of alkyl chain. Thus, under this non–polar mobile phase, the expected products could migrate much better than standard sugars. The distance traveled by the substance divided by the distance traveled by the mobile phase was known as Rf value. This value was used to identify the substance.

The general objective of the present work was to synthesize longer chain alkyl glycoside. In this respect, to confirm the migration of the transglycosylated products on the TLC under this mobile phase, the reactions of CGTase with C_4(s) with 0 and 24 hours incubation were performed. From Figure 22, Lane 1 was β–CD donor, Lane 2–4 was glucose, maltose and maltotriose, respectively. Lane 5–6 was reaction of CGTase without alcohol at 0 and 24 hours. It was found that β–CD was hydrolyzed resulting to the mono–di– and tri– saccharides. The reaction mixtures of 0 and 24 hours incubation of CGTase with 1–butabol (Lane7–8), isobutanol (Lane 9–10), 2–butanol (Lane 11–12) and *tert*–butanol (Lane13–14) were determined. The results revealed that at 0 hour incubation with CGTase, only a spot of β–CD donor was observed. The products at higher Rf values could not be detected by TLC. Thus, this can be confirmed that the products with higher Rf values than standard sugars were the expected products from the action of CGTase.

The reactions from each alcohol acceptors with good coupling activity were selected for the determination of the ability of CGTase to use them as acceptors. The reaction without alcohol acceptor was also analyzed with TLC. This was to confirm that the hydrolysis activity of CGTase using water as an acceptor could occur in the absence of alcohol. From TLC chromatogram (Figure 23), lanes 1–4 were β–CD, glucose, maltose and maltotriose respectively. With this mobile system, β–CD (substrate) could not migrate and, hence was observed at the origin. Glucose, maltose and maltotriose are products from hydrolysis reaction with Rf of 0.16, 0.10 and 0.05, respectively. Other spots with greater Rf values than sugar standard were believed to be the alkyl glycosides which were synthesized by CGTase catalyzed

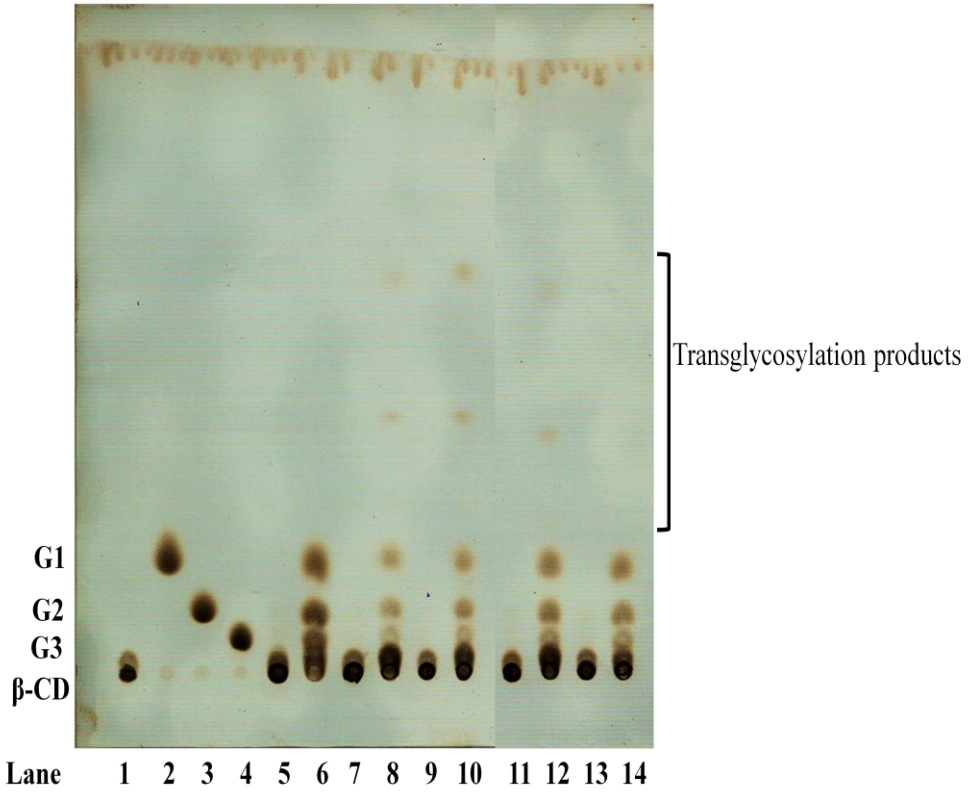


Figure 22 **TLC chromatogram of the transglycosylation products from β–CD to C_4 alcohols by CGTase.** TLC condition was ethyl acetate : acetic acid : water (3:1:1).

Lane 1–4: standard β–CD, glucose, maltose and maltotriose (25 μg).

Lane 5–6: reaction mixture without alcohol (0, 24 hours).

Lane 7–8: reaction mixture with 8% (v/v) butanol (0, 24 hours).

Lane 9–10: reaction mixture with 8% (v/v) isobutanol (0, 24 hours).

Lane 11–12: reaction mixture with 8% (v/v) 2–butanol (0, 24 hours).

Lane 13–14: reaction mixture with 8% (v/v) *tert*–butanol (0, 24 hours).

transglycosylation reaction from β–CD to alcohol acceptor. As can be seen in lane 5 the hydrolysis products appeared with the same Rf values of the standard sugars. Thus, these hydrolysis products were suggested as glucose, maltose and maltotriose by Rf relationship. The reactions performed in the presence of various alcohols were shown in lane 6–19.

Methanol, ethanol and propanol at 40% (v/v) were studied. It was found that the numbers of transglycosylation products observed on the TLC were varied. Methanol (Figure 23, lane 6) gave only one transglycosylation product (Rf = 0.30). Two transglycosylated products were obtained from ethanol and 1–propanol (Figure 23, lane 6 and 7). Other alcohols were used at lower concentration due to the fact that they had stronger effect on CGTase activity. Alcohols containing 4–6 carbons on alkyl chain were used at 5% (v/v) where those of carbons 7–8 residues were done at 2% by volume. The number of products from C_4 and C_5 alcohols had three products with different intensity. Thus, to reduce the confusion, the spot with the highest Rf value from each alcohol acceptor was named as product I, another products with lower Rf value were named as product II and III, respectively. Here, product I was proposed as an alkyl monoglucoside, product II was proposed as alkyl maltoside, and product III was proposed as alkyl maltotrioside.

1–Butanol and isobutanol gave 3 transglycosylation products with the same Rf value (0.66, 0.39 and 0.24) (Figure 23, lane 10 and 11). While the three products from 2–butanol acceptor were observed at slightly lower Rf values (Figure 23, lane 12). Two transglycosylation products from pentanol and isopentanol were observed at the same Rf values (0.74 and 0.44) (Figure 23, lane 14 and 15). The transglycosylation products from 2–hexanol and cyclohexanol were found to be very low (Figure 23,

lane 16 and 17). While 1–heptanol and 1–octanol could not be used as direct acceptors for CGTase catalyzed transglycosylation reaction for the synthesis of alkyl glycosides (Figure 23, lane 18 and 19). However, the hydrolysis products of CGTase in the presence of long chain alcohols (1–heptanol and 1–octanol) could be observed. On the basis of this result, it was thus suggested that heptanol and octanol were not able to be functional acceptor for intermolecular transglycosylation reaction catalyzed by CGTase from *Paenibacillus* sp. RB01. This might be due to the fact that their structures are not compatible with the catalytic site of the enzyme. The Rf values of the transglycosylated products were summarized in Table 4.

From the TLC (Figure 23), the number and the intensity of the expected transglycosylated products were different. Also they could not be distinguished by eye observed. Therefore, to select an appropriate acceptor for transglycosylation of CGTase, the product spot intensities thus were measured by GeneTools program. Glucose spot (15 μg) was set as standard value.

3.4 Determination of the spot intensity using GeneTool program

The amount of transglycosylation product obtained after 24–hour incubation from the catalysis ability of CGTase was preliminary determined from spot intensity on the TLC. The intensities were measured using GeneTool program from Genesnap®, and were calculated using glucose as a standard on the same TLC. Reaction mixtures of various alcohol types and concentrations were applied in triplicate to the TLC and run with non–polar mobile system. TLC was developed as previously described. The intensities of all transglycosylation products were expressed in terms of relative intensity Relative intensities of all alkyl glycoside products from each alcohol acceptors at various concentrations were shown in Figure

24. From Figure 24, the percentage of intensity referred to the amount of transglycosylation products obtained from the action of CGTase. The amount and number of products observed on the TLC from each alcohol acceptor were different. It was found that, the highest yield was obtained when short chain alcohol acceptor (methanol) was used (Figure 24). Ethanol also gave good yield at the concentration of 30% (v/v). As can be seen in Figure 24 that the amount of transglycosylation products obtained did not relate as a linear function of acceptor concentration in the reaction.

In the group of medium chain acceptor (Figure 25), isobutanol at 5% (v/v) gave the best yield (> 90% intensity). 1–Propanol, 1–butanol, 1–pentanol and isopentanol gave about 50% intensity at one phase system. 2–Butanol and 2–hexanol gave about 20% intensity at 30% (v/v). No transglycosylated product were detected when *tert*–butanol was used as an acceptor. In addition, cyclohexanol–a secondary alcohol with aromatic side chain was also able to be used as an acceptor. This confirmed the capability of CGTase for catalyzing intermolecular transglycosylation reaction to the aromatic alcohol. Reaction was performed by varying concentration of cyclohexanol between 2–30% (v/v). The result showed that CGTase had ability to catalyze transglycosylation reaction using an aromatic alcohol as acceptor at low concentration (2–5 v/v) although small yield was obtained. When reaction with cyclohexanol was in two phase system, the products from cyclohexanol could not be found (Figure 26).

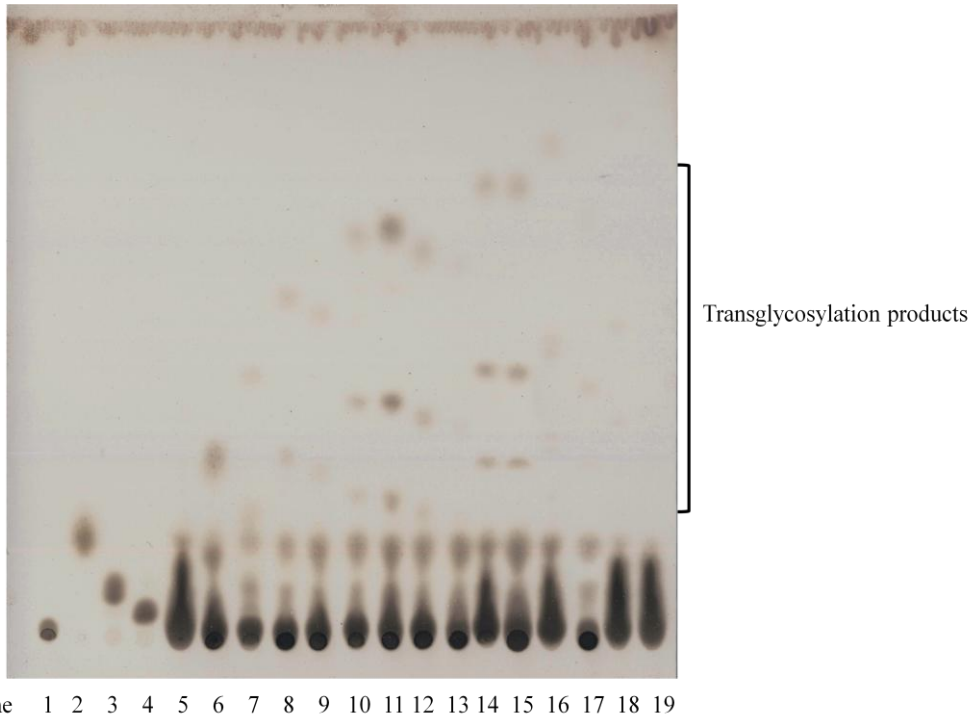


Figure 23 **TLC chromatogram of the transglycosylation products from β–CD to various alcohols by CGTase.** TLC condition was ethyl acetate : acetic acid : water (3:1:1). Reactions were from incubation for 24 hour.

Lane 1–4: standard β–CD, glucose, maltose and maltotriose (25 μg).

Lane 5: reaction mixture without alcohol acceptor.

Lane 6–8: reaction mixture with methanol, ethanol, propanol (40%, v/v).

Lane 9: reaction mixture with 2–propanol (10%, v/v).

Lane 10–17: reaction mixture with butanol, isobutanol, 2–butnol, *tert*–butnol, pentanol, isopentanol, 2–hexanol, and cyclohexanol (5%, v/v).

Lane 18–19: reaction mixture with heptanol and octanol (2%, v/v).

Table 4 Separation of transglycosylation products on the TLC using non–polar mobile phase, ethyl acetate:acetic acid:water (3:1:1 by volume).

Alcohol acceptors	Number of products observed on TLC	R_f value
Methanol	1	0.30
Ethanol	2	0.43
		0.22
Propanol	3	0.44
		0.30
2–Propanol	–	0.54
		0.28
Butanol	3	0.66
		0.39
		0.24
Isobutanol	3	0.66
		0.39
		0.24
2–Butanol	3	0.43
		0.36
		0.21
tert–Butanol	0	–

Table 4 Separation of transglycosylation products on the TLC using non–polar mobile phase, ethyl acetate:acetic acid:water (3:1:1 by volume). Continued.

Alcohol acceptors	Number of products observed on TLC	R_f value
Pentanol	3	0.74
		059
		0.25
Isopentanol	2	0.74
		059
		0.25
2–Hexanol	3	0.81
		0.43
		–
Cyclohexanol	3	0.70
		0.42
		–
Heptanol	0	–
Octanol	0	–

Long chain primary alcohols (1–heptanol and 1–octanol) were used to study the ability of enzyme to catalyze the transferring of glucose molecule to long linear alkyl chain alcohol which has high potential for detergent industries. 1–Heptanol is slightly soluble in water while 1–octanol is insoluble. They were used at the concentration of 0.8–30% (v/v). However, no transglycosylation products were observed among all concentration studied.

From these results it could preliminary be summarized that the activity of CGTase was affected by alcohol to some extent. In addition, alkyl glycoside products from short chain and medium chain alcohols can be produced either in one phase or two phase reaction system. However, CGTase was not able to use tertiary alcohol and long chain alcohol as acceptor for transglycosylation reaction.

From these results, the production yield represented by spot intensities from isobutanol was higher than those obtained from other alcohols. Thus, it was selected as an acceptor for further experiments.

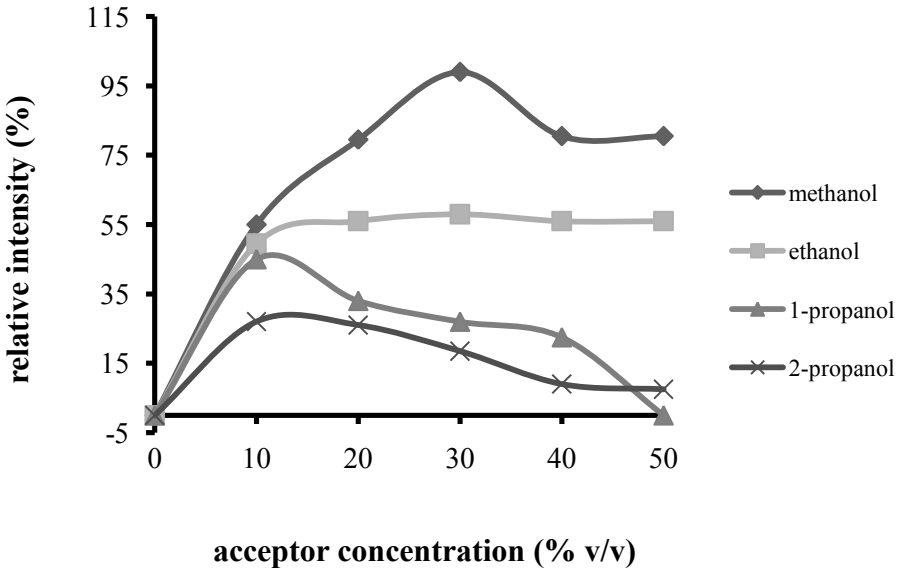


Figure 24 **Relative intensities of transglycosylation products from short chain alcohol acceptors. (**0.6% β– CD substrate incubated 200 units/mL CGTase for 24 hours. Glucose 15 µg was used as a standard). Data were from two separate duplicated experiments.

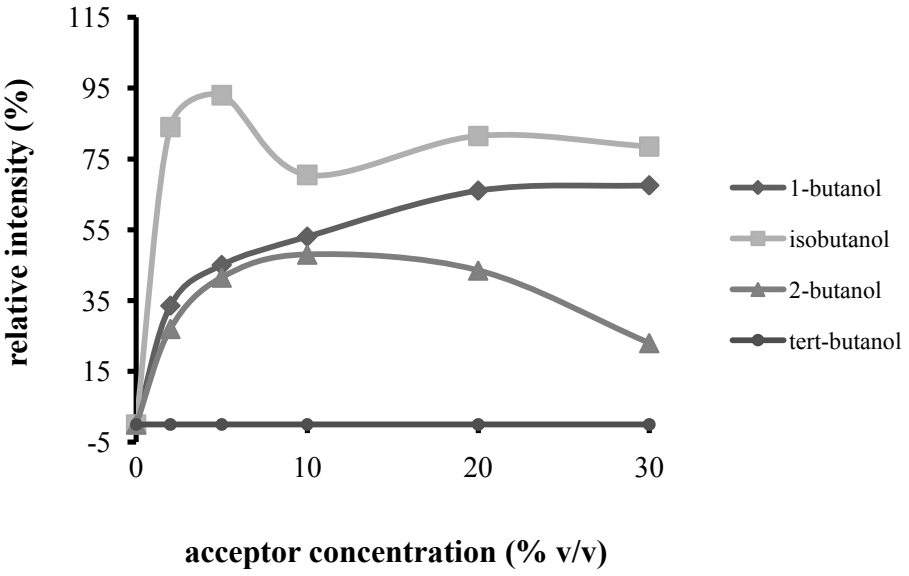


Figure 25 **Relative intensities of transglycosylation products from C_4 alcohol acceptors.** (0.6% β– CD substrate incubated with 200 units/mL CGTase for 24 hours. Glucose 15 µg was used as a standard). Data were from two separate duplicated experiments.

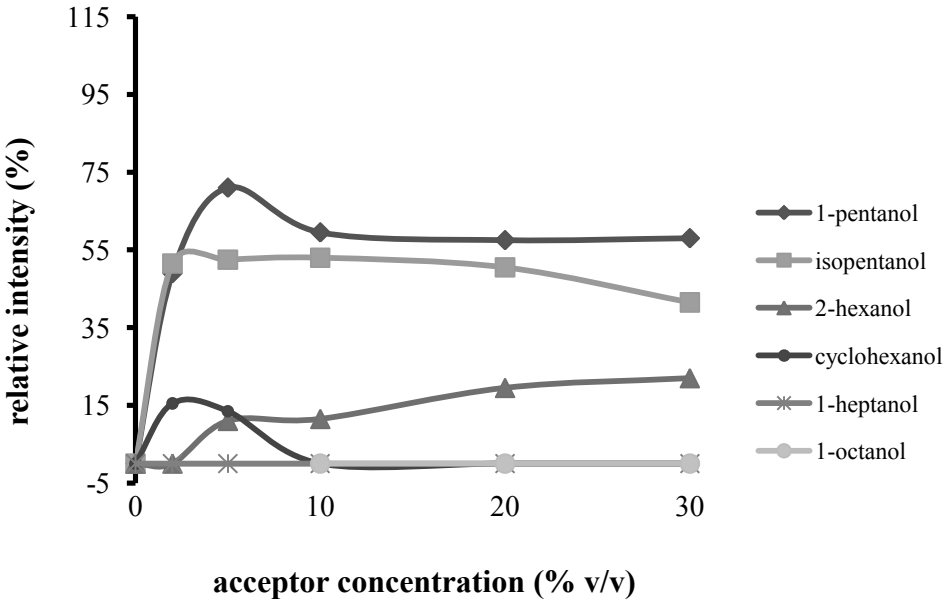


Figure 26 **Relative intensities of transglycosylation products from C_5, C_6, C_7 and C_8 alcohol acceptors. (**0.6% β–CD substrate incubated with 200 units/mL CGTase for 24 hours. Glucose 15 µg was used as a standard). Data were from two separate duplicated experiments.

3.5 Donor specificity

Transglycosylation ability of CGTase for the production of a novel isobutyl glycosides was determined using various carbohydrate donors including β–CD, soluble starch both from potato and cassava–flomax® and raw starch from cassava (commercial grade).The reactions were performed at the substrate final concentration of 0.6% (w/v). Raw starch is insoluble, therefore, it was boiled until it was completely gelatinized. The raw starch gel was used immediately in an hour of preparation. The selected alcohol acceptor–isobutanol at 5% (v/v) was incubated with 200 units/mL of CGTase in 50 mM acetate buffer pH 6.0 at 40 ºC for 24 hours. Reactions were analyzed by TLC to determine the suitable glycosyl donor (Figure 27). The reaction without glycosyl acceptor incubated with CGTase for 24 hour was used as a control (Lane 4, 7, 10 and 13 Figure 27). The reactions without incubation in the presence of 5% isobutanol and various glycosyl donors were also checked (Lane 5, 8, 11 and 14 Figure 27). Qualities were identified from the Rf values observed on the TLC. It was found that hydrolysis and all transglycosylation products from each donor were observed with the same Rf values, suggesting that the same product types were produced. The three main transglycosylation products from all starch donors were the same as those found when β–CD was used as a donor. The transglycosylated products were named as followed: at Rf = 0.74 as isobutyl monoglucoside (ISG1), Rf = 0.56 as isobutyl–maltoside (ISG2) and Rf = 0.36 as isobutyl maltotrioside (ISG3).

Quantitative analysis was determined by the spot intensities and calculated as (%) mole with the calibration curve of standard methyl–α–D–monoglucoside. The amount of yield was summarized in Table 4. The results revealed that β–CD gave highest total production yield (7.2%). The products obtained from cassava soluble

starch were quite similar with those obtained from potato soluble starch (7.0% and 6.8%, respectively). Raw starch gave a slight lower yield (6.1%) than those of other glycosyl donors. However, it is inexpensive and easily obtained from renewable agricultural resources. Hence, it might have high potential for application in the manufacturing process.

The appropriate glycosyl donor for the synthesis of isobutyl glycosides was β–CD. The ratio of the amount of ISG1 and ISG2 from this donor was about 1:1. While, ISG3 was found two times less. This could be explained by the fact that CGTase has starch hydrolysis activity. Thus, it suggested that the ISG3 was then used as a substrate by hydrolysis activity of CGTase. Therefore, it was hydrolyzed and released as a shorter product. An HPLC analysis was carried out using reverse–phase C18 column with polar mobile phase (system was previously described in section 2.13.2). HPLC profile of transglycosylation reaction of selected alcohol acceptor (isobutanol) and glycosyl donor (β–CD) incubated with CGTase is shown in Figure 28. From HPLC profile, after 24 hours of incubation, the three separated peaks were detected. The isobutanol peak was still detected (Rt ~12 min) although it was previously evaporated before reaction was subjected to analysis. According to the separation condition, in which the separation of substances is dependent on their polarity, the longer retention times, the less polarity. Therefore, ISG1 was suggested as peak Rt ~9 min, ISG2 was at 8 min and ISG3 was at 7 min. However the hydrolysis products from the action of CGTase and β–CD substrate which were previously observed on TLC could not be separated under this condition.

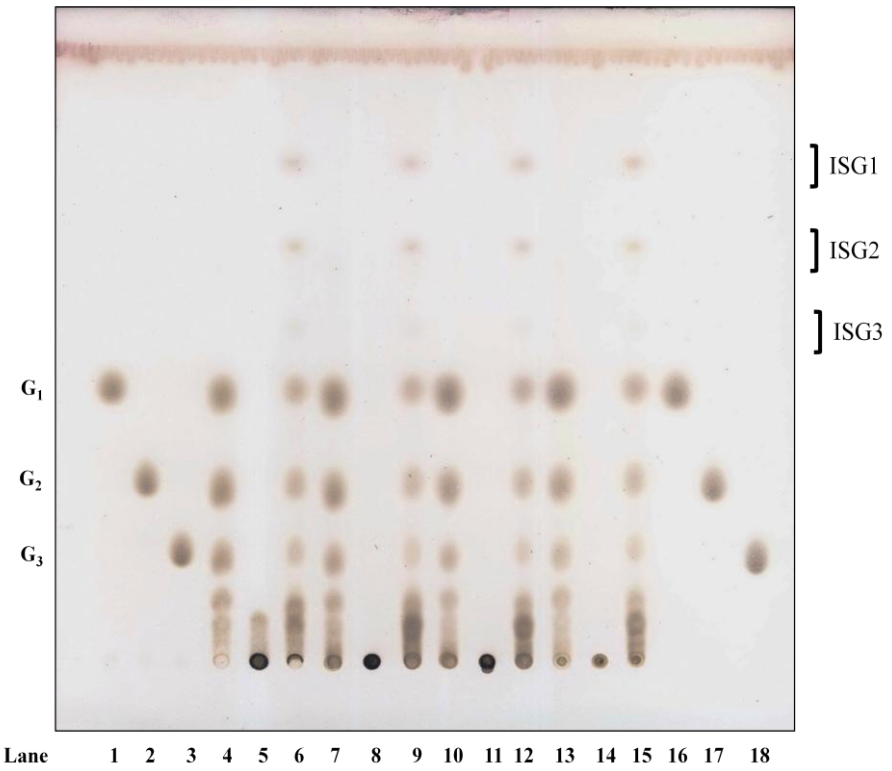


Figure 27 TLC chromatogram of reaction products of CGTase incubated with isobutanol and various donors. TLC condition was ethyl acetate/acetic acid/water, 3:1:1.

Lane 1, 16: Standard glucose (25 μg)

Lane 2, 17: Standard maltose (25 μg)

Lane 3, 18: Standard maltotriose (25 μg)

Lane 4: Control reaction (β–CD + CGTase)

Lane 5–6: Reaction mixture of β–CD as a donor, 0 and 24 h

Lane 7: Control reaction (potato soluble starch + CGTase)

Lane 8–9: Reaction mixture of potato soluble starch as a donor, 0 and 24 h

Lane 10: Control reaction (cassava soluble starch + CGTase)

Lane 11–12: Reaction mixture of cassava soluble starch as a donor, 0 and 24 h

Lane 13: Control reaction (raw cassava starch + CGTase)

Lane 14–15: Reaction mixture of raw cassava starch as a donor, 0 and 24 h

Table 5 Transglycosylation production yield from various glycosyl donors incubated with 5% (v/v) of isobutanol acceptor.

Glycosyl donor	Production yield (%) (Mean)			
	Isobutyl monoglucoside	Isobutyl maltoside	Isobutyl maltotrioside	Total
β–Cyclodextrin	3.2	2.6	1.4	7.2
Potato soluble starch	3.0	2.6	1.2	6.8
Cassava soluble starch	3.2	2.6	1.2	7.0
Raw cassava starch	2.7	2.2	1.2	6.1

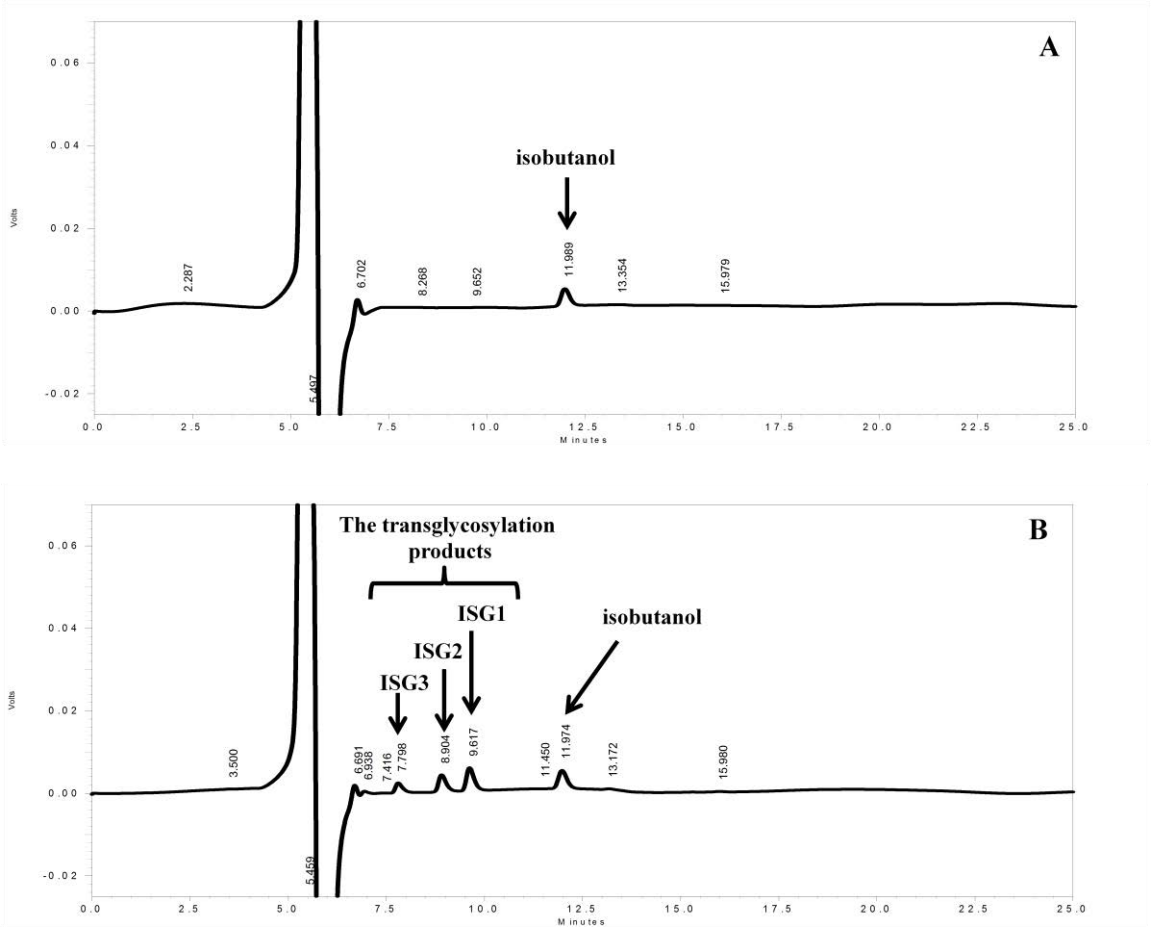


Figure 28 **HPLC profile of transglycosylation reaction mixture catalyzed by CGTase** (mobile phase was mixture of 40% methanol in water containing 0.2% (v/v) acetic acid with a flow rate of 0.5 mL/min at 50 °C) **A :** No incubation and **B:** 24 hours incubation.

3.6 Characterization of reaction products using glucoamylase and α–glucosidase

The transglycosylation products from CGTase catalyzed the transferring of glycosyl unit from donor to isobutanol had been preliminary characterized. The reaction mixture of CGTase catalyzed transglycosylation reaction for 24 hours was treated with two amylolytic enzymes –glucoamylase and α–glucosidase. In this research, it was used to confirm that the products from the action of CGTase were composed of the maltooligosyl unit. The transglycosylation reaction of CGTase reaction mixture was evaporated to remove the isobutanol substrate remained after the reaction. After removing isobutanol, reaction was then subjected to treatment with glucoamylase (final concentration of 30 units/mL) at 40ºC for 1 hour. The glucoamylase treated reaction was stopped by boiling for 10 minutes. The TLC chromatogram is shown in Figure 29. Lane 1–4 was methyl–α–D–glucopyranoside, glucose, maltose and maltotriose, respectively. The reaction mixture of CGTase with β–CD and isobutanol with 0 hour incubation was in lane 5 while the reaction of CGTase catalyzed transglycosylation for 24 hours was in lane 6 (Figure 29). The hydrolysis and the transglycosylation products from the action of CGTase were produced which were observed on the TLC chromatogram (Lane 6, Figure 29) and the HPLC profile (Figure 28). The amount of the transglycosylated products observed from TLC and HPLC were ISG1 ≈ ISG2 > ISG3. When the reaction mixture from CGTase catalyzed transglycosylation reaction was treated with glucoamylase, the intensity of ISG1 and glucose observed on TLC (Figure 29, lane 7) and HPLC (Figure 30) were increased. Whereas the oligosaccharides (maltose and maltotriose) from hydrolysis activity and the transglycosylated products attached with oligosaccharides

(ISG2 and ISG3) were disappeared on TLC (Figure 29, Lane 7). The HPLC profile (Figure 31) showed dramatically decreased of ISG2 at Rt ~8 min where the signal of ISG3 at Rt ~7 min disappeared. This can be clearly seen by comparison the spot intensity with lane 6 and HPLC profile in Figure 31. However, glucose and others hydrolysis product had the same retention time. These results could be explained by the hydrolysis activity of glucoamylase between glucose residues of oligosaccharides resulting in monosaccharide–glucose as a final product. With the same activity of glucoamylase, transglycosylation products composed of oligosaccharide conjugated with isobutanol had also been further hydrolyzed. Thus, the final products after treatment with glucoamylase were glucose and isobutyl monoglucoside (ISG1).

α–Glucosidase (EC 3.2.1.20) is one of the amylolytic enzyme which can be used to preliminary characterize the transglycosylation products. Treatment of the transglycosylation products with α–glucosidase was performed at 40 °C for 3 hours at the final concentration of 30 unit/mL. With the hydrolysis activity of α–glucosidase, it was found that only the spot of glucose was observed on TLC and the intensity of ISG1 observed on TLC was disappeared (Figure 29, Lane 8). This corresponded to the dramatically decrease of a peak at Rt ~9 min in HPLC (Figure 32). Thus, it was concluded that the ISG1 was transglycosylation products from CGTase with α–configuration structure.

After treatment of the 24–hour CGTase catalyzed reaction with glucoamylase for 1 hour and further hydrolyzed with α–glucosidase for 3 hours, Figure 29 (lane 9), the intensity of ISG1 disappeared which corresponded to the decrease of peak at Rt ~9 min (Figure 33). This result was similar to those obtained from the treatment with only α–glucosidase. From the treatment of these amylolytic enzymes, the results

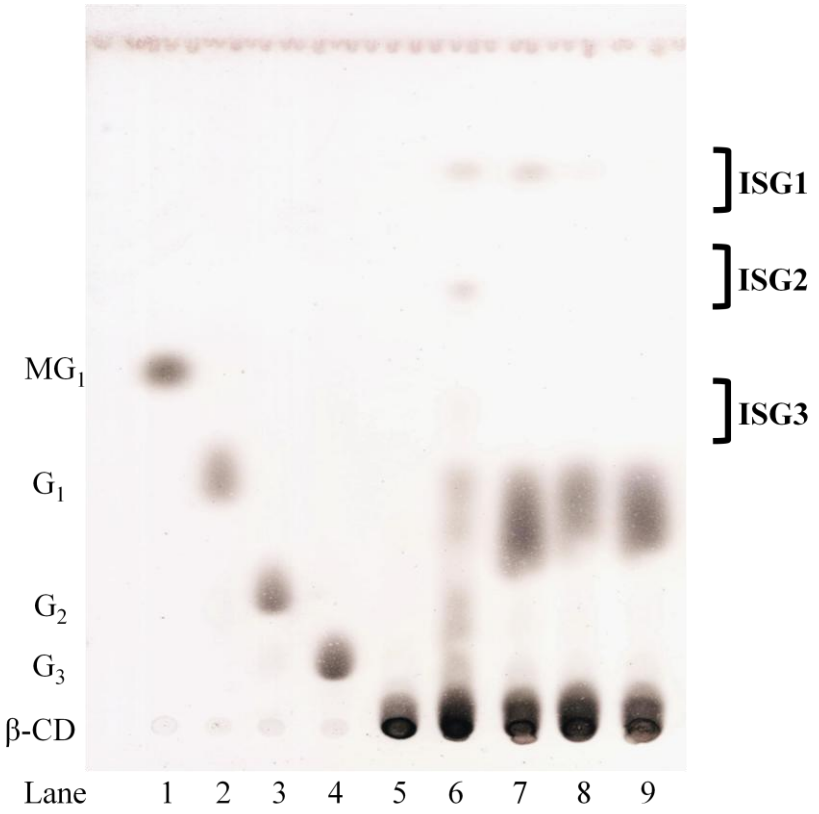


Figure 29 **TLC chromatogram of amylolytic enzymes treatment of transglycosylation products.**

Lane 1: Standard glucose(25μg)

Lane 2: Standard maltose (25 μg)

Lane 3: Standard maltotriose (25 μg)

Lane 4: Standard methyl glycoside (25 μg)

Lane 5: Transglycosylation reaction incubated for 0 hour

Lane 6: Transglycosylation reaction incubated for 24 hours

Lane 7: Transglycosylation reaction (24 hours) incubated with glucoamylase

Lane 8: Transglycosylation reaction (24 hours) incubated with α–glucosidase

Lane 9: Transglycosylation reaction (24 hours) incubated with glucoamylase and α–glucosidase

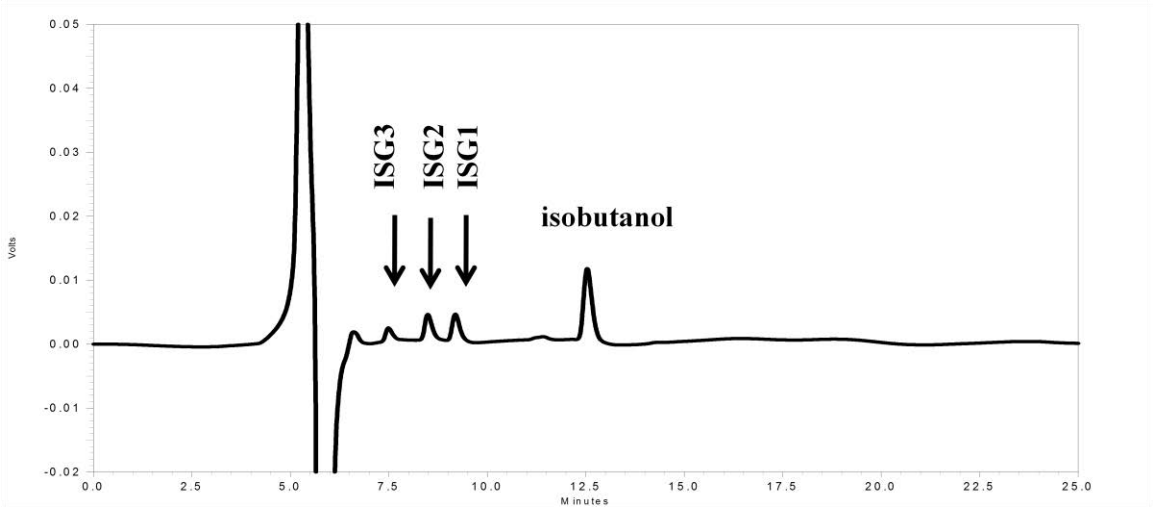


Figure 30 **HPLC chromatogram of the transglycosylation products from the CGTase catalyzed transglycosylation reaction from β–CD to isobutanol.**

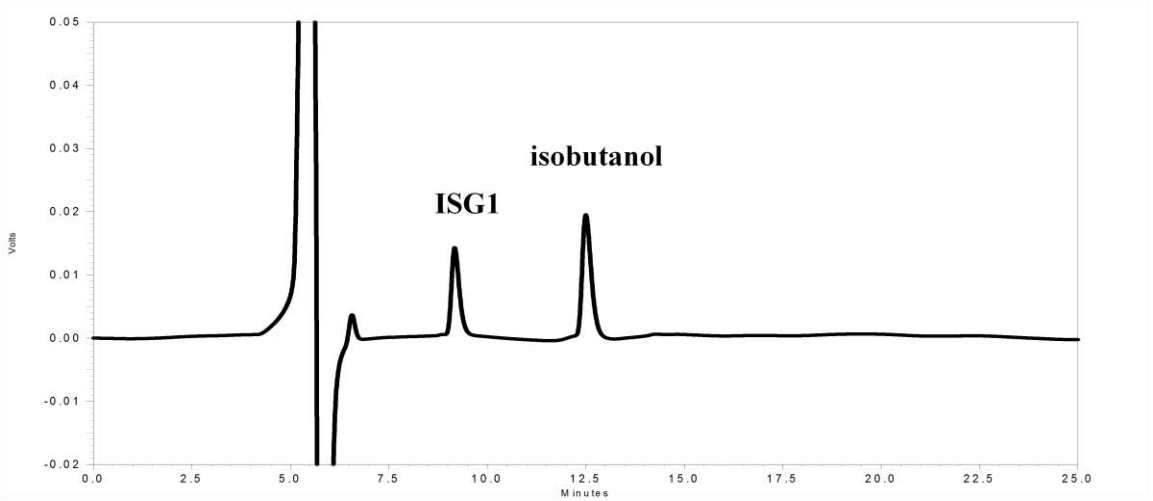


Figure 31 **HPLC chromatogram of the transglycosylation products treated with glucoamylase (final concentration of 30 U/mL)**.

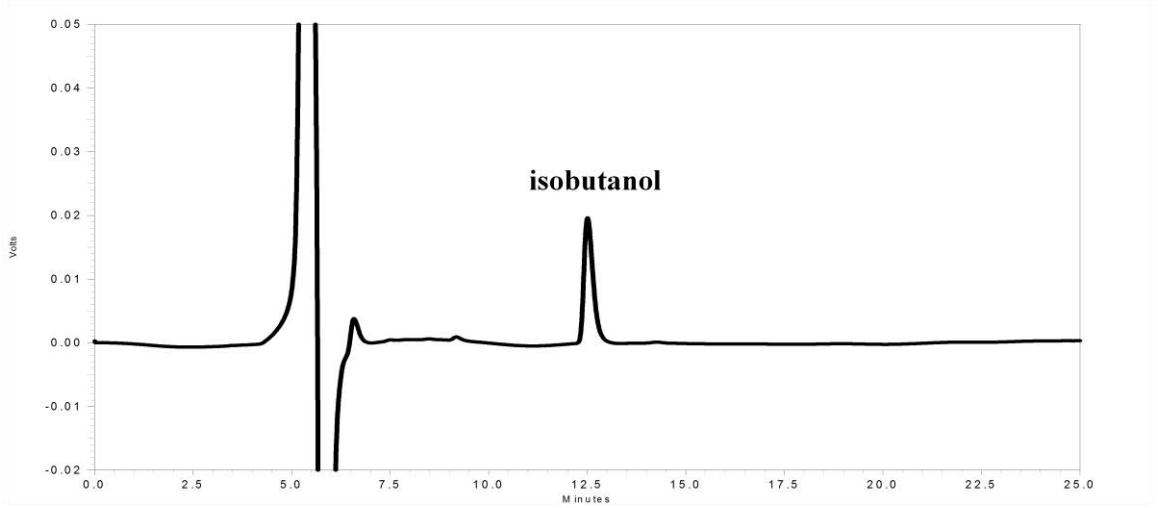


Figure 32 **HPLC chromatogram of the transglycosylation products treated with α–glucosidase (final concentration of 30 U/mL).**

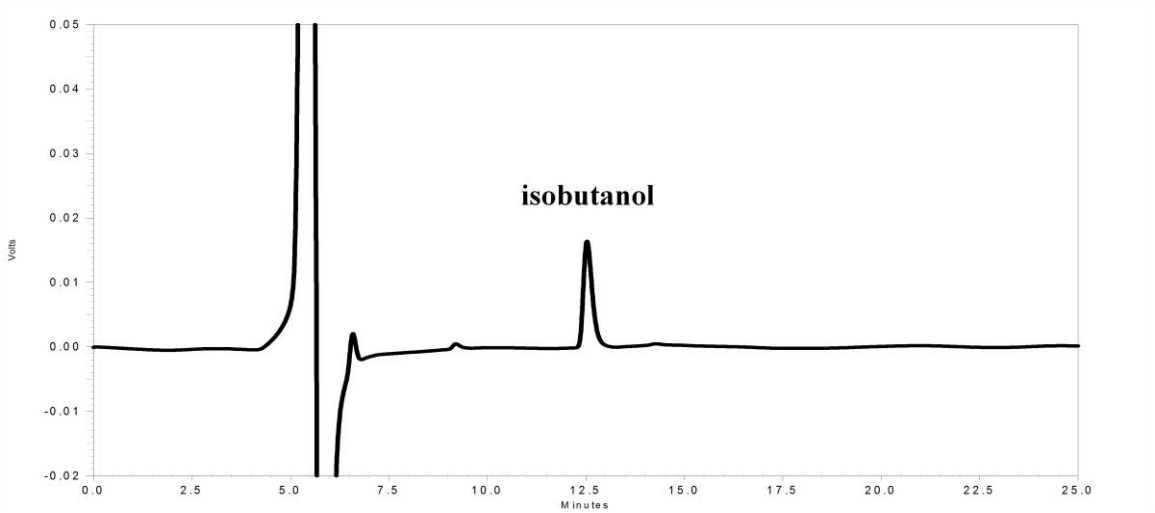


Figure 33 **HPLC chromatogram of the transglycosylation products treated with glucoamylase and α–glucosidase (both amylolytic enzymes used at final concentration of 30 U/mL).**

obtained could preliminary confirm that the transglycosylation products from β–CD to isobutanol were isobutyl glycoside derivatives.

3.7 Optimization condition for transglycosylation products

Synthesis of the isobutyl glycosides by one–step catalysis of partial purified CGTase under the optimum conditions transglycosylation reaction was reported. The optimum pH and temperature of CGTase from *Paenibacillus* sp. RB01 for transglycosylation reaction followed previous report by Chotipanang (2010). The optimum pH was 50 mM acetate buffer pH 6.0, and the suitable temperature was 40 °C. Other conditions (concentration of isobutanol and β–CD, the amount of enzyme, and the incubation time) were then optimized to achieve the highest yield. The amount of the product was analyzed from total peak area of transglycosylated products

3.7.1 Isobutanol concentration

Isobutanol which was previously selected as a glycosyl acceptor was varied from 0–30% (v/v). The reactions mixtures were incubated with the final concentration of 0.6% (w/v) β–CD in 50 mM acetate buffer pH 6.0. The reaction was started with the addition of 200 U/mL CGTase and incubated at 40 ºC with continuous shaking for 24 hours. Production yield were calculated from the total peak area from HPLC. Yield was calculated as described in section 2.14.1 (see Eq.2).

Alkyl glycoside yield was highest when 5% (v/v) of isobutanol was used. Increasing isobutanol concentration did not increase the production of alkyl glycosides (Figure 34, A) In addition, the concentration of organic alcohol acceptor used to achieve the highest amount of product was relatively low and thus, is

applicable to be applied in environmental care products and green production process. The suitable concentration of isobutanol was at 5% (v/v) and was further used in the next experiment.

3.7.2 β–CD concentration

Final concentration of β–CD substrate from 0–1.8% (w/v) was done. The reaction of various β–CD concentrations were incubated with the optimized isobutanol concentration (5%, v/v) in 50 mM sodium acetate buffer pH 6.0. The final concentration of 200 U/mL CGTase were added and incubated for 24 hours at 40 °C. Reaction termination was employed by boiling the reaction mixture for 10 minutes. The amounts of products obtained were calculated as previously described. The appropriate donor concentration was found to be 1.2% (w/v), (Figure 34 B).

3.7.3 Enzyme concentration

The optimization of the enzyme concentration for the synthesis of isobutyl glycosides catalyzed by CGTase was studied. The reaction mixture of 5% (v/v) isobutanol and 1.2% (w/v) β–CD were incubated with 0–500 units/mL of dextrinizing activity of CGTase. The reactions were performed in 50 mM acetate buffer pH 6.0 at 40° C for 24 hours. The suitable enzyme concentration was judged by HPLC. The optimum amount of enzyme for the production of isobutyl glycoside was 450 units/mL (Figure 34 C).

3.7.4 Incubation time

The incubation time for the CGTase catalyzed intermolecular transglycosylation from β–CD donor to isobutanol acceptor was also checked. The reaction mixture of optimized concentration of donor, acceptor and enzyme was performed in 50 mM sodium acetate buffer pH 6.0 at 40 ºC. After addition of the final

concentration of 450 units/mL of CGTase, sample of the interval time was withdrawn to determine the amount of products obtained. The higher production yield was obtained after incubation for 2–3 days and reached plateau (Figure 34 D).

Thus, the suitable conditions for the production of isobutyl glycosides were to use 5% of isobutanol acceptor, 1.2% β–CD as glycosyl donor incubated with 450 units/mL (dextrinizing activity) of CGTase for 48 hours at 40 °C, pH 6.0. The HPLC chromatogram of transglycosylation reaction incubated with CGTase before and after optimization condition is shown in Figure 35. After optimization, total yield was increased about 3.2 times and the ratio of ISG1, ISG2 and ISG3 was 4.7:2.6:1.

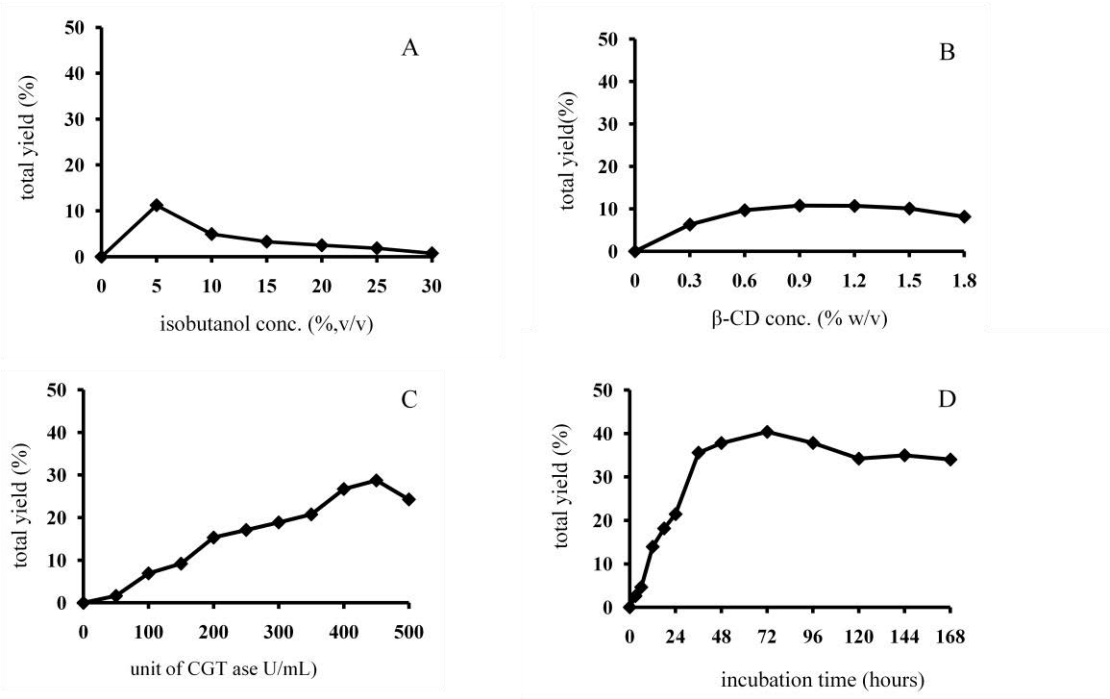


Figure 34 **Optimization conditions for the production of isobutyl glycoside by CGTase**. **(A)** isobutanol concentration, **(B)** β–CD concentration, **(C**) Enzyme activity and **(D**) incubation time. The transglycosylation reactions were performed in 50 mM acetate buffer at 40°C.

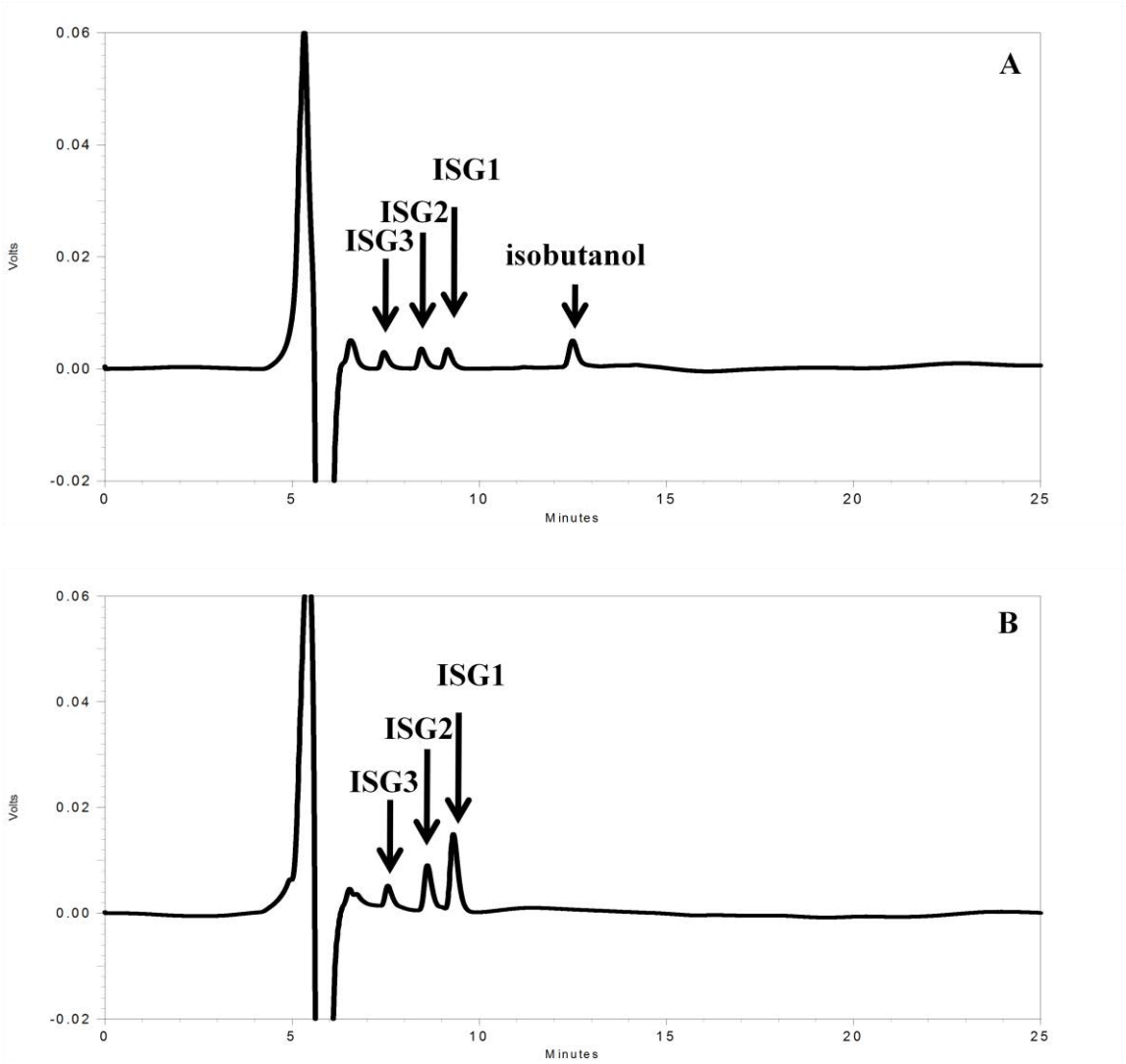


Figure 35 **HPLC chromatogram of the transglycosylation products catalyzed by CGTase**. **(A)** before optimization **(B)** after optimization.

3.8 Time course production of isobutyl glycosides

Isobutyl glycosides synthesized from the CGTase catalyzed intermolecular transglycosylation reaction between β–CD substrate and isobutanol acceptor was identified as time dependent. Reaction mixture was prepared under the optimum conditions obtained from previous results (section 3.6). The amount of transglycosylation products were analyzed by HPLC. From Figure 36, at the initial of the production process the main products were isobutyl monoglucoside (ISG1), isobutyl maltoside (ISG2) and isobutyl maltotrioside (ISG3), respectively. The amount of isobutyl polyglucosides (ISG2 and ISG3) was found to decrease after 2 days of incubation. This can be described by the glucoamylase-like activity of CGTase which removed glucose units sequentially from the non–reducing end of oligosaccharide moiety conjugated to ISG1.

3.9 Mass spectrophotometry

To investigate the nature of the transglycosylation products produced by CGTase, mass spectroscopy (MS) was employed to determined mass/charge ratios of samples.

Reaction mixtures which were analyzed by MS had been prepared by 2 conditions. In the first condition, transglycosylation reaction was proceed by the use of 200 units/mL CGTase, 0.6% β–CD (w/v) and 5% (v/v) of isobutanol acceptor at 40 °C for 24 hours. For the second reaction condition, it was prepared under optimized condition which was obtained from previous studies, which was (v/v) of isobutanol, 1.2% (w/v) of β–CD, and 450 units/mL of CGTase and incubated for 48 hours at 40 °C. The results were illustrated in Figure 37 and 38, respectively.

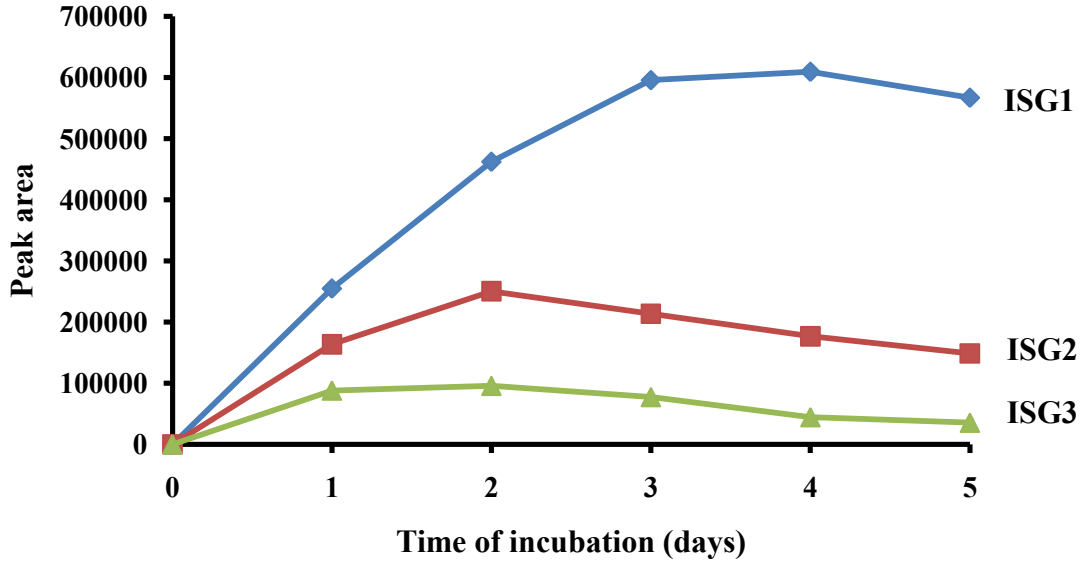


Figure 36 **Time course production of isobutyl glycosides from CGTase catalyzed transglycosylation reaction.**

The positive ion–mode MS spectrum of the reaction mixture gave 2 main hydrolysis products at *m/z* : 203. 05 and 365.10 which were confirmed to be glucose–$Na^+$and maltose–Na^+, respectively. The 2 main transglycosylation products were observed at *m/z* : 259.11 and 421.16 which were ISG1–Na^+ and ISG2–Na^+, respectively.

Preparative TLC (PLC) was employed to purify transglycosylation products by application of an intense reaction mixture on the Merck preparative plate (1 mm). Mobile phase used here was the same as in TLC analysis. The expected products at the indicated Rf value on PLC was scraped out separately then, it was subjected to solvent extraction with methanol. Prior to the MS analysis, the isolated products were checked by TLC and HPLC. The reaction mixture from the catalysis of CGTase was in Lane 1 and glucose was in Lane 2 and 5 (Figure 39). The expected products isolated at individual Rf on PLC were in Lane 3 and Lane 4. The TLC showed that the isolated products from PLC were purified. Furthermore, the expected products which were isolated from PLC and methanol extraction were also checked by HPLC (Figure 40). The signal at Rt ~9 and 8 min were observed from reaction of Lane 3 and 4, respectively. The peak at Rt ~5 min was found suggesting that it was a signal from the remaining methanol or silica contamination (Figure 40). The products separated through this chromatography method were then subjected for mass determination by MS analysis. The results confirmed that the products at Rf = 0.74 (Rt ~9 min) was isobutyl monoglucoside (259.1 m/z) (Figure 41 A) and the product at Rf = 0.56 (Rt ~8 min) was isobutyl maltoside (421.67m/z) (Figure 41 B).

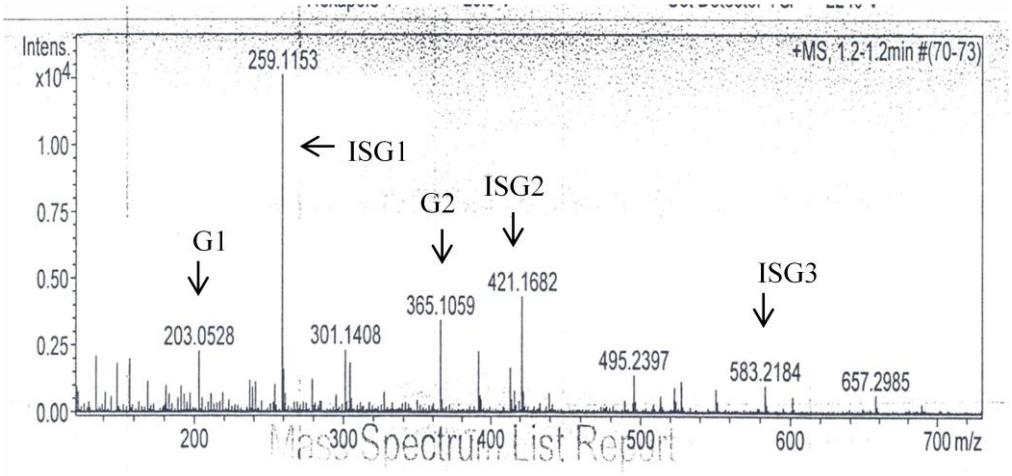


Figure 37 **ESI–TOF of CGTase catalyzed reaction** (transglycosylation reaction from β–CD (0.6%, w/v) to 5% (v/v) isobutanol incubated with 200 units/mL of CGTase for 24 hours).

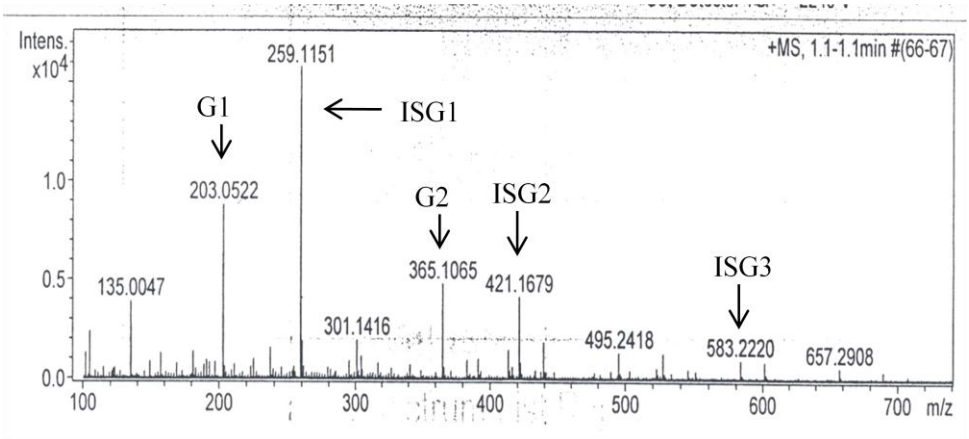


Figure 38 **ESI–TOF of CGTase catalyzed reaction** (transglycosylation reaction from β–CD (1.2%, w/v) to 5% (v/v) isobutanol incubated with 450 units/mL of CGTase for 48 hours).

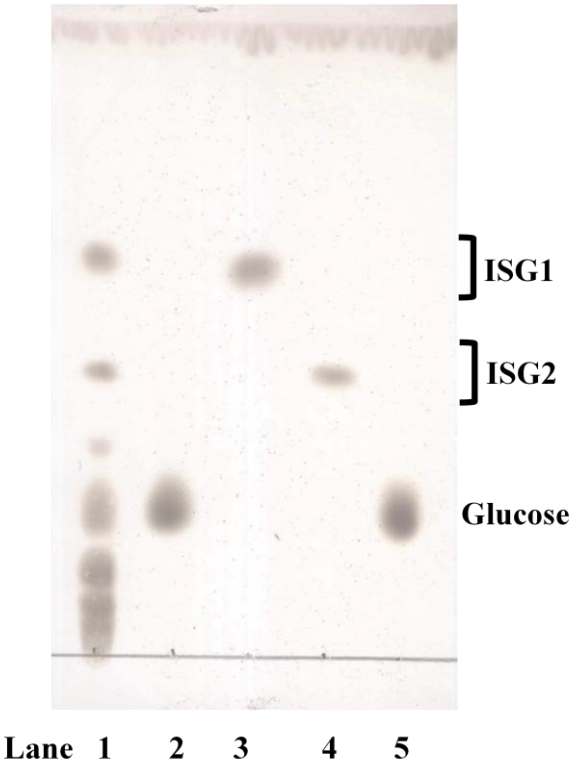


Figure 39 TLC chromatogram of the products isolated from the PLC.

Lane 1: Transglycosylation reaction of CGTase from β–CD to isobutanol

Lane 2: Standard glucose (15 μg)

Lane 3: The expected isobutyl–α–monoglucoside isolated from PLC

Lane 4: The expected isobutyl–α–maltoside isolated from PLC

Lane 5: Standard glucose (15 μg)

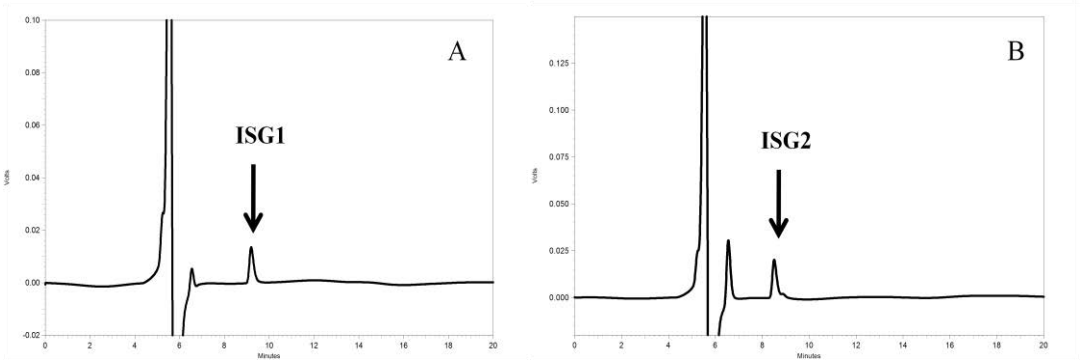


Figure 40 **HPLC profile of the products isolated from the PLC (A)** the expected product at Rf value of 0.66 and at 0.39 (**B**).

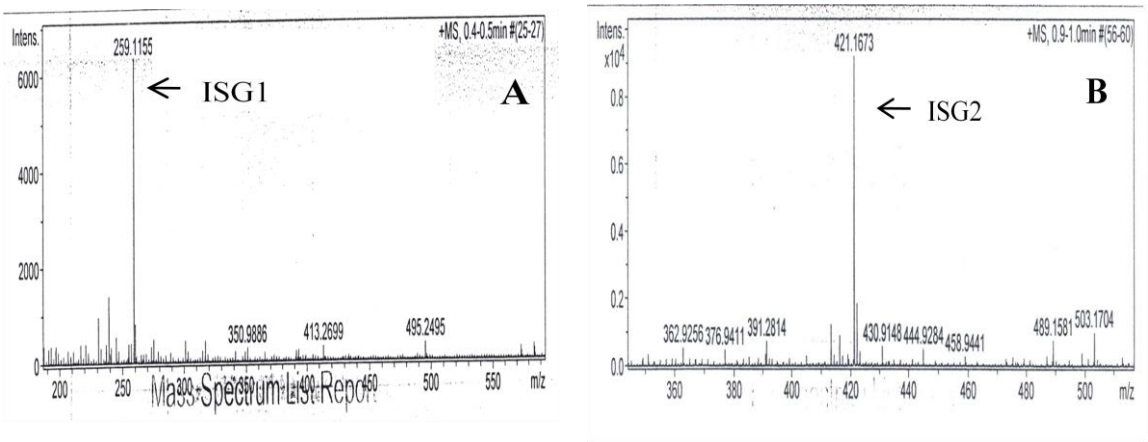


Figure 41 **ESI–TOF of isobutyl–α–monoglucoside (A) and isobutyl–α–maltoside (B) synthesized by CGTase catalyzed reaction**.

3. 10 Large scale production of isobutyl glycoside

Large scale production (12.5 mL) was performed after the reaction conditions were optimized as described in materials methods section 2.15. The reaction mixture was concentrated by evaporation at 40°C and the final volume was then adjusted to 1 mL with distilled water. The concentrated reaction was applied onto Amberlite IRA–900 column.

3. 11 Purification of alkyl glycosides using strong anionic column chromatography

Transglycosylation products from large scale production (12.5 mL) were then purified using strong anionic column chromatography. The collected samples were checked the total sugar by treatment with phenol sulfuric acid solution. The absorbance was then read at 485 nm. The purification profile from phenol–sulfuric acid test is shown in Figure 42, 5 peaks (a–e) were observed. These fractions were then checked by TLC and HPLC. From the TLC, lane 1 was a mixture of standard glucose, maltose and maltotriose. Lane 2 was standard methyl–α–D–glucopyranoside. The fractions of peak a to peak e were in lane 3–7, respectively. The purified products were obtained at peak a (Rf 0.77) and c (Rf 0.58). Peak b was a mixture of 2 products and no products were detected from peak d and e (Figure 43). Peak a and c were ISG2 respectively. Peak b, when it was checked by HPLC, it gave two signals at Rt ~9 and 8 min, therefore it was a mixture of ISG1 and ISG2 (Figure 44). From these results, it was summarized that the sugars from the hydrolysis activity of CGTase were successfully removed. The two expected transglycosylated products purified through

this chromatography method were isobutyl–α–monoglucoside and isobutyl–α–maltoside. Isobutyl–α–maltotrioside could not be collected. This maybe because of the lower yield of this was produced from the action of CGase. The purified products were then characterized for the emulsification properties.

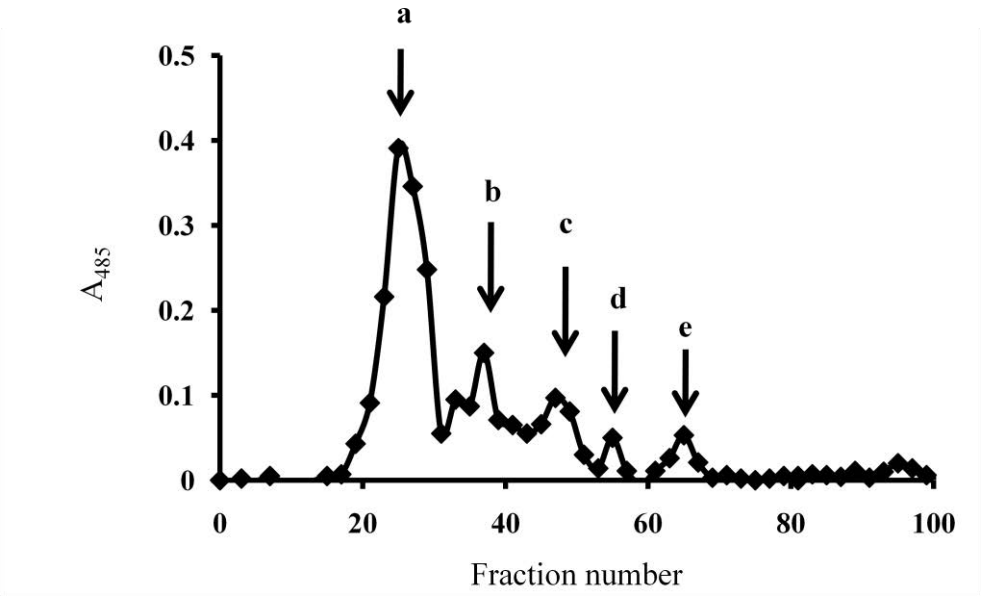


Figure 42 **Phenol–sulfuric test of the transglycosylation reaction of CGTase** (β–CD donor and isobutanol acceptor) **by Amberlite IRA900 Cl^- column chromatography** (Eluent: ultrapure water, Flow rate of 0.5 mL/min, and Fraction volume of 2 mL).

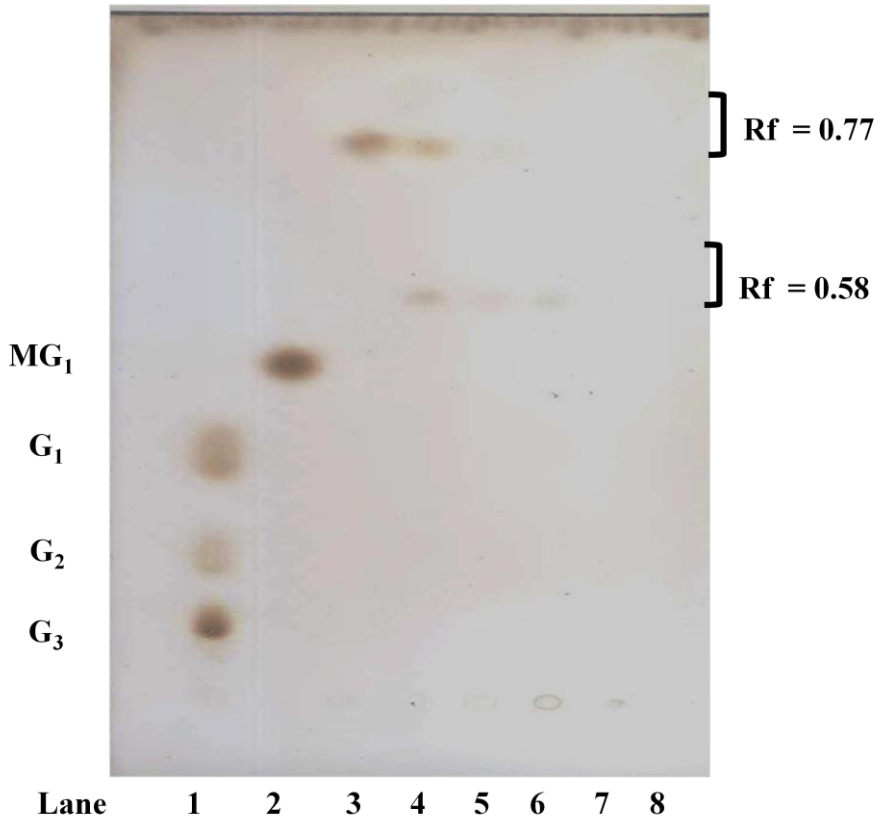


Figure 43 **TLC chromatogram of purified products from Amberlite IRA900 Cl^-column chromatrography** (Eluent: ultrapure water, Flow rate of 0.5 mL/min, and Fraction volume of 2 mL).

Lane 1: Standard mixture (glucose, maltose and maltotriose) 15 ug

Lane 2: Standard methyl glycoside (15 ug)

Lane 3: fraction number 24 (peak a)

Lane 4: fraction number 36 (peak b)

Lane 5: fraction number 39

Lane 6: fraction number 46 (peak c)

Lane 7: fraction number 53 (peak d)

Lane 8: fraction number 66 (peak e)

3. 12 Characterization of the alkyl glycoside products

3.12.1 Emulsification activity

Emulsification activities of purified isobutyl–α–monoglucoside and isobutyl –α–maltoside were characterized from their ability to form oil in water emulsion (O/W) with n–hexadecane substrate. The mixture was mixed on a vortex mixer. The resulting uniform emulsion was determined by spectrophotometer at 540 nm. Standard methyl–α–D–monoglucoside as well as isobutanol was also checked for emulsification activity. Triton X–100®, a commercial nonionic surfactant was also studied and set as 100 percent activity. The turbidity of other glycosides were measured and compared with the turbidity of Triton X–100®. The results revealed that methyl–α–D–monoglucoside had better emulsification activity than isobutyl–α–monoglucoside. This result suggested that the emulsification activity of linear alkyl–α–monoglucoside decreased with an increasing number of hydrophobic alkyl chain length. While isobutyl–α–maltoside showed higher emulsification activity than those of methyl–isobutyl–α–monoglucoside (Figure 45), suggesting that the behavior of isobutyl–α–maltoside and isobutyl–α–monoglucoside were different. The hydrophobic interactions of alkyl–α–manoside is stronger than that of alkyl–α–monoglucoside although these two isomers differ only by the position of one hydroxyl group. Therefore, this suggested that the water molecules around alkyl–α–mannoside are more ordered (Brown et al., 1970). From these results, it could be concluded that purified isobutyl glycosides derivatives showed effect to O/W emulsion depending on the number carbons on alkyl chain and the number of sugar residues.

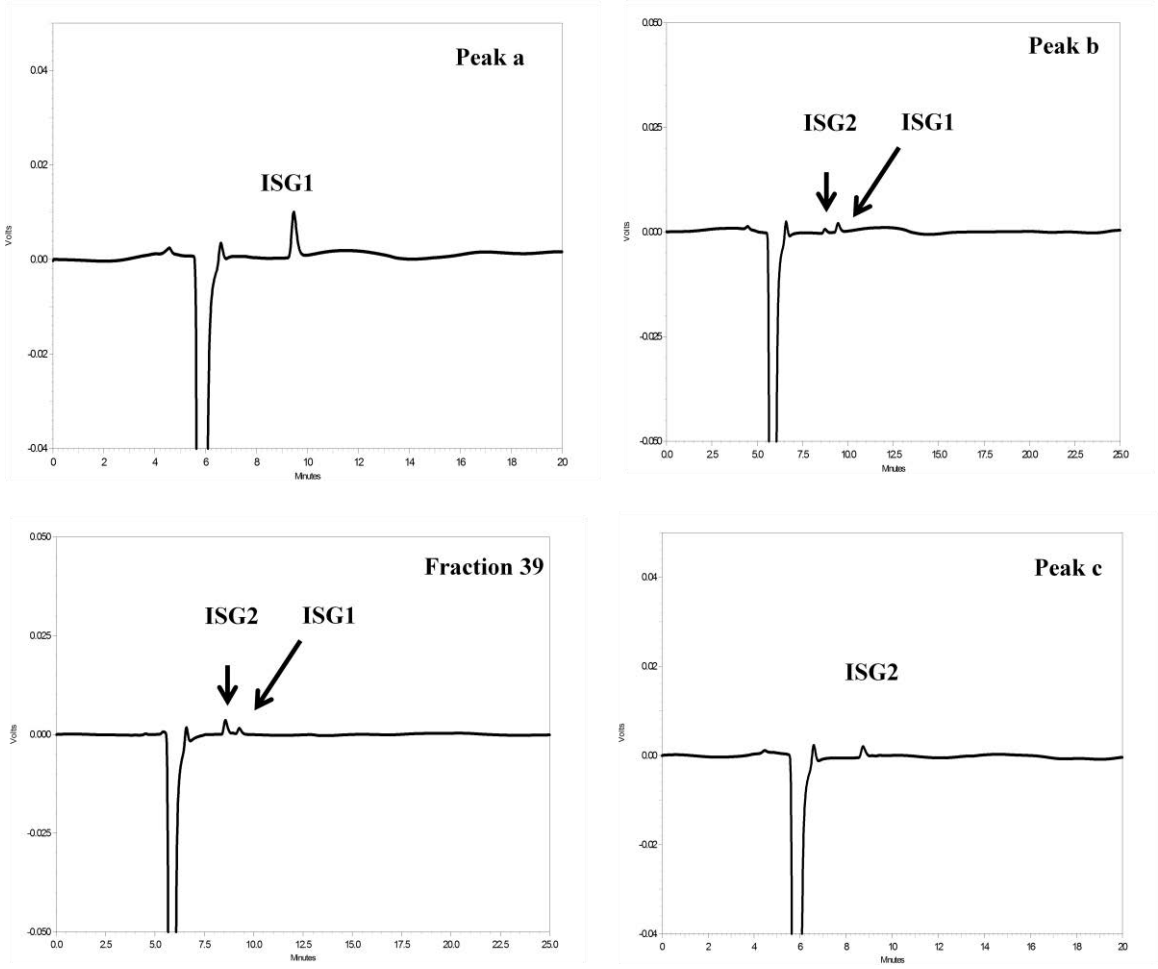


Figure 44 **HPLC analysis of alkyl glycoside derivatives purified through column chromatography (**Using Amberlite IRA column equilibrated with 0.1 M NaOH. Fractions were collected with 2 mL at the flow rate of 0.5 mL/min under room temperature).

3.12.2 Emulsification stability

The stability of emulsion formed by alkyl glycoside was studied as function of time. Emulsion of n–hexadecane was performed and the turbidity was studied for 60 minutes by spectrophotometry at 540 nm. The emulsification stability was calculated from the dissociation constant (K_d) obtained from logarithmic plot of absorbance versus time (Figure 46). The smaller K_d the greater the stability. Triton X–100® was the most effective in stabilizing n–hexadecane, thus the stability of Triton X–100® to stabilize O/W emulsion was set as 100% emulsification stability. It was found that glycoside derivatives reduced the emulsion decay over 60 minutes time period examined. n–hexadecane forms emulsion with water in the presence of isobutanol. Nevertheless, these emulsion rapidly separated after 10 minutes. The addition of isobutyl glycoside derivatives to the emulsion showed that they were able to stabilize the emulsion over period of time tested. Isobutyl glycosides showed better ability to stabilize O/W stability than methyl–α–monoglucoside (Table 6).

From these results, isobutyl–α–maltoside exhibits good emulsification properties than isobutyl–α–monoglucoside and standard methyl–α–monoglucoside. This product composes the maltosyl group as a hydrophilic moiety conjugated with alkyl group from isobutanol. It is a novel product from CGTase which cannot be obtained from using glucosidase enzymes.

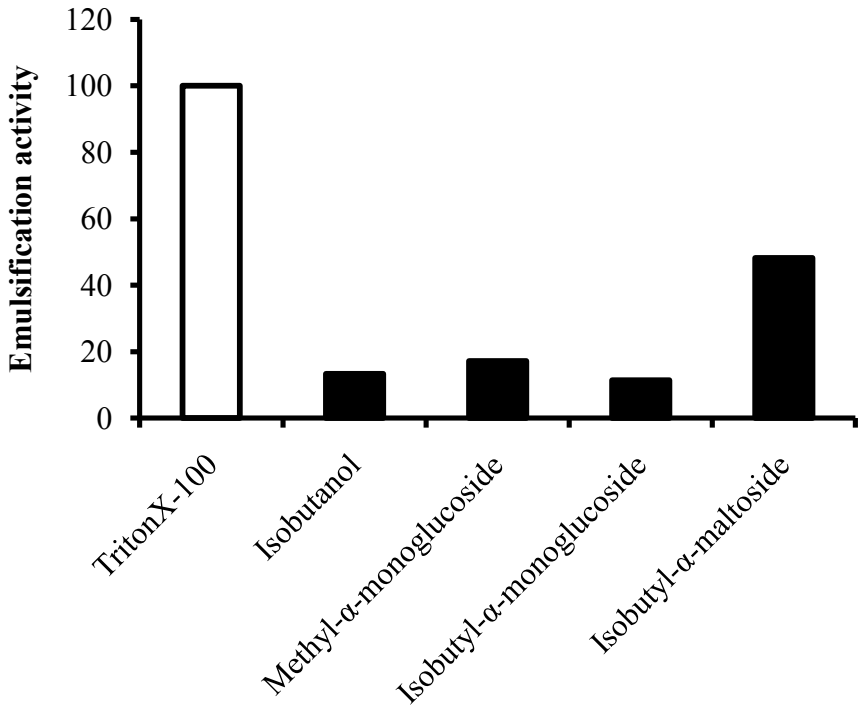


Figure 45 **Emulsification activities of isobutyl glycosides synthesizing from CGTase catalyzed transglycosylation reaction.**

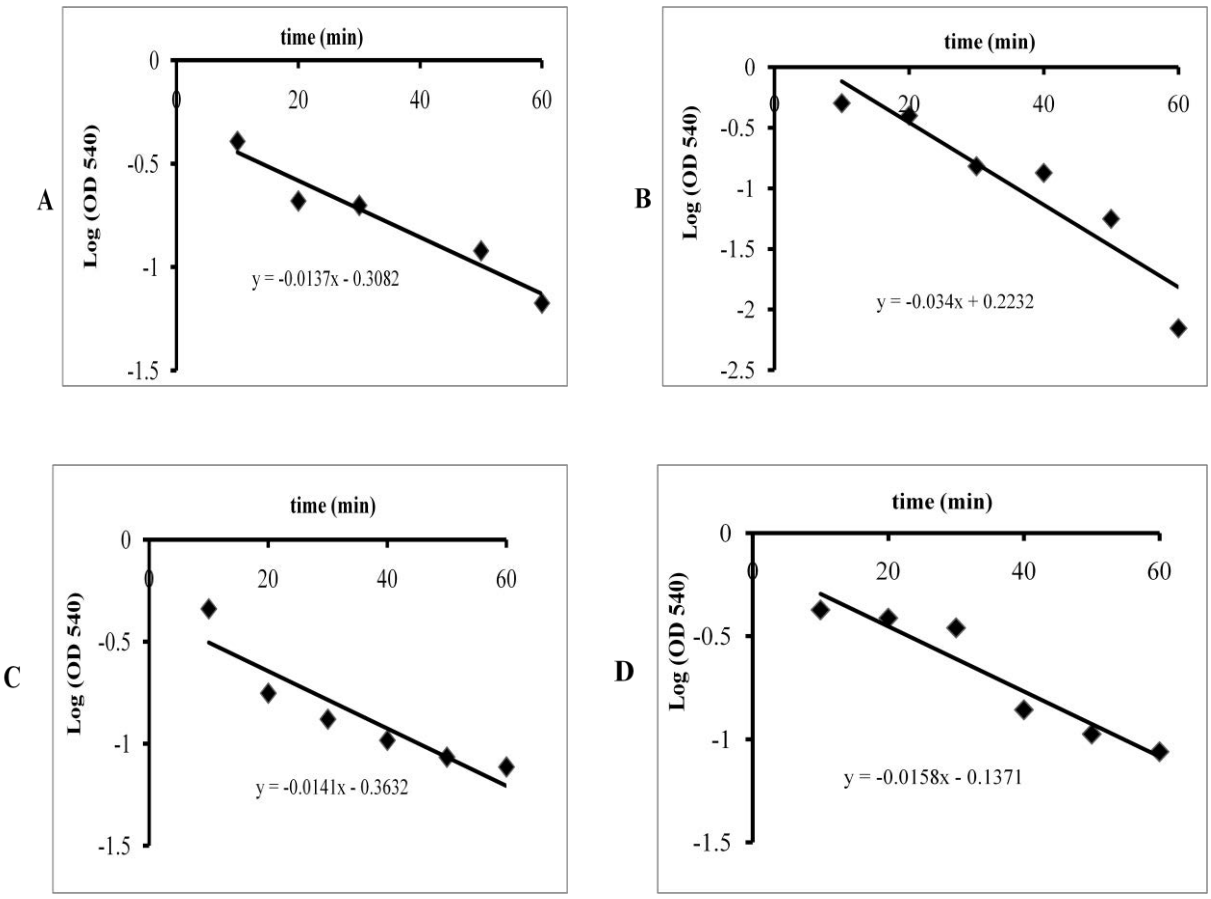


Figure 46 **Stabilization of n–hexadecane oil emulsion by; (A) TritonX–100, (B) Methyl–α–monoglucoside, (C) Isobutyl–α–monoglucoside and (D) Isobutyl–α–maltoside**.

Table 6 **The ability of transglycosylated products to stabilize emulsion of n–hexadecane.**

Surfactant	**K_d**	**Emulsification stability (%)**
Triton X–100	–0.0137	100
Isobutanol	–	–
Methyl–α–monoglycoside	–0.0340	40
Isobutyl–α–monoglucoside	–0.0141	93.5
Isobutyl–α–maltoside	–0.0158	86.6

CHAPTER IV

DISCUSSION

The enzymes as we know it today are bio–catalysts. The effective properties of enzymes have already promoted their introduction into several industrial processes such as detergent industry, fuel alcohol production, feed industries and textile applications (Kirk et al., 2002). Enzymes catalyze chemical reactions with great specificity and rate enhancements (Beilen and Li, 2002).

CGTase normally catalyzes transglycosylation reaction. Transglycosylation reaction is kinetically controlled, thus it becomes possible to overshoot the equilibrium conversion of the reaction into products (Mladenoska et al., 2008). CGTase from *Bacillus macerans* had been reported that the enzyme required a pyranose structure as an effective acceptor (Kitahata, 1988). The alkalophilic *Bacillus* species A2–5a had wide acceptor specificity as well as rhamnose (Kometani et al., 1996). Using alcohol as an acceptor to produce alkyl glycoside from short chain alcohol by CGTase from *Paenibacillus* sp. RB01 had been reported by Chotipanang (2010). Alkyl glycoside showed high stability over a wide range of pH (Papanikolaou, 2001). They are non–ionic surfactant. Since they had high surface activity, low toxicity and good biocompatibility (Turner et al., 2007), they were suitable for use in surfactant as well as in food industry.

4.1 Partial purification of CGTase from *Paenibacillus* sp. RB01 using starch adsorption

CGTase is an extracellular enzyme produced from the thermotolerant bacteria wild type stain, *Paenibacillus* sp. RB01. This strain was isolated from hot spring soil

area in Rachburi, Thailand. The optimal conditions for growth and for the production of CGTase were reported previously by Tesana (2001). Since CGTase secreted from the cells, crude CGTase was harvested after the removal of cells by centrifugation. Crude enzyme was partially purified by starch adsorption (Kato and Horikoshi, 1995 with some modification by Laloknam, 1997). This purification method is based on the binding affinity of enzyme and its substrate. CGTase and other enzymes that were capable of using starch as a substrate were adsorbed to starch while other enzymes were then removed by centrifugation. Adsorbed CGTase was further separated from starch by switching substrate to the higher binding affinity substrate, maltose to compete for CGTase. Consequently, enzyme–maltose that presented in the soluble fraction was separated from starch. Maltose was then removed by dialysis against 3 changes of buffer at 4 °C. The enzyme obtained was concentrated with UF membrane concentrator and aquasorb, respectively.

Partial purified CGTase was then assayed for starch degradation property by iodine test using soluble starch as a substrate. From purification table, partial purified CGTase had 120 purification fold with 50.6 % yield obtained. Specific activity of purified CGTase was 4800 units/mg of protein. From previous work, the specific activity of CGTase from *Paenibacillus* sp. RB01 that was purified through starch adsorption method was 4437 units/mg of proteins. The specific activity was increased to 7268 units/mg of protein when enzyme was subsequently purified through DEAE−cellulose and Bio−Gel P−100 column chromatography (Yenpech, 2000).

The purity of this partially purified CGTase was checked by gel electrophoresis. SDS was added into the protein sample to provide negative charges on the polypeptide chain. Binding of SDS to polypeptide chain allows an even

distribution of charge per mass. Therefore, the separation is dependent on only size. From Figure 19, the enzyme showed the one major band with estimated size of about 70 kDa. From previous report, CGTase size was found approximately to be 65 kDa Yenpech (2000). Other proteins were removed after purification as determined from the decrease of number of protein bands. Thus, the purity of this partially purified CGTase was high enough for further transglycosylation study. To confirm that the obtained protein was CGTase, starch degrading property was also checked by Native–PAGE. The soluble starch substrate was introduced into the polyacrylamide gel. This gel was then developed by the addition of iodine solution. The distinct clear zone represented the location of CGTase which corresponded to the protein band observed from Coomassie blue–protein staining, Figure 20. Thus, these results suggested that the protein obtained from this purification method was CGTase. The 3 bands observed by activity staining revealed that this enzyme had 3 isoforms with different conformation and net charge. This was in good agreement with previous report (Yenpech, 2000).

4.2 Effect of alcohols on the CGTase activity.

Organic solvent is widely utilized as a media for enzyme-catalyzed organic syntheses (Brink et al., 1987). For transglycosylation reaction catalyzed by CGTase from *Paenibacillus* sp.RB01, organic solvent like miscible alcohol had been used as an acceptor substrate to produce alkyl glycosides (Chotipanang et al., 2011). The main restriction to the use of enzyme in reaction media containing alcohol was the effect of alcohol on the activity of enzyme. The synthesis of alkyl glycoside from low water organic solvent by glycosidases has thus performed in two phase system (Vulfson et al., 1990). In this work, the effect of alcohols on the transglycosylation

reaction of CGTase in both one phase and two phase reaction system was investigated. The effect of alcohols on CGTase was determined from coupling activity remaining after treatment the enzyme with buffer containing various alcohols for 24 hours.

The alcohols used here were classified in three groups: short chain (C_1–C_3), medium chain (C_4–C_6) and long chain (C_7–C_8) alcohols. In a group of short chain alcohols, the reaction mixtures were one phase (homogeneous). The coupling activities remained after incubation of CGTase with short chain alcohols was found to be high at the alcohols concentration of ~10–20% (v/v). However, the remaining coupling activity was dramatically decreased when the alcohol concentration was increased (Figure 21 A).

The medium chain alcohols (C_4–C_6) and long chain alcohols (C_7–C_8) had low water solubility (except *tert*–butanol). Therefore, when increasing alcohol concentrations to over their water solubility, the reaction mixtures were separated into two phases. The effects of these alcohols at high concentration were found to be lower than those of short chain alcohols at the same concentration tested (Figure 21 B and C). Although the reaction were performed with shaking, it seems unlikely that an enzyme and alcohols could react uniformly. Unlike, *tert*–butanol, a tertiary alcohol, which is well soluble. After incubation of enzyme with various concentrations of *tert*–butanol, the remaining coupling activity was dramatically decreased. When increasing the concentration of *tert*–butanol to more than 10 % (v/v), the coupling activity was not recovered. There are many causes of lower enzymatic activity in organic solvent compared with that in water such as active center blockage, conformational change and transition state stabilization (Klibanov, 1997). The impact of alcohol on the direct

glycosylation of β–glucosidase catalyzed synthesis of alkyl–β–glucoside from alcohol was dependent on the alcohol chain length. To facilitate the dispersion of enzyme into reaction media, the immobilized enzyme was developed (Papanikolaou, 2001). The literatures suggested that factors that restricted enzymatic synthesis of alkyl–glycoside in organic solvent include the poor solubility of substrate in alcohol media (Vulfson et al., 1990). The hydrolysis activity of enzyme may take place parallel to the transglycosylation reaction (Stevenson et al., 1993). Finally, the mass transfer limitation can give a possible explanation of the poor reactivity in the synthesis of the alkyl–glucoside by water immiscible alcohols (Vulfson et al., 1990).

4.3 Transglycosylation reaction for the synthesis of alkyl glycosides

After incubation of CGTase with various alcohols and β–CD donor, the expected transglycosylated products were detected by TLC. This chromatography composed of the silica gel (polar stationary phase), where alcohols cannot be adsorbed. The polar compound–oligosaccharides, were strongly adsorbed to silica gel (smaller Rf) when non–polar mobile system was used (ethyl acetate: acetic acid: water (3:1:1 by volume). The expected transglycosylated products with less polar than oligosaccharide were migrated to the solvent front with larger Rf value. While in a control reaction (no alcohol acceptors), transglycosylated products were not observed. The visualization of spots on TLC was done by charring agent. This step includes spraying with sulfuric in methanol followed by heating the TLC plate at high temperature. The organic compounds were degraded into black or brown zone of carbon on white background. This charring agent was non–specific and sometimes was called universal detection (Wall, 2005).

The numbers of transglycosylated products from each acceptor were different. On TLC (Figure 23), methanol gave only one product while, ethanol and propanol gave two products. Alcohols with chain length of C_4–C_5 (excepted *tert*–butanol) gave three transglycosylated products. In addition, C_6 alcohol gave smaller yield than those from C_4 and C_5 alcohols. The lower yield obtained from C_6 alcohols might be because of low solubility of alcohol in aqueous phase where the reaction occurred. The possible solution was thus to dissolve the alcohol in a water–miscible organic medium. Bousquet and co–workers (1998) used *tert*–butanol as a co–solvent in order to enhance the solubility of medium chain alcohols. The yield was highest when using 60% (v/v) of *tert*–butanol as a water-miscible organic co–solvent. However, according to our result it suggested that *tert*–butanol caused the reduction of CGTase's coupling activity. Thus, *tert*–butanol was not suitable for CGTase catalyzed transglycosylation reaction. No transglycosylation products were obtained from either C_7-C_8 alcohol or *tert*-butanol. However, the hydrolysis products could be observed, suggesting that in the presence of alcohol, the enzymatic cleavage of the α–(1–4) linkage of donor can lead to the transfer of glucosyl residues onto either water, alcohol or dextrin. Thus, three reactions were competing in the media (Bousquet et al., 1993).

β–Glucosidase has been extensively used to synthesize alkyl glycoside in alcohol media through either reverse hydrolysis or transglycosylation reaction. β–Glucosidase from Thai–rosewood was used to synthesize alkyl glycosides from various alcohols (C_1–C_4) via transglycosylation reaction (Lirdprapamongkol and Svasti, 2000). Only one expected glycoside product (alkyl monoglucoside) was detected on TLC. However, no glycoside product was obtained when *tert*–butanol

was used as a substrate. β–Glucosidase from cassava linamarase showed great ability to transfer glucose from *p*–nitrophenyl–β–glucoside to secondary alcohol acceptor. Surprisingly, it was able to synthesize alkyl glycoside from *tert*–butanol (Svasti et al., 2003). To achieve the alkyl (poly) glycosides, α–amylase was used instead of glycosidase. The main alkyl glycosides synthesized from α–amylase from *A. aryzae* were methyl maltoside and methyl maltotrioside when methanol was used as an acceptor of glycosyl unit from starch donor (Larsson et al., 2005).

In the presence of suitable acceptors, CGTase catalyzes mainly intermolecular transglycosylation reactions. Uitdehaag and co–workers (2000) suggested that the binding of sugar at the acceptor subsites activated CGTase for catalysis and this activation was lower when water molecule was bound. Coupling reaction was taken place via double displacement mechanism involving a covalent enzyme–intermediate complex. Therefore, the compatible structure of enzyme and substrate in the ternary complex should be discussed. As a consequence of enzyme–substrate complex, Nakamura et al (1994) reported that the coupling activity of CGTase required subsite +2 and +3 for acceptor bound to form the random ternary complex for enzyme mechanism. However, β–CD ring interfered to the binding process at the acceptor binding site of enzyme. Catalysis mechanism of enzyme is dependent on the compatibility of the structure of enzyme and its substrate in the active site. Hence, enzymatic production of alkyl glycosides depends not only the influence of alcohol substrate but also structural compatibility of enzyme and its substrate. The proposed model of event occurs in the CGTase catalyzed transglycosylation reaction is shown in the Figure 47. CGTase from *B. stearothermophilus* was reported to transfer glucosyl residue to the hydroxyl group of L–ascorbic acid (Aga et al., 1991). *B.*

ohbensis CGTase transferred glucosyl residue to inositol (Sato et al., 1992). Therefore, the acceptor specificities for the intermolecular transglycosylation reaction depended on the enzyme origin. The acceptor specificity of fourteen alcohol acceptors to CGTase from *Paenibacillus* sp. RB01 was determined from relative intensity on TLC. This quantitated method was previously reported by Lirdprapamongkol et al., (2000), Svasti et al., (2003), and Chotipanang (2010). The intensities were calculated by average of tripicated sample spot intensities then compared with the glucose standard on the same TLC plate. According to the relative intensity on the TLC, the highest yield was obtained from short chain alcohol substrates (methanol) (Figure 24). On the contary, *tert*–butanol and long chain alcohols could not function as acceptors for transglycosylation reaction from β–CD catalyzed by CGTase. These results suggested that the structures of long chain alcohols were not suitable for ternary complex formation during the catalysis. In the case of *tert*–butanol, the results suggested that its hindering structure interrupted the ternary complex formation. (Figure 25). Moreover, linear alkyl glycosides have been extensively studied. Thus, isobutanol, a branch chain primary alcohol with medium alkyl chain length was selected as an appropriate acceptor for further experiments.

The use of low–cost carbohydrate as glycosyl donor has been purposed for the synthesis of alkyl glycoside by transglycosylation (Stevenson et al., 1993). Here, soluble starch from cassava and potato, raw starch and β-CD were tested as a glycosyl donor and isobutanol was used as an acceptor in transglycosylation reaction with

In a group of medium chain alcohol acceptors, isobutanol gave the highest yield in comparison with that of straight chain primary and secondary alcohols CGTase. TLC showed the same glycosylated products (Figure 27). The amounts of

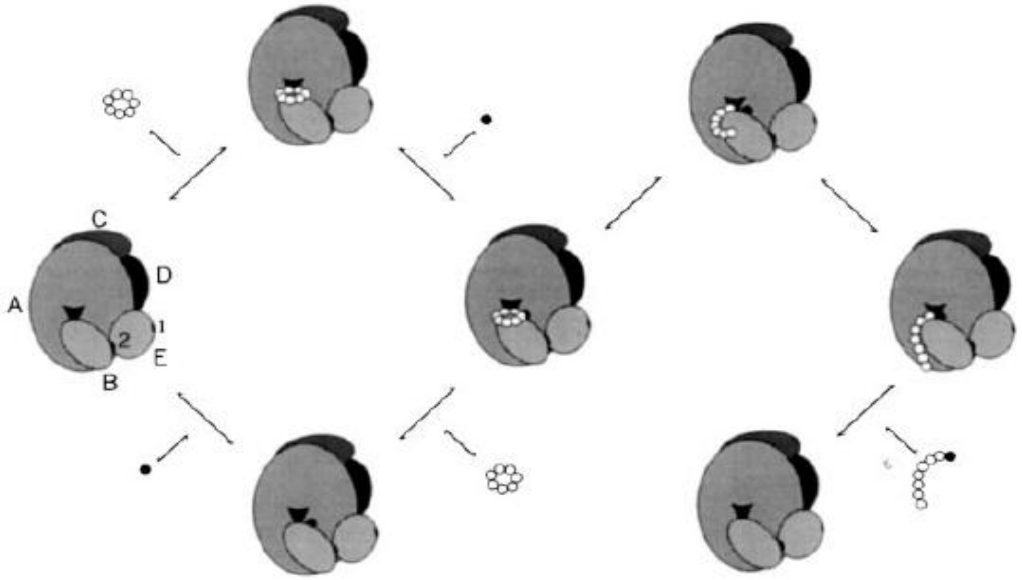


Figure 47 The proposed model of the events taking place in the CGTase–catalyzed coupling reactions. The different CGTase domains are indicated (A, B, C, D, and E). 1 and 2 indicate the maltose binding sites on the E–domain. The triangle indicates the cleavage site in the active site. Circles represent glucose residues; acceptor residues are represented in black (van der Veen et al., 2000).

total glycosylated products were determined from the spot intensity. Mole (%) of glycosylated products were calculated from the calibration curve of intensity of standard methyl–α–D–glucopyranoside. The yield from β–CD donor was found to be higher than others (Table 5). Therefore, β–CD was selected as an appropriate donor for the transglycosylation reaction catalyzed by CGTase from this bacterial strain. Raw starch was found to have an ability to function as a glycosyl donor although smaller amount was obtained.

Since separation of di–and tri–saccharides on Aminex column with refractive index detector had low sensitivity (Srisomsap et al, 1999). Thus, this method was not suitable for the analysis of alkyl glycoside (Lerdprapamongkol et al, 2000). HPLC analysis was then tried using reverse phase C–18 column with refractive index detector (RI). The use of 40 % methanol as a mobile phase was employed but the pressure was too high. The addition of 0.2 % glacial acetic acid into the water was made to reduce the pressure. This method has been previously reported by Gargouri et al., (2004). The HPLC system was operated with the flow rate of 0.5 mL/min at controlled temperature of 50 °C. From TLC chromatogram, two major spots (namely ISG1 and ISG2) and a minor spot (namely ISG3) were detected. While, the products analyzed by HPLC showed the peak of products at Rt ~7, 8 and 9 minutes (Figure 28). These peaks were suggested from the polarity of substances as followed ISG_1 was at Rt ~9 min, ISG_2 was at Rt ~8 min and ISG_3 was at Rt ~7 min. The amount of two main products observed at Rt ~9 and ~8 min were about 1:1. In addition, hydrophilic substances from hydrolysis activity catalyzed by CGTase could not be separated under this mobile system.

4.4 Preliminary study of transglycosylation products by amylolytic enzymes

The number of glucosyl units and the configuration of the products synthesized by CGTase were characterized by the treatment with two amylolytic enzymes, glucoamylase and α–glucosidase. Glucoamylase (amyloglucosidase, EC. 3.2.1.3.) catalyzes the hydrolysis of terminal 1,4–linked α–D–glucose residues successively from the non–reducing ends of maltooligo–and polysacharides with release of β–D–glucose (Meager et al., 1989). After treatment the transglycosylation products with glucoamylase, the expected transglycosylated products and oligosaccharide from the hydrolysis activity were hydrolyzed to release monoglucoside and glucose molecules. From TLC (Figure 29), the spot of maltose (G_2), maltotriose (G_3), ISG_2 and ISG_3 disappeared. The increase in intensities of glucose and ISG_1 were observed. From HPLC, the decrease of peaks at Rt ~7 and 8 were observed. While, a peak at Rt ~9 min was observed with increasing peak area (Figure 31). Therefore, these results can confirm the previous suggestion that ISG_1 was at Rt ~9 min, $ISG_2$was at Rt ~8 min, and ISG_3 as at Rt ~7 min. Moreover, α–glucosidase (EC. 3.2.1.20) was employed to characterize the configuration between isobutanol and glucosyl unit of the expected product. Since α–glucosidase catalyzes exo–type hydrolysis to form α–anomer configuration product (Chiba, 1997), the transglycosylation products were thus hydrolyzed by α–glucosidase in order to confirm the linkage between isobutanol and glucosyl unit. From TLC chromatogram (Figure 29), the spot intensity of ISG_1 was dramatically decreased. Only spot of glucose was recovered. From HPLC (Figure 32), after treatment with α–glucosidase the peak at Rt ~9 min disappeared. Thus, this confirmed that the ISG_1 was isobutyl monoglucoside with α configuration. After treated the reaction mixture with

glucoamylase and further hydrolyzed by α–glucosidase, the intensity of ISG_1 spot disappeared and a peak at Rt ~9 min could not be detected. The result was found to be similar as the treatment with α–glucosidase.

4.5 Optimization of transglycosylation reaction

To achieve high glycosylated yield, the parameters affecting the yield including isobutanol concentration, β–CD concentration, units of CGTase and incubation time were optimized. The suitable pH of CGTase from *Paenibacillus* sp. RB01 was previously optimized and reported by Thanadolsathien (2007) that this enzyme was stable for a wide pH range (6–8). Transglycosylation to methanol gave the highest yield when acetate buffer (pH 6) was used (Chotipanang, 2010). CGTase from this thermotolerant bacterium was found to be stable over 40–65 °C. However, the optimized temperature for transglycosylation reaction for the synthesis of methyl glycoside was found to be at 40 °C. (Chotipanang, 2010). Therefore, the suitable pH and temperature from previously work were used for transglycosylation reaction catalyzed by CGTase.

The concentration of isobutanol was varied from 5–30%. The solubility of isobutanol was ~8% (v/v). The transglycosylation yield was maximal at 5% (v/v). The optimized donor concentration was 1.2% (w/v). The amount of enzyme was varied from 50–500 units determined by dextrinizing activity. The highest yield was obtained when 500 units/mL of enzyme were used. However, the amount of transglycosylated product did not differ much when 450 units/mL of enzyme was employed. Thus, to reduce the cost of enzyme, enzyme at 450 units/mL was chosen for the next experiment (Figure 34).

In order to determine the optimum time for transglycosylation reaction of glucosyl unit from β–CD to isobutanol acceptor, the reaction was performed at various time with 5% (v/v) of isobutanol, 1.2 % (w/v) of β–CD and 450 units/mL in 50 mM acetate buffer pH 6 at 40 °C. The 0.25 mL of reaction mixture was withdrawn at the interval time for 7 days. The production yield was dramatically increased at the initial stage of incubation. When the reaction was carried out for 48 hours, the yield obtained found to reach plateau. Although the maximal yield was obtained after incubation for 72 hours, the suitable time for the production was chosen at 48 hours.

The amount of transglycosylation products were varied according the size of the oligosaccharide chain because CGTase exhibits glucoamylase–like activity. From this activity, the glucose unit of oligosaccharide conjugated to isobutyl monoglucoside can be removed from non–reducing end. This enzymatic property of CGTase for the alkyl glycoside production was consistent with ascorbic acid 2–O–glucoside formation by the action of CGTase from *B. sterothermophilus* (Tanaka and Yamamoto, 1991).

4.6 Large scale production and isolation of the transglycosylated products

To prepare higher amount of the transglycosylated product for identification, the reaction mixture was performed under the optimal conditions and was scaled up 50 times. The reaction mixture was concentrated with evaporation. The concentrated reaction mixture was passed through the Amberlite–IRA 900 column. Amberlite IRA 900, product from Sigma, carried the quaternary ammonium as a functional group on molecule. The positive–functional group was substituted with negative charges by equilibration the column with 0.1 M NaOH. The flow rate of system was kept constant at 0.5 mL/min at room temperature. The method takes advantage of the

affinity between ionized group(s) on the saccharide at alkaline pH and a pellicular quaternary amonium stationary phase (Townsend et al., 1988). The anion–exchange affinity, and thus retention time, follows the order sugar alcohols < monosaccharides < disaccharides < oligosaccharides (White and Widmer, 1990). Ultrapure water was used as an eluent to elute the adsorbed samples from the resin metrix. Fractions were determined by phenol–sulfuric test. The purity was checked by TLC and HPLC (Figure 39 and 40). With this purification condition, transglycosylation products were separated. Based on the Rf and retention time relationship, the purified products collected from this anion–column chromatography were isobutyl–α–monoglucoside and isobutyl–α–maltoside. Isobutyl–α–maltotrioside could not be purified.

Preparative TLC method was carried out to isolate the expected transglycosylated products at the indicated Rf values. Products were then extracted from silica by methanol. Methanol was subsequently evaporated at 40 °C. The purified products were checked with TLC (Figure 39) and HPLC (Figure 40) before MS analysis. This analytical method includes the first step of ion preparation where injected sample is altered to gaseous ions before analysis and detection. The sample thus will be separated by mass/charge ratio. The molecular weight of the purified products was confirmed using ESI–MS with positive mode. There were intensive signals at the *m/z* of 259 and 421 which suggested that they were isobutyl–α–monoglucoside–Na^+ and isobutyl–α–maltoside–Na^+, repectively (Figure 41).

4.7 Determination of properties of isobutyl glycosides

The isobutyl glycoside products were determined for the emulsification activity and the emulsification stability. These properties were previously used to confirm the emulsification properties of several bio–surfactants, such as bio–

surfactant produced by *Candida lipolytica* (Cirigliano and Carman, 1985) and *Klebsiella* sp. Y6–1 (Lee et al., 2007). Moreover, this method was slightly modified by Kim et al. (2009) to study the emulsification properties of methyl–and ethyl–α–D–monoglucoside. The O/W emulsion was performed by using n–hexadecane as substrate. The emulsion was allowed to stand for 10 minutes. The turbidity of the emulsion was measured by spectrophotometer at 540 nm. A reference test was done without the addition of purified isobutyl glycoside. The emulsification activity of transglycosylation products was compared with Triton X–100®, a commercial non–ionic surfactant. Here, the properties of commercial methyl–α–monoglucoside were also checked. The emulsification activity of isobutyl–α–monoglucoside and methyl–α–monoglucoside, after 10 minutes of forming emulsion, were 11.3% and 17% of Triton X–100®, respectively. It has previously been reported that the emulsification activity of methyl–α–monoglucoside and ethyl–α–monoglucoside, after 30 minutes of forming emulsion, were 31% and 26% of Triton X–100®, respectively (Kim et al., 2009). Thus, the emulsification activity of alkyl–α–D–monoglucoside might be impacted by the alkyl chain length. The shorter chain showed better activity with n–hexadecane. The 3 times lower emulsification activity of methyl–α–monoglucoside obtained compared with those of previous work could be because of the different time used for O/W forming emulsion. However, the purified isobutyl–α–maltoside gave about 4 times higher emulsification activity than that from isobutyl–α–monoglucoside (Figure 44). This can preliminary summarize that the emulsion–forming properties have relationship with physiochemical structure of surfactants (emulsifier). Hydroxybenzyl–α–D–glucoside did not show emulsifying properties

when n–hexadecane and 2– methylnapthalein were used as substrates (Shin et al., 2000). However, it showed high tyrosinase inhibitory activity.

Emulsions are unstable because of the large free energy required to increase the surface area between oil and aqueous phase. They tend to break down into oil and aqueous phase with time (McClement and Dungan, 1995). In the present studies, the emulsification stability of transglycosylation products was determined in comparison with a commercial non–ionic surfactant (Triton X–100®), isobutanol and standard methyl–α–monoglucoside. For control test, surfactant was replaced by the addition of buffer. The ability of surfactant to stabilize the emulsion of n–hexadecane was determined for 60 minutes by spectrophotometry. The log of the absorbance was plotted over time and the emulsification stability was calculated from the slope of the graph. It was found that isobutanol did not present the ability to stabilize the emulsion during a period of time tested. The emulsification stability of Triton X–100® was found to be the best. Therefore, the emulsification stability of Triton X–100® was then set as 100%. Among the group of alkyl glycoside, isobutyl glycoside derivatives gave better the emulsification stability than that from standard methyl–α–monoglucoside. This suggested that the different number of alkyl chain length had an effect on the stability of the emulsion. Long hydrophobic alkyl chain can stabilize the emulsion better than short chain. This may be due to the difference form (droplet oil/water interface, micelle, or individual molecule) of surfactant presented in the emulsion (McClement and Dungan, 1995). From these results isobutyl–α–maltoside had better emulsification activity and emulsification stability

than isobutyl–α–monoglucoside. This indicated that isobutyl–α–maltoside could be useful as an emulsion stabilizing agent.

In this work, CGTase from *Paenibacillus* sp. RB01 was shown to be able to use short chain, medium chain with primary and secondary alcohols as acceptors for intermolecular transglycosylation reaction to yield alkyl glycosides. The alkyl glycoside produced from the action of CGTase are alkyl monoglucoside and alkyl (poly)glycosides. Isobutyl glycosides exhibited good emulsification properties when n–hexadecane was used as an emulsion–forming substrate. The products produced could be applied to be used in almost every sector in various industries and also in household uses.

CHAPTER V

CONCLUSIONS

1. CGTase from *Paenibacillus* sp. RB01 was partially purified through starch adsorption method. The specific activity was 4800 U/mg of protein. The obtained yield was 50.6% with 120 fold increase in purity.
2. Alcohols had an effect on the enzyme activity. The effect was dependent on the structure of alcohol, concentration and whether the reaction was in one or two phase system.
3. CGTase had ability to use short chain alcohols and medium chain alcohols (except tertiary alcohol) as an acceptor for transglycosylation reaction to yield alkyl glycosides. Here the suitable acceptor was isobutanol.
4. β-CD was reported as an appropriate donor for transglycosylation reaction. Raw starch, however, was also function as a donor. Therefore, it was proposed as a donor in case of the objective was to reduce the production cost.
5. The synthesized products were confirmed by TLC, HPLC and MS. The configuration was confirmed by the action of amylolytic enzymes. The three transglycosylation products produced by CGTase were isobutyl–α–monoglucoside, isobutyl–α–maltoside and isobutyl–α–maltotrioside.
6. The optimum condition for CGTase catalyzed transglycosylation reaction were to incubate isobutanol at the concentration of 5% (v/v) and 1.2 % (w/v) of β– CD with 450 units/mL of CGTase in 50 mM acetate buffer pH 6.0 at 40°C for 48 hours.

7. Only two transglycosylation products (isobutyl–α–monoglucoside and isobutyl–α–maltoside) were successfully purified by strong anionic column chromatography (Amberlite IRA 900 Cl^-).
8. The emulsification properties (emulsion activity and emulsion stability) of synthesized products were determined. Isobutyl–α–maltoside showed better emulsification properties than that of isobutyl–α–monoglucoside.

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APPENDICES

Appendix A Preparation for polyacrylamide gel electrophoresis

1) Stock reagents

Solution A (40% (w/v) acrylamide monomer solution containing 5% (w/v) bis-acrylamide, ready for use)2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 mL with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 mL with distilled water.

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 mL with distilled water.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 mL with distilled water.

10% (w/v) SDS

Sodium dodecyl sulfate 10 g

Adjusted volume to 100 mL with distilled water.

1% (w/v) Bromophenol blue

Bromophenol blue 0.1 g

Added in 10 mL distilled water and mixed well. Then, solution was filtered to

eliminate the aggregated dye.

10% (w/v) Ammonium persulfate ($(NH_4)_2S_2O_8$)

$(NH_4)_2S_2O_8$	0.5 g

Adjusted volume to 5 mL with distilled water.

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) $(NH_4)_2S_2O_8$	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) $(NH_4)_2S_2O_8$	25 µL
TEMED	3 µL

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

One part of sample buffer was added to four parts of sample.

3) Working solutions (SDS-PAGE)

7.5% separating gel

Solution A	1.41 mL
Solution B	2.50 mL
Distilled water	3.49 mL
10% (w/v) $(NH_4)_2S_2O_8$	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) $(NH_4)_2S_2O_8$	25 µL
TEMED	3 µL

Sample buffer

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

One part of sample buffer was added to four parts of sample. The mixture was heated for 5 minutes in boiling water prior to loading to the gel.

Electrophoresis buffer

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 L with distilled water (pH should be approximately 8.3)

Appendix B Preparation for buffer solution

50 mM Acetate buffer pH 5.0 (50 mL)

50 mM Sodium acetate (CH_3COONa)	31.75 mL
50 mM Acetic acid (CH_3COOH)	18.25 mL

50 mM Acetate buffer pH 6.0 (50 mL)

50 mM Sodium acetate (CH_3COONa)	47.28 mL
50 mM Acetic acid (CH_3COOH)	2.72 mL

50 mM Phosphate buffer pH 6.0 (50 mL)

50 mM di-Potassium hydrogen phosphate (K_2HPO_4)	6.52 mL
50 mM Potassium dihydrogen phosphate (KH_2PO_4)	43.48 mL

50 mM Phosphate buffer pH 7.0 (50 mL)

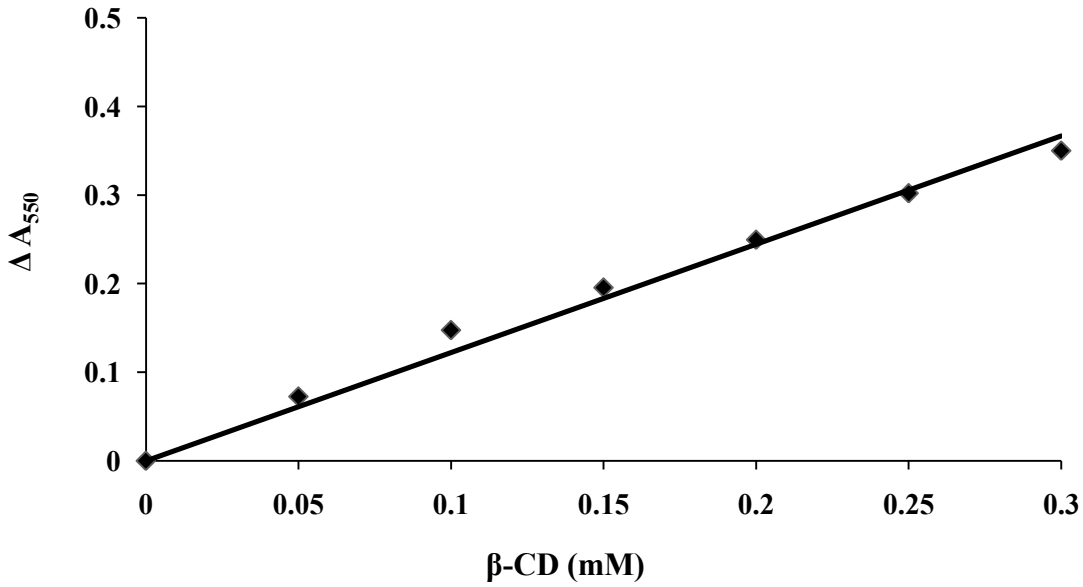
50 mM di-Potassium hydrogen phosphate (K_2HPO_4)	30.08 mL
50 mM Potassium dihydrogen phosphate (KH_2PO_4)	19.92 mL

50 mM Tris-glycine buffer pH 7.0 and 8.0 (50 mL)

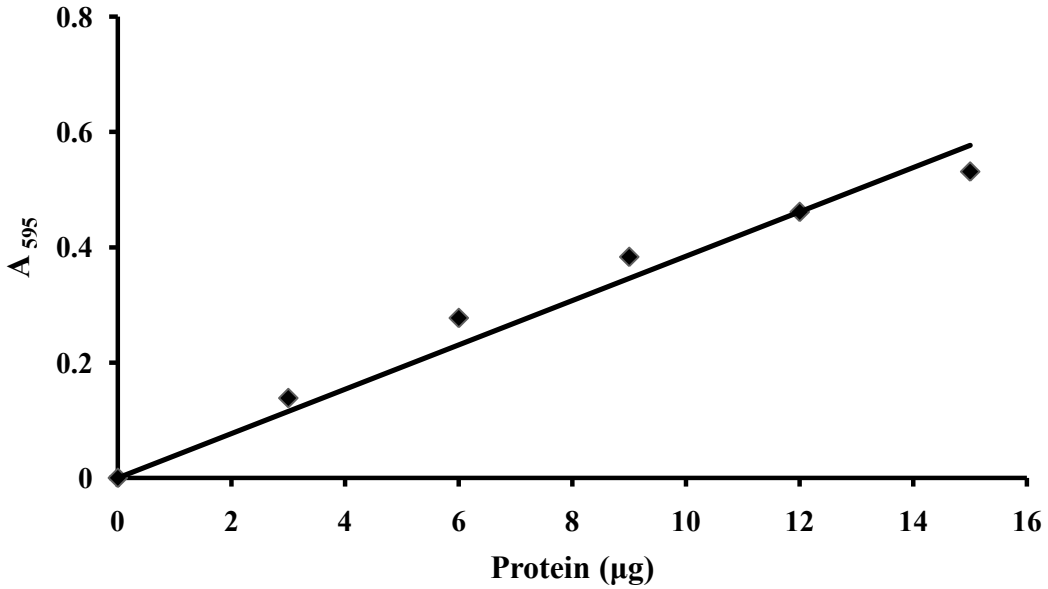
Tris (hydroxymethyl)-aminomethane	0.303 g
Glycine	0.188 g

Adjust pH to 7.0 and 8.0 by 1 M NaOH and adjusted volume to 50 mL with distilled water.

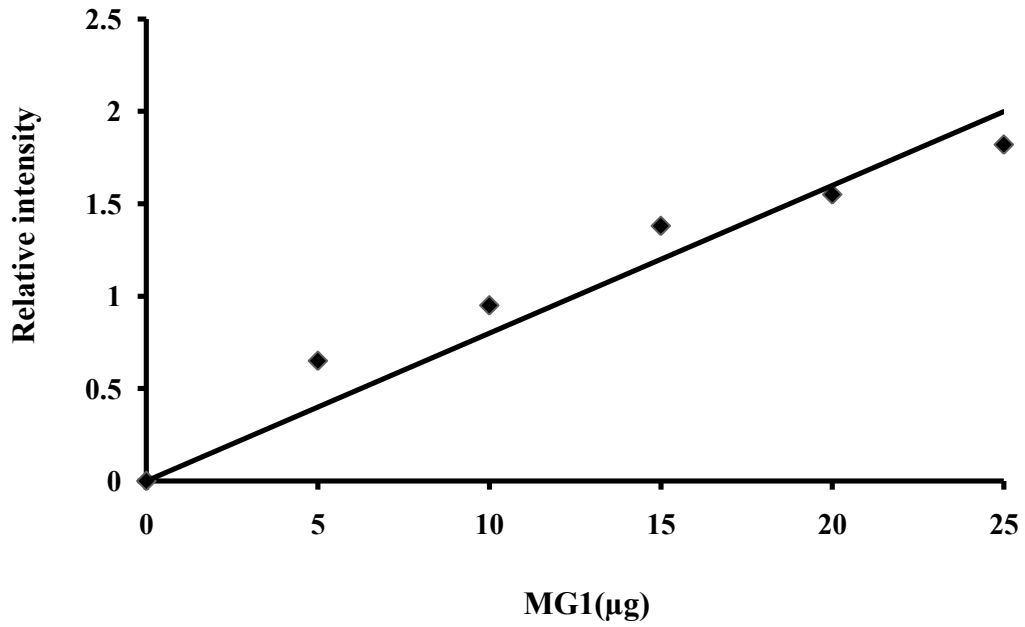
Appendix C Standard curve for β-CD determination by Phenolphthalein method



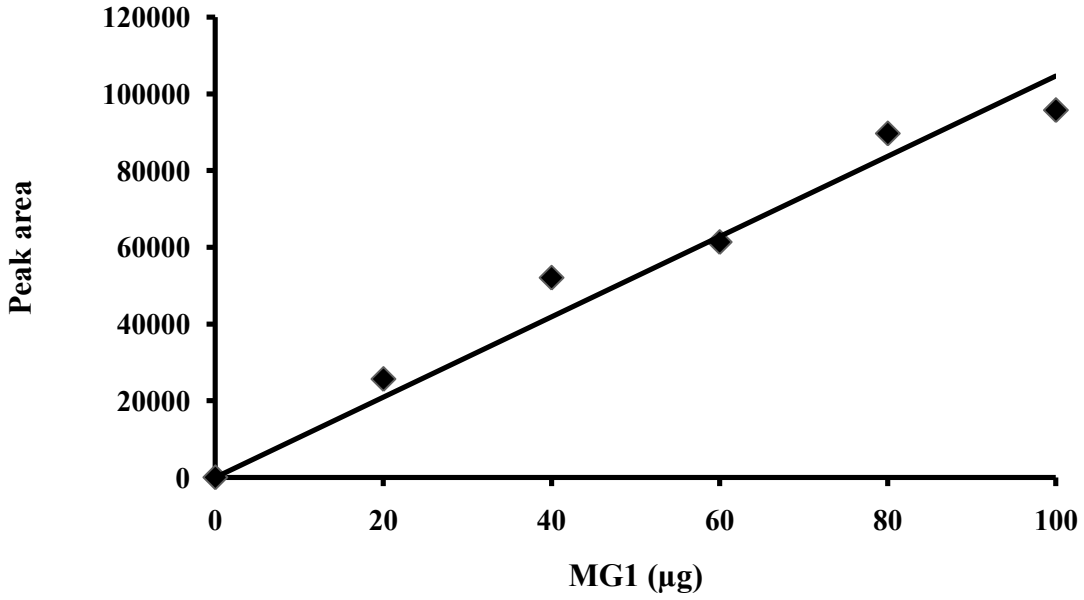
Appendix D Standard curve for protein determination by Bradford's method



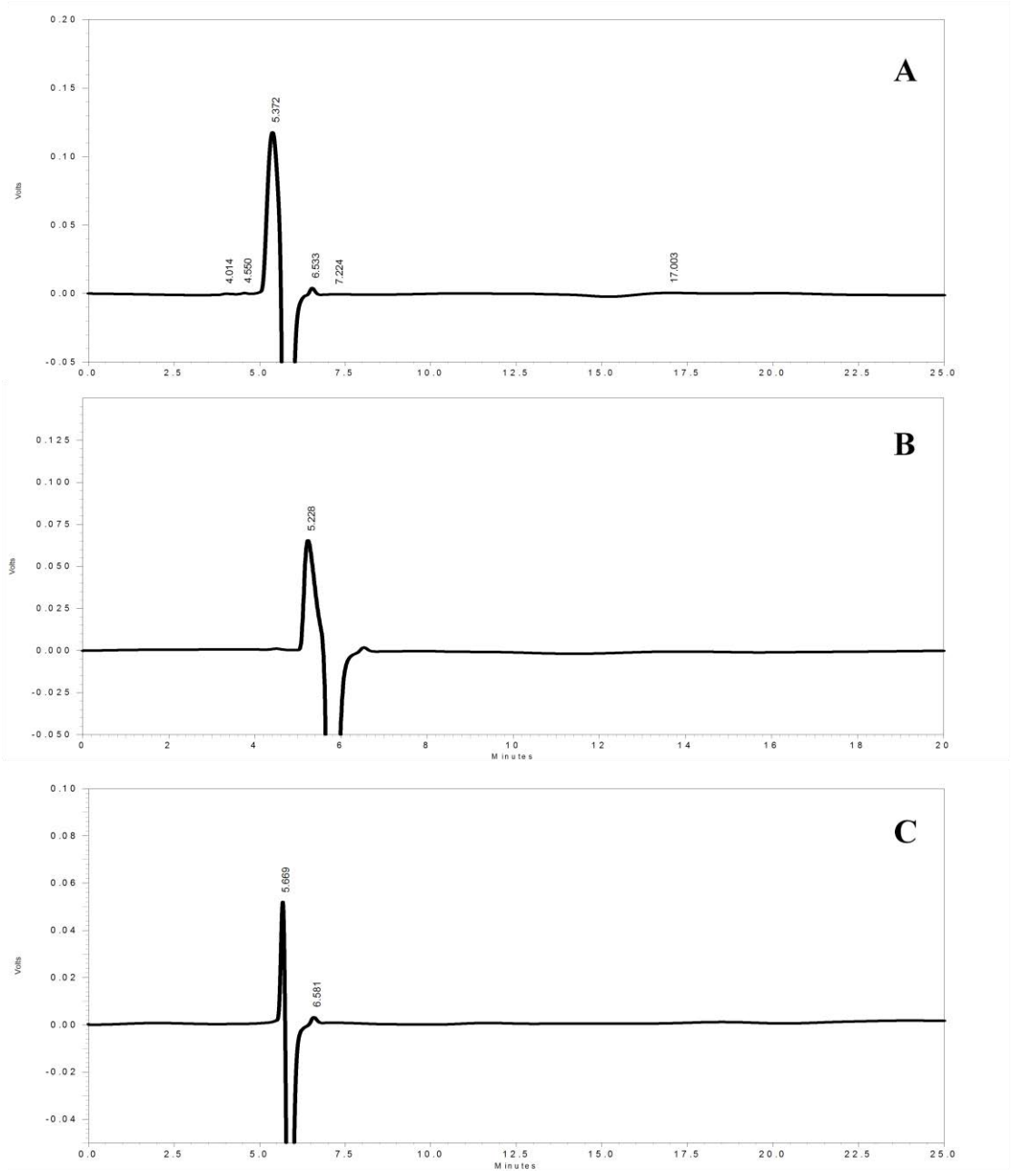
Appendix E Standard curve of standard methyl–α–monoglucoside concentration by determination of spot intensity on TLC plate



Appendix F Standard curve for standard methyl–α–monoglucoside concentration by HPLC



Appendix G HPLC chromatogram of (A) standard G1-G3, (B) standard β–CD and (C) standard methyl–α–monoglucoside



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