การสังเคราะห์แอลคิลไกลโคไซด์โดยไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจาก *Paenibacillus* sp. RB01

นางสาวขวัญใจ โชติพนัง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

SYNTHESIS OF ALKYL GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2010

Thesis Title	SYNTHESIS OF ALKYL GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01
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ขวัญใจ โชติพนัง : การสังเคราะห์แอลคิลไกลโคไซด์โดยไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจาก *Paenibacillus* sp. RB01. (SYNTHESIS OF ALKYL GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. มัญชุมาส เพราะสุนทร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร. วรวรรณ พันธุมนาวิน, 112 หน้า.

แอลคิลไกลโคไซด์ถูกนำไปใช้เป็นสารชะล้างอย่างมีประสิทธิภาพ เนื่องจากมีสมบัติเป็นสารลดแรงตึงผิวที่มีความเป็นพิษต่ำและสามารถย่อยสลายได้ตามธรรมชาติ ความก้าวหน้าทางด้านการประยุกต์ใช้เอนไซม์ในการผลิตสารลดแรงตึงผิวจำพวกนี้แสดงให้เห็นถึงข้อดีที่มีมากกว่าการใช้วิธีทางเคมี งานวิจัยชิ้นนี้ได้สังเคราะห์แอลคิลไกลโคไซด์จากบีต้า-ไซโคลเดกซ์ทรินและแอลกอฮอล์ที่สามารถละลายน้ำได้หลายชนิดโดยปฏิกิริยาการโยกย้ายหมู่ไกลโคซิลของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส (CGTase) จาก *Paenibacillus* sp. RB01 แอลกอฮอล์ที่ใช้เป็นตัวรับหมู่ไกลโคซิลประกอบด้วย เมทานอล เอทานอล 1-โพรพานอล 2-โพรพานอล 1-บิวทานอล และ 2-บิวทานอล ผลการศึกษาพบว่าปริมาณผลิตภัณฑ์สูงสุดเกิดขึ้นเมื่อใช้ 30% เมทานอล 20-30% เอทานอล 10-20% 1-โพรพานอล 10% 2-โพรพานอล 8% 1-บิวทานอล และ 5-10% 2-บิวทานอล เมทานอลเป็นตัวรับที่ประสิทธิภาพสูงสุดต่อเอนไซม์ชนิดนี้ ภาวะที่เหมาะสมในการสังเคราะห์เมทิลไกลโคไซด์จากไซโคลเดกซ์ทรินคือการบ่ม 1.2% (w/v) บีต้า-ไซโคลเดกซ์ทริน กับ เอนไซม์ 240 ยูนิตต่อมิลลิลิตรในระบบที่มี 30%(v/v) เมทานอล pH 6.0 อุณหภูมิ 40°ซ เป็นเวลา 96 ชั่วโมง จากการวิเคราะห์ผลิตภัณฑ์ที่เกิดขึ้นด้วยเทคนิค MS พบว่าขนาดโมเลกุลของผลิตภัณฑ์หลักเท่ากับ 194, 356 และ 518 ดาลตัน ซึ่งสอดคล้องกับเมทิลไกลโคไซด์ที่ประกอบด้วยหน่วยมอนอแซ็กคาไรด์หนึ่งถึงสามหน่วยเชื่อมต่อกับหมู่เมทิลของเมทานอล เมื่อเพิ่มปริมาณการสังเคราะห์เมทิลมอนอกลูโคไซด์ (MG_1) แล้วทำการแยกผลิตภัณฑ์ด้วยคอลัมน์แอมเบอร์ไลต์ IRA-900 และทำการพิสูจน์โครงสร้างของผลิตภัณฑ์ด้วยเทคนิค ^{13}C-NMR พบว่า MG_1 มีโครงสร้างเป็น methyl α-D-glucopyranoside

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....ชีวเคมี.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์.....
ปีการศึกษา....2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5072224523 : MAJOR BIOCHEMISTRY
KEYWORDS : ALCOHOL / ALKYL GLYCOSIDE / CYCLODEXTRIN GLYCOSYLTRANSFERASE / *Paenibacillus* sp / TRANSGLYCOSYLATION

KWANJAI CHOTIPANANG : SYNTHESIS OF ALKYL GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01. ADVISOR : ASST. PROF. MANCHUMAS PROUSOONTORN, Ph.D., CO-ADVISOR : ASST. PROF. WORAWAN BHANTHUMNAVIN,Ph.D., 112 pp.

Alkyl glycosides have potential use as biodegradable detergents due to their high surface activity with low toxicity. Recent progress in the application of enzymes to the preparation of these surface-active compounds demonstrates the advantages to the chemical synthesis. In this work, alkyl glycosides were synthesized from β-cyclodextrin (β-CD) and various soluble alcohols by transglycosylation reaction using cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp. RB01. Several alcohols (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol and 2-butanol) as glycosyl-acceptor substrates were evaluated. It was found that the reaction products which were analyzed by TLC were maximum for 30% methanol, 20-30% ethanol, 10-20% 1-propanol, 10% 2-propanol, 8% 1-butanol and 5-10% 2-butanol. Methanol was the most efficient acceptor toward this enzyme. From product analysis by HPLC, optimal reaction conditions for methyl glycoside synthesis from CD were to incubate 1.2% (w/v) β-CD and 240 U/mL of CGTase in a water/methanol system containing 30% (v/v) methanol, pH 6.0 and a temperature of 40°C for 96 hours. The molecular weights of the main products were 194, 356, and 518 Daltons which were in accordance with methyl glycosides having one to three monosaccharide units attached to methyl group of methanol. The methyl monoglucoside (MG_1) was prepared in large scale and isolated by Amberlite IRA-900 column. The structure of MG_1 was confirmed as methyl α-D-glucopyranoside via ^{13}C-NMR analysis.

Department : Biochemistry Student's Signature .....
Field of Study : Biochemistry Advisor's Signature .....
Academic Year : 2010 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assistant Professor Dr. Manchumas Prousoontorn, for her excellent instruction, guidance, encouragement, attention and support throughout this thesis.

My gratitude is also expressed to my co-advisor, Assistant Professor Dr. Worawan Bhanthumnavin, for her valuable suggestions.

Sincere thanks and appreciation are also extended to Professor Dr. Piamsook Pongsawasdi, Associate Professor Dr. Tipaporn Limpaseni and Assistant Professor Dr. Jarunee Kaulpiboon who serve as the members of the master committees, for their helpful suggestions and comments.

My appreciation is given to all staff members and friends of the Biochemistry Department, for their assistance and friendship especially Mr. Wanchai Yenpetch, Miss Wiraya Srisimarat, Miss Pitchanan Nimpiboon, Miss Siriwipa Saehu and Miss Prapai Hongsa Moreover, my thanks must be expressed to Mr. Supawiriya Saranarak, the member of Organic Synthesis Research Unit (OSRU) at Chemistry Department, for his assistance and helpfulness.

Finally, the greatest gratitude is expressed to my parents for their encouragement, willpower and heartiness support throughout my life.

This research was supported by Ratchadapiseksompote Research Fund granted to Starch and Cyclodextrin Research Unit, Department of Biochemistry, Faculty of Science, Chulalongkorn University. The Support from the Thai Government Stimulus Package 2 (TKK2555) under the project PERFECTA is also acknowledged.

CONTENTS

PAGE

PAGE

PAGE

PAGE

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

FIGURE **PAGE**

FIGURE **PAGE**

LIST OF ABBREVIATIONS

A	Absorbance
BSA	bovine serum albumin
CDs	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
cm	centimeter
°C	degree Celsius
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
ESI-TOF-MS	Electrospray Ionization-Time of Flight Mass Spectrometry
et al.	Et. Alii (latin), and others
FT-NMR	Fourier Transform Nuclear Magnetic Resonance
g	gram
h	hour(s)
HPLC	High Performance Liquid Chromatography
kDa	kiloDalton
L	Liter
µg	Microgram
µL	Microliter
µmole	Micromole
M	Molar
min	minute(s)
ma	Milliampere
mg	Milligram
mL	Milliliter

mM	Millimolar
MS	Mass Spectrometry
nm	nanometer
NMR	Nuclear Magnetic Resonance
PAGE	Polyacrylamide gel electrophoresis
rpm	revolution per minute
R_f	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

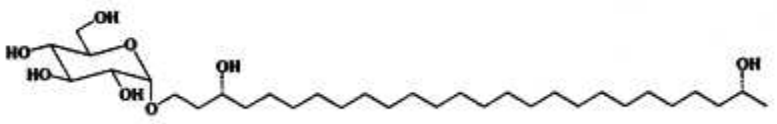
1.1 Alkyl glycosides

Alkyl glycosides are composed of the hydrophilic sugar headgroup (innumerable different sugar units) connected via an ether bond to the hydrophobic part which could consist of one, two or even more chains. Alkyl glycosides with two or more hydrophobic chains are usually referred to as glycolipids (Stubenrauch, 2001). Natural alkyl glycosides are biosynthesized as glycolipids by living organisms. In nitrogen-fixing cyanobacteria, they take part in the protection of these cells. Lambein and Wolk (1973) isolated and purified glycolipids from heterocysts of *Anabaena cylindrica*. One of them is shown in Figure 1 Other forms with different chain length and functional groups were found in other species. For instance, bidensyneosides from plant *Bidens parviflora* WILLD (Wang *et al.*, 2001) and simplexides from the marine sponge *Plakortis simplex* (Costantino *et al.*, 1999) (Figure 1).

1.1.1 The Advantages of alkyl glycosides

Since alkyl glycosides are the amphipathic molecules with hydrophobic and hydrophilic moieties, they confer several abilities on surfactants. Surfactants are one of the most ubiquitous and important families of organic compounds that have two essential properties: (i) their ability to lower the surface or interfacial tension, and (ii) their capacity to solubilize water insoluble compounds. Especially, because alkyl glycosides are unique nonionic surfactants produced from the reactions of fatty

A



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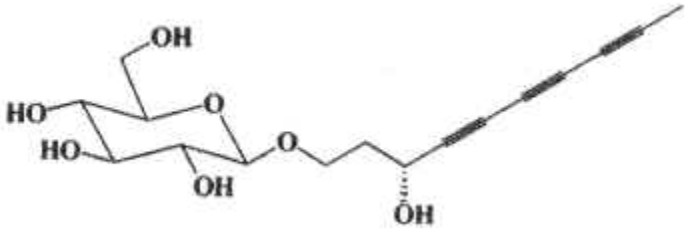

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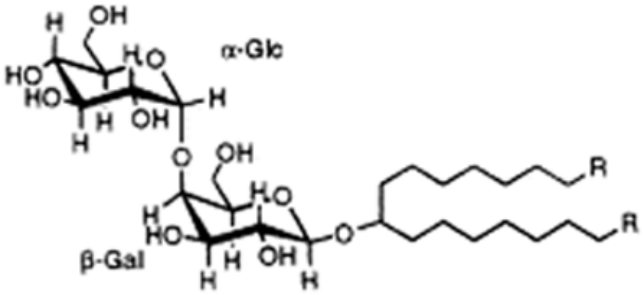


Figure 1 **Examples of glycolipids from living organisms.**

A: glycolipids from heterocysts of *Anabaena cylindrical* (Lambein and Wolk, 1973)

B: bidensyneosides from plant *Bidens parviflora* WILLD (Wang *et al.*, 2001)

C: simplexides from the marine sponge *Plakortis simplex* (Costantino *et al.*, 1999)

alcohols and saccharides, they are rapidly biodegradable and nontoxic. Increased environmental awareness among consumers has prompted more serious consideration of biological surfactants as possible alternatives to existing products. Therefore, alkyl glycosides will undoubtedly constitute an important part not only in industrial chemicals widely used in almost every sector of various industries but also in household uses (Sarney and Vulfson, 1995). Surfactant types used in modern industries are shown in Table 1.

1.1.2 Commercial production of alkyl glycosides

The commercial production of alkyl glycosides is primarily the production of alkyl glycosides from a primary alcohol or primary alcohol mixture containing 8-18 carbon atoms and a glucose source. Two of the most common processes are described as follow:

The direct (or one-step) reaction

The glucose source reacts directly with an excess of the alcohol or alcohol mixture. The reaction is carried out at ~95 to 115°C under relatively high vacuum (relatively low pressure) in the presence of a strong acid catalyst. The water formed during the acetalization reaction is removed as a distillate. When the reaction is complete, the catalyst is neutralized and the product is recovered from the excess alcohol.

The transacetalization (or two-step) reaction

The glucose source reacts first with an excess of a lower molecular weight, more polar alcohol than that of the final alkyl glucoside product. A low-molecular-

Table 1 Types of surfactants used in modern industry (Sarney and Vulfson, 1995)

Surfactant type	Examples	Percentage of total production	Major uses
Anionic	Carboxylates Sulphonates Sulphuric acid esters	66	Washing powders
Cationic	Amine oxides Monoamines Quaternary ammonium salts	9	Fabric softeners Shampoos
Nonionic	Carboxylic acid esters Glycerides Carbohydrate esters and their ethoxylated derivatives	24	Laundry co-surfactants Washing-up liquids Personal-care products Foods
Amphoteric	Alkylbetaines Alkyldimethylamines Imidazonlinium derivatives	~1	Speciality uses

weight alcohol such as butyl alcohol is typically used. The reaction is carried out at ~95 to >140°C under relatively low vacuum to pressures greater than atmospheric pressure in the presence of a strong acid catalyst. The bulk of any free water initially present in the glucose source or formed during acetalization is removed as a distillate. Glucose sources initially containing increasingly higher levels of glucose oligo or polysaccharides require increasingly higher reaction temperatures and pressures. Under appropriate conditions, these glucose sources can be converted by transacetalization into low-DP butyl glucosides. The butyl glucosides, by transacetalization with an excess of a higher-molecular-weight alcohol or alcohol mixture, can then be converted into the final alkyl glucoside product. The reaction conditions for this transacetalization are similar to those of the direct or one-step reaction. During this reaction, the bulk of the low-molecular weight alcohol initially present as either free or reacted alcohol is removed by distillation. When the reaction is complete, the catalyst is neutralized and the product is recovered from the excess, high-molecular-weight alcohol. Table 2 presents the possible ways of converting the glucose sources into alkyl glucoside products.

1.1.3 Chemical synthesis of alkyl glycosides

Fischer glycosidation

Fischer glycosidation (or Fischer glycosylation) refers to the formation of a glycoside by the reaction of an aldose or ketose with an alcohol in the presence of an acid catalyst (Figure 2). The reaction is named after the German chemist, Emil Hermann Fischer, winner of the Nobel Prize in chemistry, 1902. Commonly, Fischer glycosidation has been used for the anomeric protection of monosaccharides. Since

Table 2 The possible ways of converting the glucose sources into alkyl glucoside products (Varvil *et al.*, 2009)

Glucose Product or Source	Possible Reaction Processes
Anhydrous glucose	Direct reaction, transacetalization reaction
Glucose monohydrate	Direct reaction, transacetalization reaction
High-dextrose corn-syrup solids	Direct reaction, transacetalization reaction
High-dextrose corn syrup	Direct reaction, transacetalization reaction
Corn-syrup solids	Direct reaction, transacetalization reaction
Corn syrups	Transacetalization reaction
Maltodextrins	Transacetalization reaction
Cornstarch	Transacetalization reaction

the reaction is carried out in the presence of an acid catalyst in alcohols under reflux, a mixture of α and β anomers as well as certain amounts of by-products are generally formed. However, these drawbacks were alleviated by the effective types of acid catalysts such as chlorotrimethylsilane (TMSCl) (Izumi *et al.*, 2002) and $H_3PW_{12}O_{40}$ (Deng *et al.*, 2010) which promoted Fischer glycosylation under milder reaction condition.

Koenigs-Knorr reaction

The Koenigs-Knorr reaction, named after Wilhelm Koenigs and Edward Knorr, is one of the oldest and simplest glycosylation reactions. This reaction is the substitution reaction of a glycosyl halide with an alcohol to give a glycoside. Two-step reaction involves treatment of glucose pentaacetate with HBr, followed by addition of the appropriate alcohol in the presence of silver oxide. For example, methylarbutin, a glycoside found in pears, has been prepared by reaction of tetraacetyl-α-D-glucopyranosyl bromide with *p*-methoxyphenol as shown in Figure 3.

1.1.4 Enzymatic synthesis of alkyl glycosides

Although it has been shown that their chemical synthesis was well-developed, the method still employs toxic and expensive compounds to yield α- and β-configuration products via several steps. However, advances in enzyme technology have led an interest in the development of biological method to synthesize anomerically pure alkyl glycosides under mild conditions by a simple one-step reaction process (van Rantwijk *et al.*, 1999). Two types of enzymes have been used for the synthesis of alkyl glycosides, the glycohydrolases (E.C. 3.2) and the glycosyl transferases (E.C. 2.4).

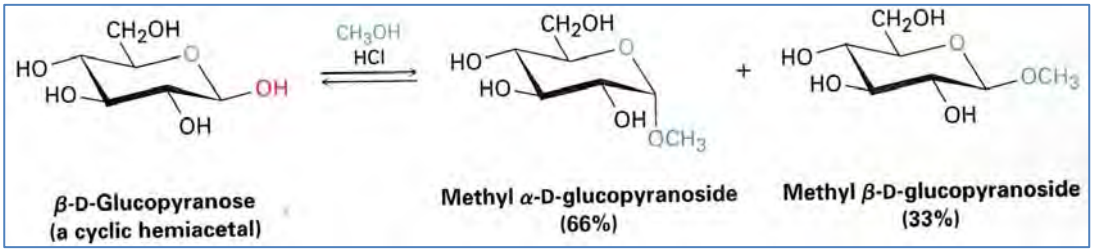


Figure 2 **The Fischer glycosidation reaction of β-D-glucopyranose with methanol in the presence of HCl as acid catalyst to produce a mixture of α and β methyl-D-glucopyranosides (McMerry, 2008).**

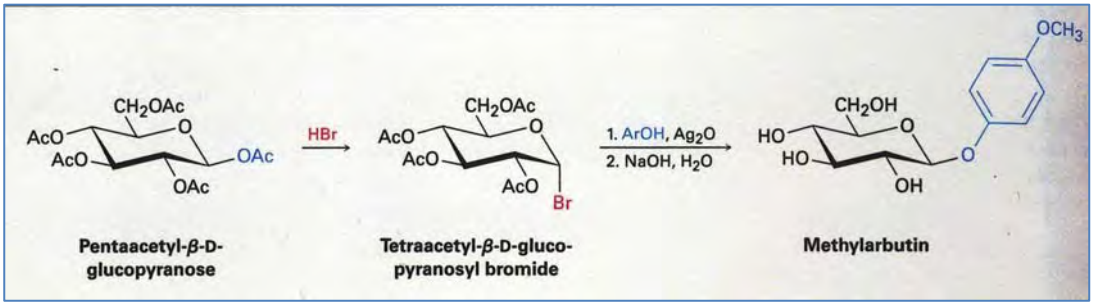


Figure 3 **The Koenigs-Knorr reaction involved two-step reaction of treatment of glucose pentaacetate with HBr, and followed by addition of ArOH in the presence of silver oxide to produce methylarbutin (McMerry, 2008).**

The glycohydrolases (E.C. 3.2)

The glycohydrolases are responsible for the hydrolytic cleavage of glycosidic bonds in nature. In general, they can be divided into two groups: the exoglycosidases, which cleave glycosidic bonds at the nonreducing end of the oligosaccharide, and the endoglycosidases, which cleave internal glycosidic bonds. Additionally, there are retaining glycosidases that preserve the stereochemistry of the anomeric center of the glycoside and inverting glycosidases that invert this center (Ichikawa *et al.*, 1992). Most reports on enzymatic synthesis of alkyl glycosides have used various glycohydrolases as shown in Table 3.

The glycosyl transferases (E.C. 2.4)

The glycosyl transferases catalyze the stereo- and regiospecific transfer of a monosaccharide from a donor substrate to an acceptor substrate. They are classified by the sugar transferred from donor to acceptor and by the acceptor specificity. The regio- and stereospecificity, the high selectivity for the acceptor substrate and the high yields achieved are attractive features of these catalysts (Nilsson, 1988). On the other hand, although glycosyl transferase enzymes have widely been studied for the synthesis of oligosaccharide by transfer reactions between donor and various kinds of acceptors (Pereira *et al.*, 1998; Lee *et al.*, 1997; Sangeetha *et al.*, 2005), for alkyl glycoside synthesis, they have been explored to a minor extent. Only levansucrase (Kim *et al.*, 2000) and dextransucrase (Kim *et al.*, 2009) have been reported to synthesize alkyl glycosides (Table 4).

Table 3 Examples of alkyl glycoside synthesis by glycohydrolases

Enzyme type	Source	Donors	Acceptors	Products	Reference
α-amylase (E.C. 3.2.1.1)	*Aspergillus oryzae*	starch	methanol ethanol 1-propanol 1-butanol	alkyl-α-glucosides having two to five glucose unit	Larsson *et al.*, 2005
			1-hexanol 1-octanol	no product occurred	
			benzyl alcohol	benzyl glucosides having two to five glucose unit	
α-rhamnosidase (E.C. 3.2.1.40)	*Aspergillus niger*	rhamnose, rhamnosides	methanol ethanol 1-propanol 2-propanol	alkyl-α-rhamnosides	Martearena *et al.*, 2003
β-galactosidase (E.C. 3.2.1.23)	*Pseudoaiteromonas* sp. 22b	lactose	2-propanol 2-butanol	alkyl galactosides	Makowski *et al.*, 2009
			t-butanol	no product occurred	
			1-pentanol 1-hexanol cyclohexanol 1-heptanol 1-octanol 1-nonanol	alkyl galactosides	

Table 3 (cont.) **Examples of alkyl glycoside synthesis by glycohydrolases**

Enzyme type	Source	Donors	Acceptors	Products	Reference
β-glucosidase (E.C. 3.2.1.21)	Thai rosewood (*Dalbergia cochinchinensis* Pierre)	pNP-Glc	methanol ethanol 1-propanol 2-propanol 1-butanol 2-methyl-1-propanol 2-butanol	alkyl-β-glucosides	Lirdprapamongkol and Svasti, 2000
			2-methyl-2-propanol	no product occurred	
			1-hexanol 1-octanol	alkyl-β-glucosides	
β-xylosidase(E.C. 3.2.1.37)	*Aspergillus niger*	xylobiose	methanol	alkyl-β-xylosides	Shinoyama *et al.*, 1988
			ethanol		
			1-propanol		
			2-propanol		
			1-butanol		
			1-pentanol		
			1-hexanol		
			2-hexanol		
			cyclohexanol		
			1-heptanol		
			1-octanol		
			benzyl alcohol		

Table 4 Examples of alkyl glycoside synthesis by glycosyl transferases

Enzyme type	Source	Donors	Acceptors	Products	Reference
levansucrase (E.C. 2.4.1.10)	*Rahnella aquatilis* ATCC 33071	sucrose	methanol	methyl β-D-fructoside	Kim *et al.*, 2000
dextransucrase (E.C. 2.4.1.5)	*Leuconostoc mesenteroides*	sucrose	methanol	alkyl α-D-glucosides	Kim *et al.*, 2009
			ethanol		
			1-propanol		
			2-propanol	no product occurred	
			1-butanol	1-butyl α-D-glucosides	

1.2 Cyclodextrin glycosyltransferase (CGTase)

1.2.1 General information of CGTase

Cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19) belong to family 13 of the glycosyl hydrolases, called α-amylase family. Most important starch processing enzymes such as α-amylases, α-glucosidases, pullulanases and isoamylases are members of this large family (Svensson, 1994). Within this family, the catalytic site residues and the α-retaining bond cleavage mechanism are well conserved, but the product and reaction specificity vary widely (McCarter and Withers, 1994). The main difference is the preference for hydrolysis or transfer reactions and specificity for α-(1,4) or α-(1,6)-glycosidic bonds. However, the structural determinants for the variation in reaction and product specificity remain to be elucidated.

CGTases convert starch into non-reducing, cyclic malto-oligosacchrarides called cyclodextrins (CDs) through an intramolecular transglycosylation called cyclization. Apart from catalyzing this reaction, CGTase presents an intermolecular transglycosylation: a coupling activity (opening of the rings of CDs and transfer to acceptors) and a disproportionation activity (transfer of linear maltooligosaccharides to acceptors) (Biwer *et al.*, 2002). The hydrolytic activity of CGTases is in general much lower than the transglycosylation activities (Penninga *et al.*, 1995 and van der Veen *et al.*, 2000).

1.2.2 Structure and mechanism of CGTase

CGTases consist of homologous $(\beta/\alpha)_8$-barrel proteins that present in all enzymes of the α-amylase family (Uitdehaag *et al.*, 2002). In fact, α-amylase is a

strongly hydrolytic enzyme, whereas CGTase is basically a transglycosylate one. It has been postulated that the transferase activity of CGTase evolved from an ancestral hydrolase (del-Rio *et al.*, 1997). CGTase has five domains, labeled A to E (Figure 4). Domain A is the catalytic $(\alpha/\beta)_8$ domain, which CGTase has in common with other α-amylase family members. Three carboxylic residues involve in catalysis: Asp230, Glu258 and Asp329. Domain B, also presented in many other α-amylase family enzymes, is an extended loop region inserted after β-strand 3 of domain A. It contributes to substrate binding by providing several amino acid side chains alongside a long groove on the surface of the enzyme that interact with the substrate. Domains C and E have a β-sheet structure, and are specialized in binding to raw starch granules. The function of domain D, which also has a β-sheet structure, remains to be elucidated (Uitdehaag *et al.*, 2002). There are two main structural features differentiate CGTases from α-amylases: first, in all the CGTases structurally characterized, an aromatic residue (Phe or Tyr) in position 196 seems to play a central role as a cyclization axis during the CD-formation (Penninga *et al.*, 1995); second, CGTases are larger, containing two additional domains (D and E). Moreover, there are other factors which may contribute to the differences in the ratio between hydrolysis and transglycosylation activities within this family. One of them is the existence of an extra acceptor subsite in CGTases, but not present in amylases. It has been postulated that an induced-fit mechanism may take place after binding of sugars (but not water) at the acceptor subsites, which activates CGTases for catalysis (Leemhuis *et al.*, 2002).

All glycosyl hydrolases are thought to act by a general acid catalysis mechanism in which two amino acid residues participate in a single-displacement or

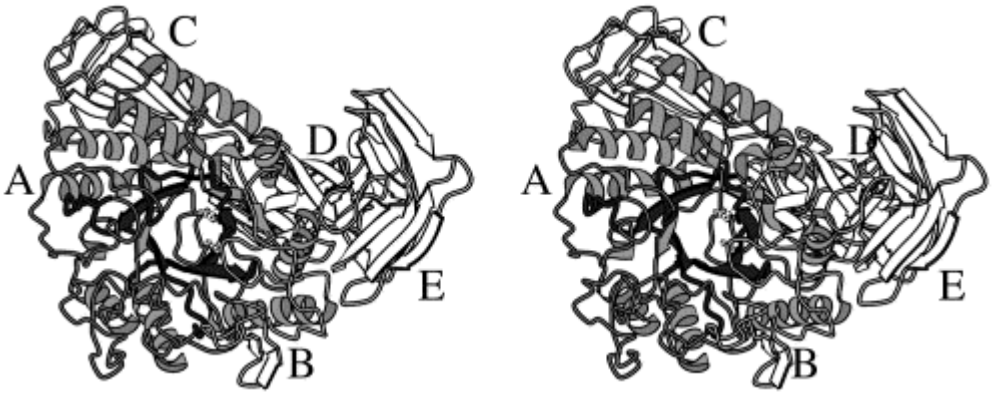


Figure 4 **Stereo-view of the structure of a representative member of the α-amylase family, *Bacillus circulans* strain 251 cyclodextrin glycosyltransferase (BC 251 CGTase) (Uitdehaag *et al.*, 2002).**

Domain A:	the catalytic $(\beta/\alpha)_8$ barrel (gray) containing the sequence regions that are conserved in the α-amylase family (black)
Domain B:	a loop protrudes that forms part of the active site (white)
Domain C, D and E:	three additional domains containing binding sites for raw starch

double-displacement reaction resulting in inversion or retention of configuration respectively at the anomeric carbon atom of the hydrolysed glycoside (Sinnott, 1990). Reaction in each case of CGTases proceeds with retaintion of anomeric configuration and is believed to involve a two-step double displacement mechanism as shown in Figure 5 (Koshland, 1953). The first step involves the attack of the catalytic nucleophile at the anomeric center of the sugar with general acid assistance to aid in the leaving group departure. This generates a glycosyl-enzyme intermediate which can then undergo transglycosylation or hydrolysis in a second step with general base assistance to facilitate attack by the incoming group. Both steps proceed via transition states with substantial oxocarbenium ion character. In addition to the two active site carboxylates functioning as the nucleophile and general acid/base catalyst, a third conserved carboxylate is believed to function by modulating the ionization state of the other catalytic residues and producing a more favorable electronic environment for stabilization of the positively charged transition state.

On the basis of this reaction scheme, two types of substrate specificity can be distinguished, acceptor and donor types. CGTase has acceptor specificity since it can use various types of acceptors with different kinetic values. Hexoses such as glucose and sorbose were better acceptors than pentoses since they showed higher observed k_{cat}/K_m values (Wongsangwattana *et al.*, 2010). The donor specificity of CGTase determines the preferred sugar chain length that is bound at the donor sites and thereby the size of cyclodextrin that is predominantly formed (van der Veen *et al.*, 2000).

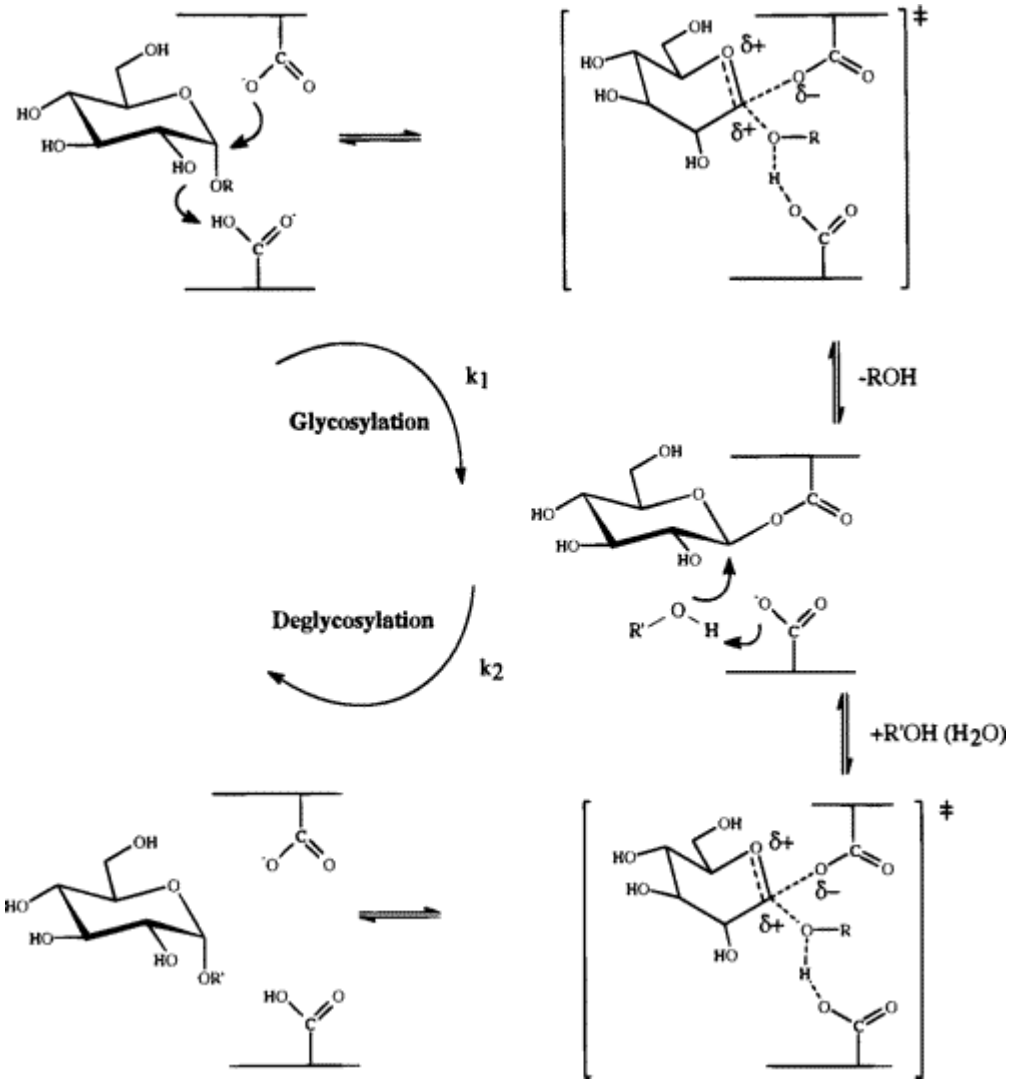


Figure 5 **Proposed mechanism for a retaining α-glycosyltransferase (Koshland, 1953)**

1.2.3 Applications of CGTases

CGTase enzymes are able to produce CDs from starch via the cyclization reaction, which is the basis of their industrial application. CDs have their systematic names of cyclic α-D-(1,4)-linked D-glucose oligosaccharides consisting of 6–8 glycosyl units, well known as α-, β- and γ-CDs. CD molecules have the ability to form inclusion complexes with a variety of compounds therefore they are used in a wide range of application in food, pharmaceutical, cosmetic and agricultural industries. However, recent developments also concentrate on the use of the CGTase catalyzed coupling and disproportionation reactions for the synthesis of gluco-conjugates of potential interest by using alternative acceptor substrates. Studies on the transglycosylation reactions of CGTases have been reported. Examples as follow:

(a) Yoon and Robyt (2002) discovered new kinds of acarbose analogues which were synthesized by the reaction of acarbose with cyclomaltohexaose and CGTase. Three major CGTase coupling products were separated, purified and characterized as D-glucopyranosyl-acarbose which the D-glucose moiety was attached to the C-4-OH group of the nonreducing-end cyclohexene ring of acarbose.

(b) Shimoda *et al.* (2007) used a two step synthesis of a series of curcumin β-maltooligosaccharides through sequential biocatalytic glycosylation using *Strophanthus gratus* cell culture and cyclodextrin glucanotransferase (CGTase). Cultured plant cells of *S. gratus* converted exogenously added curcumin into unnatural curcumin β-D-glucoside. Furthermore, four unnatural β-maltooligosaccharides, that is, α-Glc-1→(4-α-Glc-1→)$_{n-1}$4-β-D-glucosides

(n = 1–4), of curcumin were produced from curcumin β-D -glucoside in the presence of starch by CGTase-catalyzed glycosylation.

(c) Miranda-Molina *et al.* (2010) demonstrated the formation of α-D -glucopyranosyl-(1→4)-4D-*myo*-inositol and α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol by a combined enzymatic transglucosylation and hydrolysis strategy, using cyclodextrin glucosyl transferase (CGTase) from *Thermoanaerobacter* sp., followed by hydrolysis with *Aspergillus niger* glucoamylase.

The starch and cyclodextrin research unit at the Department of Biochemistry, Facuty of Science, Chulalongkorn University has been working on applications of transglycosylation of CGTase from thermotolerant *Paenibacillus* sp. RB01. This strain had previously been screened from hot spring soil in Ratchaburi and CGTase from this strain was first partially purified and characterized by Tesana (2001). Then, this enzyme was completely purified and biochemically characterized by Yenpetch (2002). Thanadolsathien (2007) used a two-step enzymatic route, a combination of α-glucosidase from *Saccharomyces cerevisiae* and CGTase from *Paenibacillus* sp. RB01, in order to improve the solubility in water of vitamin E analog (Trolox). From his research, a novel vitamin E glycoside was synthesized with the 7×10^4 times higher than Trolox. In addition, this CGTase has the wide variety of aglycone acceptor specificity. Aramsaengtienchai (2007) utilized this enzyme property to modify the structure of epicatechin, the valuable flavonoid from plant, with higher water solubility.

The aim of this research is the formation of alkyl glycosides from miscible alcohols by the transglycosylation action of CGTase from *Paenibacillus* sp. RB01. The most efficient alcohol acceptor was chosen and the glycoside products were identified. Optimized reaction conditions for maximum glycoside production were also investigated. Then, the glycoside product will be purified and structurally elucidated.

The objectives of this research were:

I) To study the transglycosylation to alcohols with CGTase from *Paenibacillus* sp. RB01

II) To determine the optimum conditions for the alkyl glycoside production

III) To purify and elucidate the structure of alkyl glycoside product

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Asahipak NH2P-50 4E (4.6 mmID × 250 mmL): Showa Denko K.K., Japan

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

Fourier Transform Nuclear Magnetic Resonance (FT-NMR):

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: MicrOTOF, Bruker Daltonics Inc., USA

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

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

2.2 Chemicals

Acetonitrile (HPLC grade): LAB-SCAN, Thailand

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

Agar: Scharlau, Spain

α-Cyclodextrin: Sigma, USA

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

di-Potassium hydrogen orthophosphate: Univar, Australia

Ethanol, absolute: Merck, Germany

Ethyl acetate: LAB-SCAN, Thailand

Ethylenediamine tetraacetic acid (EDTA): Univar, Austraria

Fiberose®: Banpong Tapioca Flour Industrial Co., Ltd., Thailand

Flomax®: National Starch, USA

γ-Cyclodextrin: Sigma, USA

Glacial acetic acid: Mallinckrodt Chemicals, Thailand

Glucose: Univar, Austraria

Glycerol: Merck, Germany

Glycine: Sigma, USA

Hydrochloric acid: Carlo Eaba Reagents, Italy

Iodine: Merck, Germany

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

85% Orthophosphoric acid: 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

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

Yeast extract: Scharlau, Spain

2.3 Bacteria

Paenibacillus sp. RB01 with CGTase activity was isolated from hot spring soil in Ratchaburi province, Thailand (Tesana, 2001) and was used for the production of CGTase.

2.4 Media preparation

2.4.1 Medium I

A liquid Medium I consisting of 0.5% (w/v) beef extract, 1.0% (w/v) peptone, 0.2% (w/v) NaCl, 0.2% (w/v) yeast extract and 1.0% (w/v) soluble starch, was prepared and adjusted to pH 7.2. For solid medium, 1.5% (w/v) agar was added. Medium I was sterilized by autoclaving at 121°C for 15 minutes.

2.4.2 Horikoshi's medium

Horikoshi's medium, slightly modified from Horikoshi (1971) by Rutchtorn (1993), was used to produce CGTase. The medium formula contained 1.0% (w/v) soluble starch 0.5% (w/v), 0.5% (w/v)yeast extract, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.75% (w/v) Na_2CO_3 which was added separately after sterilization. The pH of the medium was 10.1-10.2. Medium was sterilized as previously described.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

Paenibacillus sp. RB01 was streaked on solid Medium I and incubated at 37°C for 18 hours. Colonies of bacteria were inoculated into liquid Medium I and grown at 37°C in shaking incubator at 250 rpm until A_{660} reached 0.3-0.5.

2.5.2 Enzyme production

Starter inoculum (1.0% (v/v)) of *Paenibacillus* sp. RB01 was transferred into 300 mL of Horikoshi's medium in a 1,000 mL Erlenmeyer flask and cultivated at 40°C in shaking incubator at 250 rpm. After 72-hour incubation, cells were removed by refrigerated centrifugation at 5,000 rpm for 15 minutes at 4°C. Supernatant with crude CGTase was collected and kept at 4°C for further purification.

2.6 Partial purification of CGTase

CGTase from the culture broth was partially purified by starch adsorption method (Kato and Horikoshi, 1984) with modification by Kuttiarcheewa (1994).

Corn starch was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude CGTase supernatant to make 5% (w/v) concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 8,000 rpm for 30 minutes and washed twice with 10 mM tris-HCl pH 8.5 containing 10 mM $CaCl_2$ (TB1). CGTase was eluted from the starch cake with 0.2 M maltose in TB1 (2×125 mL for starting broth of 1 L) with stirring for 30 minutes. The eluate was collected by centrifugation at 10,000 g for 30 minutes. The enzyme solution was concentrated with ultrafiltration membrane (10,000 MWCO) and dialyzed against three changes of distilled water at 4°C.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

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

The gel was consisted of 7.5% (w/v) separating and 5.0% (w/v) stacking gels and carried out under Tris-glycine buffer pH 8.0 as electrode buffer (see Appendix A). The protein samples were mixed with sample buffer and then, the sample solutions were introduced into wells. The electrophoresis was performed at constant current of 16 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode until the dye front reached the bottom of the gel.

2.7.2 Protein staining

After electrophoresis, proteins on gel were vestured by Coomassie blue staining. Gel was stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes. The slab gel was destained

with a solution of 10% (v/v) methanol and 10% (v/v) acetic acid for 1-2 hours, followed by several changes of destaining solution until gel background was clear.

2.7.3 Dextrinizing activity staining

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

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

The denaturing gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels with Tris-glycine buffer, pH 8.0 containing 0.1% SDS as electrode buffer (see Appendix A). Samples to be analyzed were treated with sample buffer and boiled for 5 minutes prior to gel application. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode. After electrophoresis, proteins in the gel were visualized by protein staining as described above.

2.8 Enzyme assay

2.8.1 Dextrinizing activity

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

2.8.2 Coupling activity

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

2.9 Protein determination

Protein concentration was determined by Bradford's method (1976), using bovine serum albumin as the standard protein (see Appendix C). Enzyme sample (100 μL) was mixed with 1.0 mL of Coomassie blue reagent and left for 5 minutes before recording the absorbance at 595 nm. One liter of Coomassie blue reagent containing 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 dark bottle before used.

2.10 Transglycosylation reactions

To determine the synthesis ability of CGTase and an appropriate alcohol acceptor, transglycosylation reactions with varying types and alcohol content were performed using β-CD as a glycosyl donor. The reaction mixture (0.25 mL) consisted of final 0.6% (w/v) β-CD (1.5 mg), various concentrations and types of alcohols (methanol, ethanol, 1-propanol, 2-propanol: 10%-60% (v/v), 1-butanol: 5%-8% (v/v), 2-butanol: 5%-20% (v/v)) and CGTase (final dextrinizing activity of 200 U/mL) in 50 mM acetate buffer pH 6.0. The reaction mixture was incubated at 40°C for 24 hours. Aliquots were withdrawn and analyzed by TLC (System I). The coupling activity which measured the amount of β-CD disappearance was determined by the phenolphthalein method as previously described in section 2.8.2.

2.11 Donor specificity

Several glucose-rich donors were investigated for transglycosylation activity by CGTase. The reaction mixtures (0.25 mL) containing methanol (30%, v/v), 0.6%

(w/v) different glycosyl donors (β-CD, soluble starch, Fiberose®, Flomax®) and 200 U/mL CGTase in 50 mM acetate buffer pH 6.0 were incubated at 40°C for 24 hours. Samples were analyzed using thin layer chromatography. The intensity of methyl α-D-glucopyranoside (MG_1) spots was measured by MG_1 standard curve which was calculated from the intensity of standard MG_1 on TLC plate (see Appendix E).

2.12 Preliminary characterization of reaction products

The reaction mixture (0.75 mL) consisted of final concentration of 0.6% (w/v) β-CD, 30% (v/v) methanol and 200 U/mL CGTase in 50 mM acetate buffer pH 6.0 was incubated at 40°C for 24 hours. To preliminary investigate the transglycosylation products, glucoamylase was introduced to catalyze the hydrolysis of glucosidic bonds between glucose residues in the reaction mixture. After incubation, the transglycosylation reaction mixture containing methanol as an acceptor was heated to evaporate the remaining methanol by CentriVap Concentrator until the reaction volume was 0.50 mL and mixed with 0.25 mL of *A. niger* glucoamylase from stock solution of 60 U/mL (final activity of 20 U/mL). After incubation at 40°C for 1 hour, glucoamylase was inactivated by heating at 100°C for 5minutes. Moreover, to check the linkage between methanol and glucosyl unit, α-glucosidase (final activity of 20 U/mL) from *S. cerevisiae* was added to the reaction mixture after glucoamylase treatment and further incubated at 40°C for another 3 hours. The products after treatment with the two enzymes were analyzed by TLC (System II) and HPLC.

2.13 The methyl glycoside (MG_n) product determination

2.13.1 Thin Layer Chromatography (TLC)

System I (for the determination of an appropriate alcohol acceptor and donor specificity)

Reaction products were analyzed by applying 10 μL of samples on silica gel 60 F_{254} aluminum sheet (20 cm in height), resolved once in a system composing of ethyl acetate/acetic acid/water (3:1:1 by vol) for the analysis of alkyl glycoside products. Chromatograms were visualized by spraying with conc. sulfuric acid-methanol (1:2) followed by heating at 110°C for 10 minutes. The intensity of product spots of equal area was quantitated by scanning chromatograms with GeneTools program of SYNGENE. Glucose spot (35 μg) was set as standard value.

System II (for the identification of methyl glycoside products)

This solvent system was modified from System I. The TLC plate (20 cm in height) was developed twice in ethyl acetate/acetic acid/water 3:1.5:0.5 by vol as a sovent system. The glycoside products were detected by the same method as System I.

System III (for the purity determination of product fraction)

The fractions collected from column chromatography were analyzed by Thin Layer Chromatography (TLC) using silica gel 60 F_{254} (12 cm in height). Solvent system was 1-propanol/ethyl acetate/water/25% ammonia solution (6:1:3:1, v/v). After developed twice, TLC plate was detected by the same procedure as System I.

2.13.2 High Performance Liquid Chromatography (HPLC)

Products in the reaction mixture were analyzed by high performance liquid chromatography (Shimadzu, Japan) connected with Asahipak amino column (4.6×250 mm) and a refractive index detector. The adsorbed compounds were eluted with 65% (w/v) acetonitrile in ultra pure water at a flow rate of 1.0 mL/min.

2.13.3 Mass Spectrometry (MS)

The reaction mixture was concentrated by CentriVap Concentrator before subjected to the molecular mass measurement by mass spectrometry. Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was performed by a micrOTOF at the Biological Service Unit of the National Center of Genetic Engineering and Biotechnology (BIOTECH, Thailand). The compounds were ionized by electrospray ionization on the positive-ion mode.

2.14 Optimization of MG_n production

2.14.1 Optimization of temperature

The effect of temperature on the glycoside product synthesis was determined by 24-hour incubation of 0.5 mL reaction mixture consisted of 0.6% (w/v) β-CD in 50 mM acetate buffer (pH 6.0) containing 30% (v/v) methanol with CGTase (200 U/mL) at the selected temperature (20, 30, 40, 50, 60°C). After the reactions were stopped by boiling for 5 minutes, the remaining methanol was evaporated by CentriVap Concentrator until the reaction volume was 0.25 mL and 0.25 mL of glucoamylase from stock solution of 400 U/mL (final activity was raised to 200 U/mL) was added to a reaction mixture. The glucoamylase reaction was incubated at 40°C for 1 hour and

then boiled for 5 minutes. The total amount of methyl glycoside (MG_n) in the form of methyl α-D-glucopyranoside (MG_1) was analyzed by HPLC as mentioned in section 2.13.2.

2.14.2 Optimization of pH

The appropriate pH for the methyl glycoside synthesis was determined by using 30% (v/v) methanol mixed well with final concentration of 0.6% (w/v) β-CD in buffer solution and 200 U/mL CGTase in the reaction mixture of 0.75 mL. The fifty-millimolar buffers with pH ranging from 5.0-8.0 were used which were acetate buffer (pH 5.0-6.0), phosphate buffer (pH 6.0-7.0) and Tris-glycine buffer (pH 7.0-8.0). The optimum temperature determined from the previous section was used and the reaction was performed for 24 hours. Then, the reaction was stopped and treated with glucoamylase before analyzing by HPLC as described previously.

2.14.3 Optimization of methanol concentration

Methanol concentration ranging from 10-50 percent in an appropriate buffer type and pH from Section 2.14.2 was used to dissolve 0.6% (w/v) β-CD and mixed with CGTase (200 U/mL) for 24 hours at the optimum temperature from section 2.14.1. After that, the reaction mixture was treated with glucoamylase and the products were then determined by HPLC as mentioned in section 2.14.1 and 2.13.2, respectively.

2.14.4 Optimization of β-cyclodextrin concentration

The amount of β-CD which was varied from final concentration of 0.3-1.8% (w/v), was dissolved in optimum buffer solution with optimum pH from section

2.14.2. Then, the β-CD solution was mixed with optimum concentration of methanol from section 2.14.3 and 200 U/mL CGTase. The mixture was incubated at the optimum temperature from section 2.14.1 for 24 hours. The HPLC was used to analyze the reaction products after treatment with glucoamylase under the same condition as described in section 2.13.2.

2.14.5 Optimization of enzyme concentration

The substrate solution which contained the optimum concentration of β-CD (section 2.14.4) and methanol (section 2.14.3) in optimum buffer solution with optimum pH from section 2.14.2 was incubated with 40-240 units of CGTase at the optimum temperature from section 2.14.1 for 24 hours. The appropriate enzyme concentration was judged from the determination of peak area of MG_1 from HPLC analysis.

2.14.6 Optimization of incubation time

The reaction was performed by incubation of an appropriate concentration of CGTase with optimum concentration of methanol acceptor and β-CD donor at optimum pH and temperature for various times (24, 48, 72, 96 and 120 hours). The reaction was stopped by boiling for 5 minutes, and then treated with glucoamylase (section 2.14.1). The optimum incubation time for the production of methyl glycoside was analyzed by HPLC.

2.15 Determination of transglycosylation yield

All the appropriate conditions for methyl glycoside synthesis determined from section 2.14 were used to measure the efficiency of optimized reaction. Glucoamylase

was used to convert all transfer products into MG_1. Quantification was based on standard curve of MG_1 (see Appendix F).

2.16 Time course of methyl glycoside production

Time course of methyl glycoside production was established by using the reaction condition of several incubation times from section 2.14.6. The reaction products with non-treatment by glucoamylase were analyzed by HPLC to determine peak area of each methyl glycoside product at designated incubation time.

2.17 Large scale preparation, isolation and identification of MG_1

2.17.1 Scale-up for MG_1 production

In the initial experiments, methyl glycoside products were prepared and optimized in the transglycosylation reaction (section 2.14) to determine transglycosylation efficiency (section 2.15) in small scale reaction mixture of 0.5 mL. In order to produce higher amount of product for characterization, larger scale preparation (10 mL) of reaction mixture using optimum condition for transglycosylation as obtained from section 2.14 was performed. After the reaction was stopped and evaporated the remaining methanol, glucoamylase (final concentration of 200 U/mL) was added to the reaction mixture and incubated at 40°C for 1 hour. The reaction mixture was concentrated into 2 mL before further purification.

2.17.2 MG_1 purification

Enzymatically synthesized MG_1 was purified by strong anion exchange chromatography. Column (2.3 × 22 cm) was packed with Amberlite® IRA-900 pretreated with 0.1 N NaOH to substitute the functional group with OH^-. The reaction mixture of 2 mL from previous step (section 2.17.1) was loaded and eluted with distilled water at the flow rate of 0.5 mL/min. The fractions containing products were collected with 4-mL fraction size and determined for total sugar by phenol sulfuric acid assay (section 2.17.3) and TLC analysis System III (section 2.13.1). The product fractions were collected and dried by freeze-dryer for structure elucidation.

2.17.3 Phenol-sulfuric acid assay

This method was modified from Rao and Pattabiraman (1989). Fractions collected from section 2.17.2 of 0.1 mL was mixed with 2.0 mL of sulfuric acid. The solution was cooled in ice for 2 minutes. To the cooled solution, 0.5 mL of 5% (w/v) phenol was added and mixed using vortex. The reaction mixture was kept at room temperature for 10 minutes before reading the color intensity at A_{485}.

2.17.4 Nuclear Magnetic Resonance (NMR)

^{13}C NMR spectra were performed at the Organic Synthesis Research Unit of Department of Chemistry, Faculty of Science, Chulalongkorn University. The operation was at 100 MHz. The spectra were obtained with 20 mg of sample dissolved in deuterated water (D_2O).

CHAPTER III

RESULTS

3.1 Partial purification of CGTase from *Paenibacillus* sp. RB01

After cultivation of *Paenibacillus* sp. RB01 in Horikoshi's medium at 40°C with 250 rpm continuous shaking for 72 hours, the culture was centrifuged to remove cells and crude CGTase in the supernatant was obtained. Crude enzyme was partially purified by starch adsorption as described in Methods section 2.6. The purification fold and recovery of CGTase collected at each step are shown in Table 5. The % recovery of CGTase and purification fold were 38.1% and 46.3 respectively. The partial purified enzyme had a specific activity expressed in terms of dextrinizing activity per mg protein of 8,377 U/mg protein which increased through this step.

Furthermore, the purity of CGTase was checked by native polyacrylamide gel electrophoresis (Native-PAGE) as described in section 2.7.1. Protein staining revealed that the enzyme was successfully purified through one-step purification since less protein bands were observed (Figure 6A, Lane 2). The mobilities of the active bands as determined by dextrinizing activity staining (Figure 6B) coincided with those 2 bands stained with Coomassie brilliant blue (Figure 6A, Lane 2). This implies that the partial purified enzyme had 2 isoforms with different net charge and size.When sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the partial purified enzyme was performed, the molecular weight of CGTase was estimated to be 70 kDa which corresponded to previous report (Yenpetch, 2002). In addition, only

Table 5 Purification of CGTase from *Paenibacillus* sp. RB01

Purification step	Volume (mL)	Total activity* ($U \times 10^3$)	Total protein (mg)	Specific activity* (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1,700	50.1	277	181	1	100
Starch adsorption	136	19.1	2.28	8,377	46.3	38.1

* Dextrinizing activity

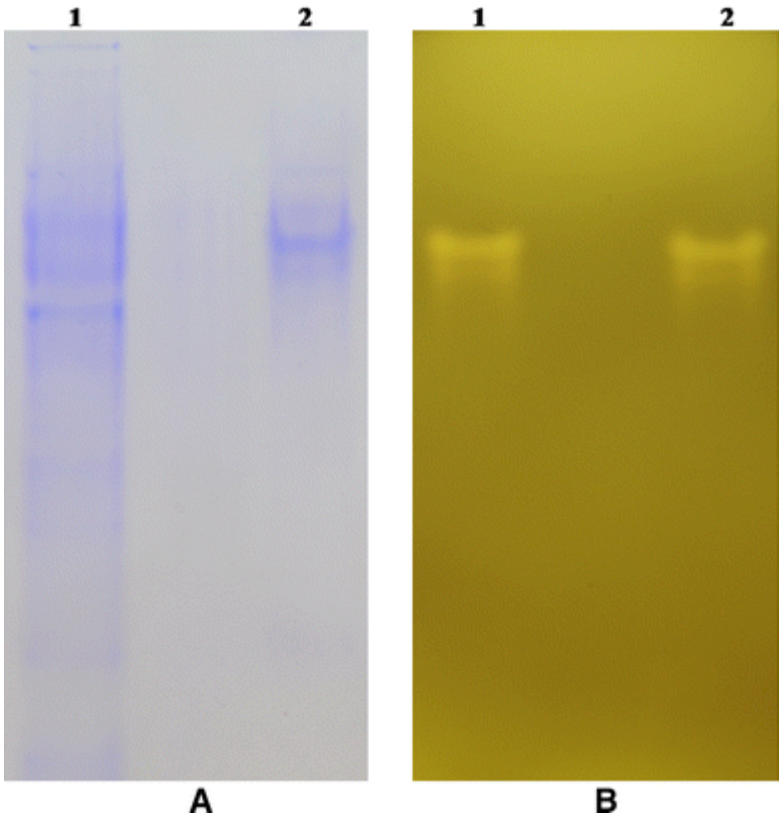


Figure 6 **Non-denaturing PAGE of *Paenibacillus* sp. RB01 CGTase in crude and starch adsorbed samples on a 7.5% acrylamide gel.**

A: Coomassie blue staining

Lane 1: Crude enzyme (50 μg)

Lane 2: Starch adsorption (20 μg)

B: Dextrinizing activity staining

Lane 1: Crude enzyme (0.05 U)

Lane 2: Starch adsorption (0.05 U)

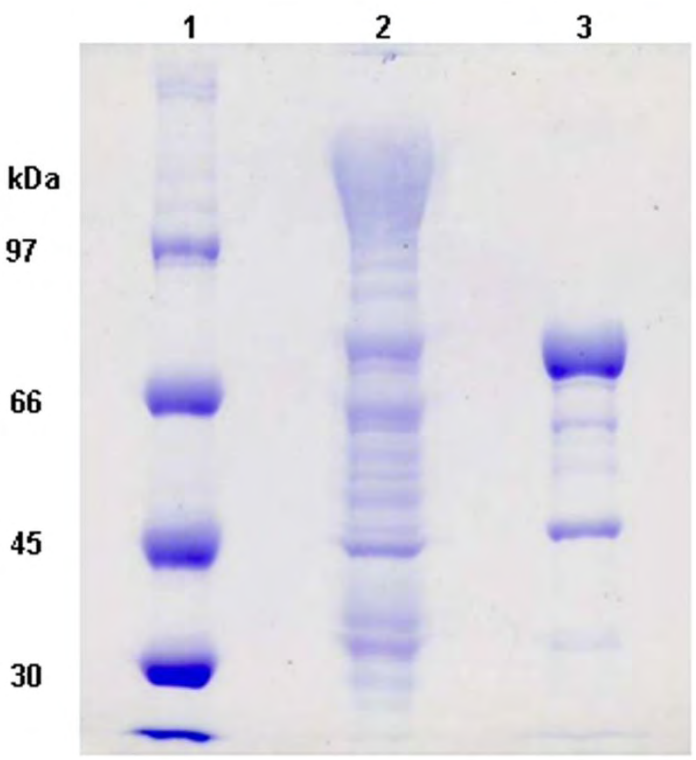


Figure 7 **SDS-PAGE of *Paenibacillus* sp. RB01 CGTase in crude and starch adsorbed samples on a 7.5% acrylamide gel.**

Lane 1: Protein molecular weight markers

Phosphorylase b (97 kDa)

Albumin (66kDa)

Ovalbumin (45 kDa)

Carbonic anhydrase (30 kDa)

Lane 2: Crude enzyme (50 μg)

Lane 3: Starch adsorbed enzyme (10 μg)

two faint protein bands of lower molecular weight were found (Figure 7). Thus, this partial purified CGTase was pure enough to be further used as a source of enzyme for the synthesis of alkyl glycosides.

3.2 Synthesis of alkyl glycosides and detection of the products

Attempts were made to synthesize alkyl glycosides to investigate the capability of CGTase to transglycosylate alkyl alcohols. Different alcohols at which concentrations give a single phase were chosen as glycosyl-acceptor substrate according to previous report when β-glucosidase from Thai rosewood was used to synthesize alkyl glucoside (Lirdprapamongkol and Svasti, 2000). The reaction was performed at the optimum pH (6.0) of the enzyme for 24 hours. Products from transglycosylation of β-CD to 40% (v/v) methanol and ethanol, 10% (v/v) 1- and 2-propanol and 5% (v/v) 1- and 2-butanol were preliminary analyzed by TLC (Figure 8). In order to assure that the product observed as a spot on TLC was transglycosylation product by the action of CGTase, the reaction mixture without alcohol acceptors and reaction mixture containing both substrates incubated with CGTase at 0 hour were used as control experiments.

At zero-incubation time, only β-CD spots which slightly moved from the origin were seen (Figure 8, Lane 5, 7, 9, 11, 13, 17). After 24-hour incubation of CGTase with various concentrations of alkyl alcohol acceptors and β-CD donor, expected alkyl glycoside spots were observed (at R_f value of 0.33-0.62) in addition to glucose and other oligosaccharides (at R_f value of 0.02-0.18) which were resulted from hydrolysis reaction (Figure 8, Lane 8, 10, 12, 14, 16, 18). The transfer products

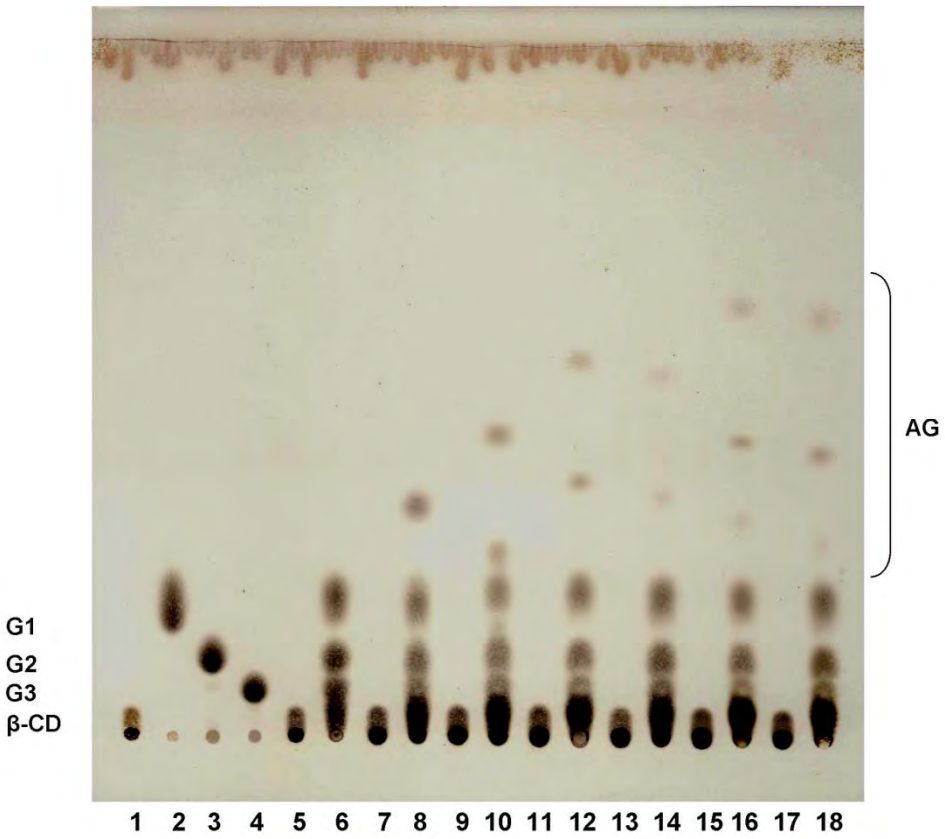


Figure 8 **TLC analysis of the transglycosylation products from β-CD to various alcohols by CGTase.** TLC condition was System I, ethyl acetate/acetic acid/water, 3:1:1.

Lane 1: Standard β-CD (25 μg)

Lane 2: Standard glucose (25 μg)

Lane 3: Standard maltose (25 μg)

Lane 4: Standard maltotriose (25 μg)

Lane 5-6: Reaction mixture without alcohol acceptor, 0 and 24 h

Lane 7-8: Reaction mixture with 40% (v/v) methanol, 0 and 24 h

Lane 9-10: Reaction mixture with 40% (v/v) ethanol, 0 and 24 h

Lane 11-12: Reaction mixture with 10% (v/v) 1-propanol, 0 and 24 h

Lane 13-14: Reaction mixture with 10% (v/v) 2-propanol, 0 and 24 h

Lane 15-16: Reaction mixture with 5% (v/v) 1-butanol, 0 and 24 h

Lane 17-18: Reaction mixture with 5% (v/v) 2-butanol, 0 and 24 h

Table 6 **R_f values from TLC analysis (System I) of standard saccharides and the reaction products**

Standard/Products			**R_f**
Standards	Saccharides	β-CD	0.02
		Glucose	0.18
		Maltose	0.12
		Maltotriose	0.07
Reaction products top ↓ bottom	Methanol	I	0.33
	Ethanol	I	0.44
		II	0.26
	1-Propanol	I	0.55
		II	0.37
	2-Propanol	I	0.51
		II	0.35
	1-Butanol	I	0.62
		II	0.43
		III	0.32
	2-Butanol	I	0.60
		II	0.41
		III	0.28

of alcohol were distinguished from sugars by larger R_f values (Table 6). Methanol gave at least one product while ethanol, 1- and 2-propanol gave at least two products. When 1-butanol and 2-butanol were used as acceptors, three alkyl glycosides were detected. In the control condition where alcohol was not added, only oligosaccharides which ran slower than those of alkyl glycosides were observed. The results suggested that CGTase from *Paenibacillus* sp. RB01 could transfer glucose residues from β-CD to short chain alkyl alcohols giving alkyl glycosides.

3.3 The effect of alcohol length and concentration on the production of alkyl glycosides

3.3.1 Determination of CGTase coupling activity

The effect of alcohols on coupling activity of CGTase was investigated in buffer and in a mixture of buffer and alcohol solution with varying alcohol length and concentration at the optimum pH (6.0) of the enzyme for 24 hours as described in section 2.8.2. The alcohol used here were 10-60% of methanol, ethanol, 1- and 2-propanol. However, for 1- and 2-butanol, their concentration used in a single phase reaction could not exceed 8 and 20%, respectively due to their low solubility in water. The coupling activity of CGTase in these alcohols was determined in relative to that in 50 mM acetate buffer, pH 6.0 which was set as 100%. As can be seen in Figure 9, when the concentration of alcohol increased, the activity of the enzyme was dramatically decreased. The activity of CGTase was highest in co-solvent system of methanol, followed by ethanol, 1-propanol ≈ 2-propanol, and 1-butanol ≈ 2-butanol, respectively. These results showed that the alcohol length and concentration had significant effect on CGTase activity. The enzyme retained appreciable activity (more

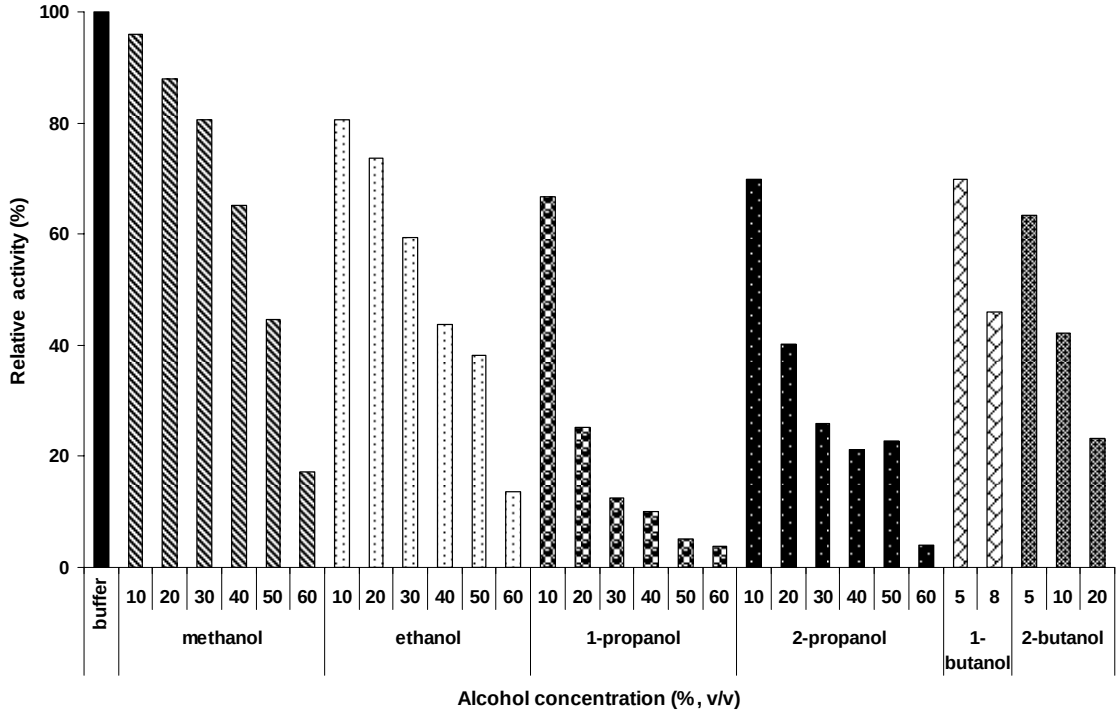


Figure 9 **Relative coupling activity of CGTase in alcohol solvent mixtures.**

Residual activity (%) was relative to the coupling activity of CGTase incubated with 0.6% (w/v) β-CD and 5-60% (v/v) alcohols in 50 mM acetate buffer solution (pH 6.0) at 40ºC for 24 hours. All values were average from two separate experiments.

than 50%) only in the methanol-containing medium when its concentration was raised to 40% (v/v). This result suggested that methanol was an appropriate solvent for transglycosylation reaction by CGTase.

3.3.2 Determination of glycoside product by TLC

The effect of alcohols on alkyl glycoside synthesis by CGTase was determined by TLC method and the product spot intensities were measured with GeneTools program of SYNGENE as described in section 2.13.1. Only a product spot from each alcohol acceptor which gave the highest R_f value was quantitated to determine acceptor specificity because different numbers of products were detected from each alcohol on TLC plate (Figure 8). The degree of intensity of alkyl glycosides, which reflected the production yield, synthesized from primary alcohols was highest in methanol, followed by ethanol and 1-propanol (Figure 10). In the case of secondary alcohols, 2-propanol and 2-butanol, alkyl glycoside yield was much lower, indicating that the length of alkyl group as well as the structure of alcohols affected the level of transglycosylation. In particular, for the alkyl glycoside production using 2-propanol as an acceptor, the yield was lower than that of 1-propanol although the enzyme activities in both reaction mixtures were approximately at the same level (see Figure 9). There was little product yield at lower concentration of alcohol, but when the level of alcohol was raised, the product yield increased until its concentration exceeded 50% which could be due to the inactivation of the enzyme. From these results, methanol showed the highest acceptor specificity toward CGTase from *Paenibacillus* sp. RB01.

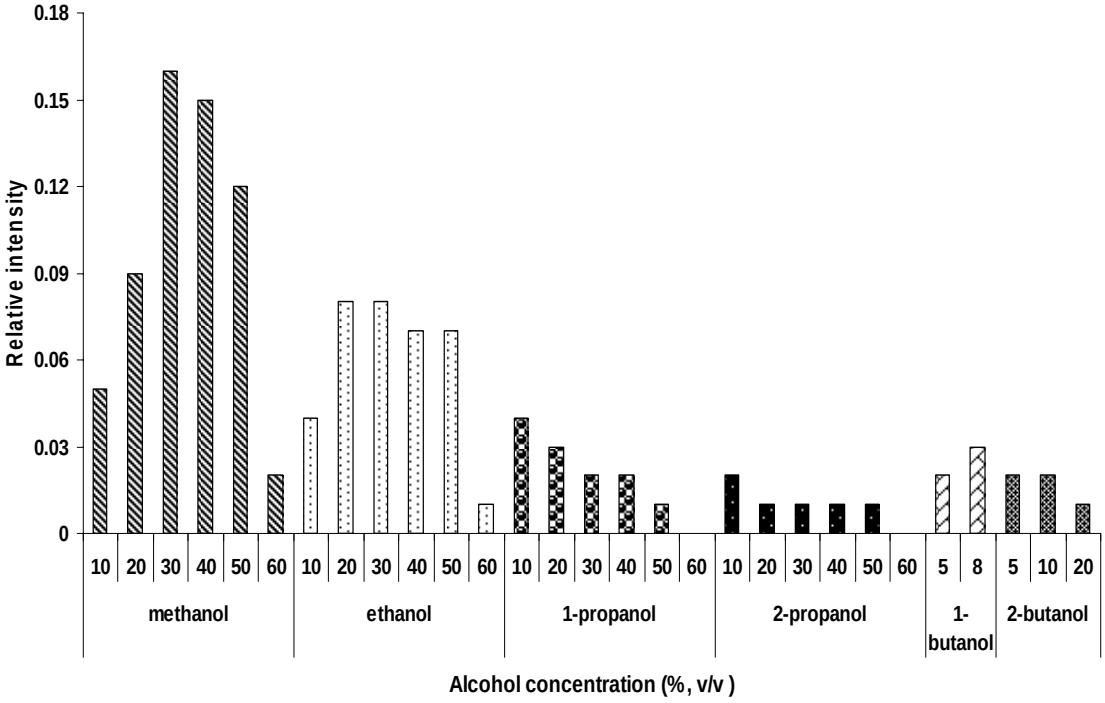


Figure 10 **Relative intensity of alkyl glycoside spots from transglycosylation reaction of β-CD to various alcohol acceptors**.

Alkyl glycosides of each spot were quantitated relative to glucose spot in the same TLC plate. The intensity of glucose spot was set to 1. All values were average from duplicate TLC plates.

3.4 Donor specificity

In order to study the synthesis of alkyl glycosides by CGTase, the reaction with methanol as an acceptor was chosen here as a model for donor specificity investigation. β-Cyclodextrin and various modified starch (soluble starch, Flomax® and Fiberose®) were selected as candidate glycosyl donors. The concentration of all the glycosyl donors was controlled at final concentration of 0.6% (w/v). After incubation as described in section 2.11, the products were detected by TLC (System I). Although different types of substrates were used, only one product spot was observed which corresponded to that of methyl-α-D-glucopyranoside (MG_1) standard (Figure 11). Therefore, the product was believed to be MG_1. The intensities of the glycoside spots were analyzed by GeneTools program. When β-CD, Fiberose® (M.W. = 12,000-25,000 Daltons), soluble starch and Flomax® were used as donors, the concentrations of MG_1 products were 1.37, 1.06, 1.15, 0.87 mg/mL, respectively when compared with MG_1 standard curve. Thus, β-CD was chosen as a glucoside donor for transglycosylation to methanol by CGTase.

3.5 Preliminary characterization of reaction products

3.5.1 Product characterization by TLC and HPLC

From Figure 8, only one glycoside product was detected by TLC System I (ethyl acetate/acetic acid/water, 3:1:1) when methanol was used as an acceptor. It is possible that other methyl glycosides were also produced but they were probably shielded by hydrolysis products formed in large amounts with similar R_f values. In order to elucidate the unobserved products, TLC running condition was then

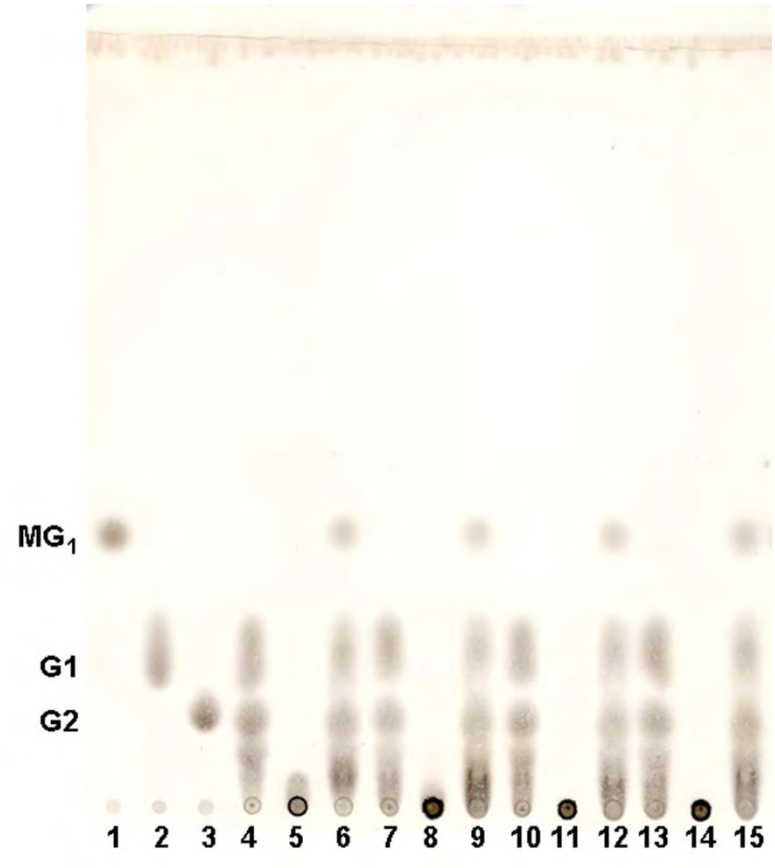


Figure 11 **TLC chromatogram of reaction products of CGTase incubated with methanol and various donors.** TLC condition was System I, ethyl acetate/acetic acid/water, 3:1:1.

Lane 1: Standard methyl monoglucoside (25μg)

Lane 2: Standard glucose (25 μg)

Lane 3: Standard maltose (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 (Fiberose® + CGTase)

Lane 8-9: Reaction mixture of Fiberose® as a donor, 0 and 24 h

Lane 10: Control reaction (soluble starch + CGTase)

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

Lane 13: Control reaction (Flomax® + CGTase)

Lane 14-15: Reaction mixture of Flomax® as a donor, 0 and 24 h

modified. The degree to which the solute interacts with the mobile phase as opposed to the sorbent will determine its distribution between the two phases. The mobile phase in the optimized TLC condition was to use ethyl acetate/acetic acid/water = 3:1.5:0.5.

The transglycosylation to methanol by CGTase using β-CD as a glycosyl donor was performed as described previously in section 2.12. After the incubation was completed, the aliquot was then analyzed by TLC (System II) and HPLC as described in section 2.13. With the optimized TLC condition, at least three glycoside products and other sugars (glucose, maltose and maltotriose) were detected as spots on TLC (Figure 12, Lane 5) with R_f values as shown in Table 7. In addition to MG_1 product (R_f = 0.74, the expected glycoside products having long chain glucosyl residues were observed as Product II (R_f = 0.51) and III (R_f = 0.29).

From HPLC profile of the reaction mixture containing methanol, β-CD, and CGTase at time zero in Figure13A, only β-CD peak at retention time (Rt) of 31.71 minutes was observed. However, when the reaction was allowed to proceed for 24 hours, the β-CD peak disappeared whereas other saccharide peaks at Rt 5.50, 6.94, 9.11 and 12.33 minutes were found to be glucose, maltose, maltotriose and α-CD, respectively. Apart from standard sugars, additional 5 product peaks at Rt 3.91, 4.70, 5.91, 7.69 and 10.33 minutes referred to as Product I to V were detected (Figure 13B). Product I at Rt 3.91 corresponded to standard MG_1 (see Appendix G). The series of methyl glycoside product II-V were presumed to be methyl glycoside having one to many glucose residues attached to methyl group of methanol (MG_n).

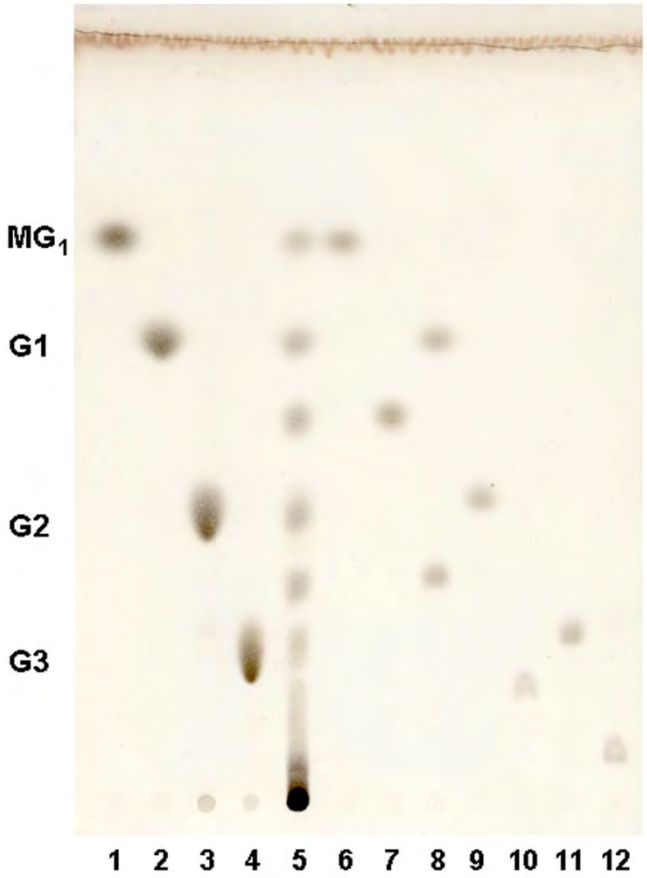


Figure 12 **TLC chromatogram of methyl glycoside products separated by HPLC.**
TLC condition was System II, ethyl acetate/acetic acid/water, 3:1.5:0.5.

Lane 1: Standard methyl monoglucoside (25μg)

Lane 2: Standard glucose (25 μg)

Lane 3: Standard maltose (25 μg)

Lane 4: Standard maltotriose (25 μg)

Lane 5: Reaction mixture after 24 h incubation

Lane 6: Product I (methyl monoglucoside, MG_1) at Rt 3.91 min

Lane 7: Product II at Rt 4.70 min

Lane 8: Product III at Rt 5.91 min and glucose (Rt 5.50 min)

Lane 9: Maltose (Rt 6.94 min)

Lane 10: Product IV at Rt 7.69 min

Lane 11: Maltotriose (Rt 9.11 min)

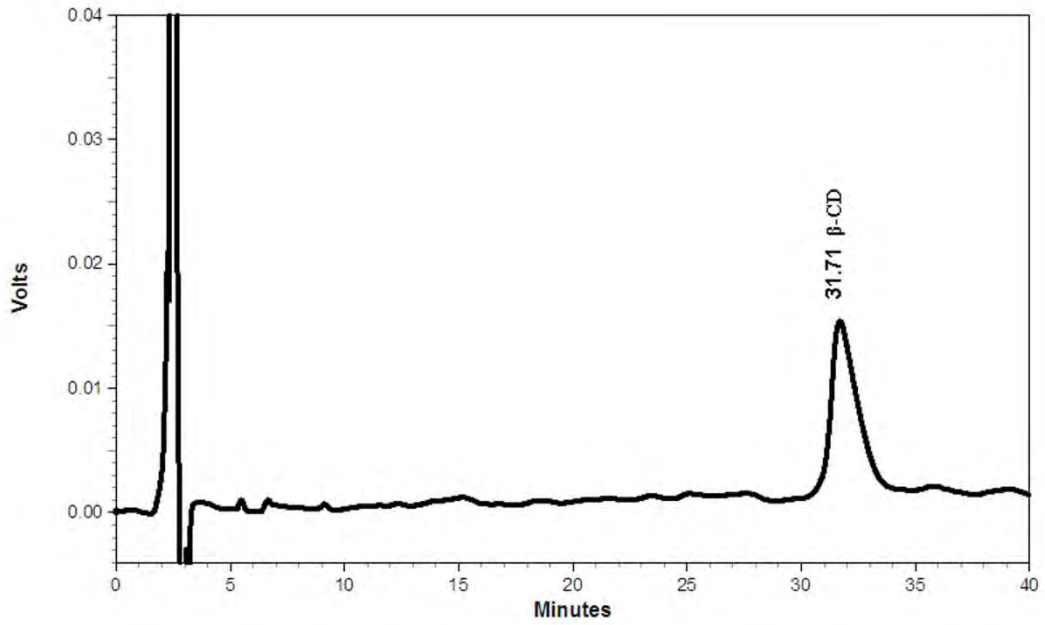
Lane 12: Product V at Rt 10.33 min

Table 7 **R_f values of TLC analysis (System II) of standard saccharides and methyl glycoside products**

Standards/Products		**R_f**
Standards	MG_1	0.74
	G1	0.61
	G2	0.38
	G3	0.19
Products	I (MG_1)	0.74
	II	0.51
	III	0.29
	IV*	-
	V*	-

* Product IV and V from the reaction mixture were not observed from TLC (Figure 12, Lane 5).

A



B

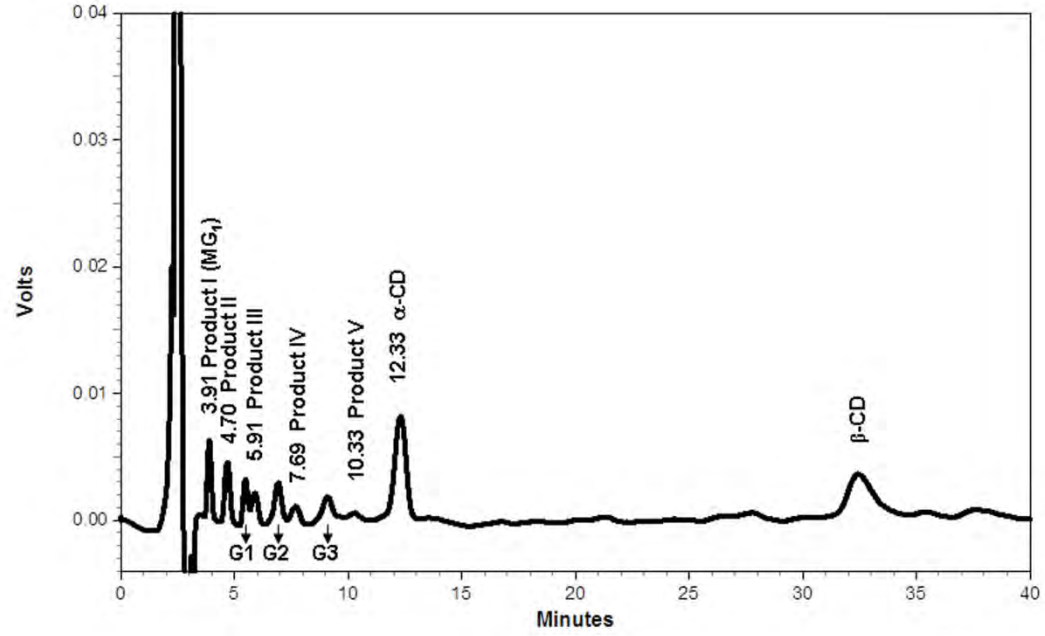


Figure 13 **HPLC chromatogram of reaction products of CGTase with methanol and β-CD.**

A: Reaction mixture at 0 h

B: Reaction mixture at 24 h

In order to indicate which HPLC peaks (at Rt 3.91, 4.70, 5.91, 7.69 and 10.33 minutes) corresponded to the spots on TLC, fractions from these peaks were collected by HPLC fraction collector and then analyzed by TLC (Figure 12, Lane 6-12). From TLC chromatogram, the three major products at Rt 3.91, 4.70 and 5.91 minutes had R_f equal to Product I (MG_1), II, and III, respectively. However, Product IV (Lane 10) and V (Lane 12) could not be separated by TLC.

3.5.2 Glucoamylase/α-glucosidase-catalyzed degradation of reaction products

To confirm that the product was a glycoside derivative, the reaction mixture was treated with glucoamylase from *A. niger*. This enzyme cleaves the glycosidic bond of α-1,4-glucan. After the reaction mixture was incubated for 24 hours, glucoamylase (final concentration of 20 U/mL) was added and further incubated at 40°C for 1 hour and analyzed by TLC (Figure 14, Lane 7). The results showed that after treatment with glucoamylase, the spots of transfer products (Product II and III) and hydrolysis product (maltose and maltotriose) disappeared and only the intensities of MG_1 and glucose increased. This result agreed with HPLC chromatogram from Figure 15B in which the peak areas of MG_1 and glucose increased whereas other transfer products dramatically decreased. Hence, it is confirmed that the peak of Product II to V was the conjugate of methanol and oligosaccharides corresponding to MG_n where $n > 1$.

In addition, the glycoside product was also checked for the configuration between methanol and glucosyl unit with α-glucosidase. This enzyme hydrolyzes the α-linkage of the non-reducing terminal of glucoside substate. The CGTase's reaction

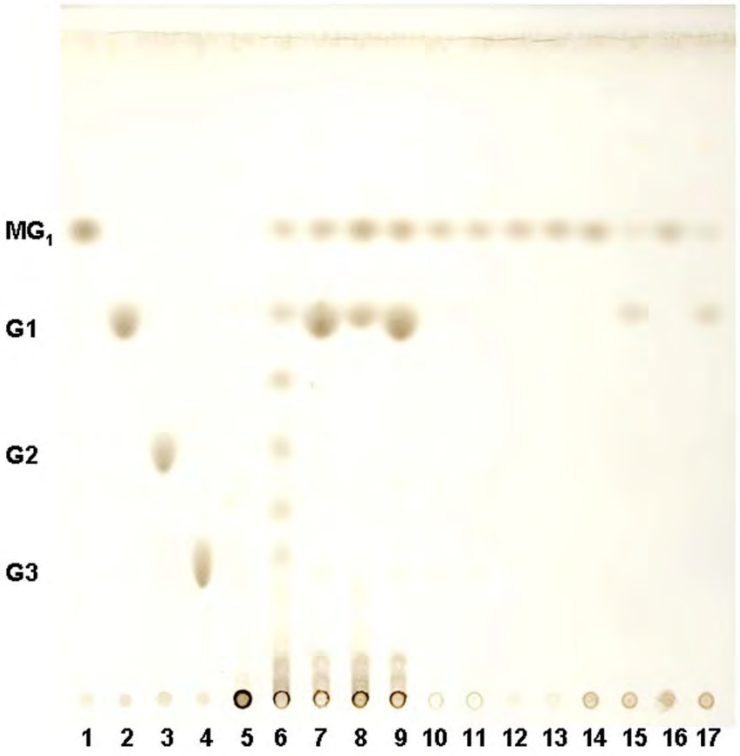


Figure 14 **TLC chromatogram of amylolytic enzyme treatment of CGTase's reaction products.** TLC condition was System II, ethyl acetate/acetic acid/water, 3:1.5:0.5.

Lane 1: Standard methyl monoglucoside (25μg)
Lane 2: Standard glucose (25 μg)
Lane 3: Standard maltose (25 μg)
Lane 4: Standard maltotriose (25 μg)
Lane 5: Reaction mixture at 0 min incubation
Lane 6: Reaction mixture after 24 h incubation
Lane 7: Reaction mixture (24 h) with glucoamylase
Lane 8: Reaction mixture (24 h) with α-glucosidase
Lane 9: Reaction mixture (24 h) with glucoamylase and α-glucosidase
Lane 10-11: Reaction mixture of 0.1% (w/v) MG_1 with CGTase, 0 and 24 h
Lane 12-13: Reaction mixture of 0.1% (w/v) MG_1 with glucoamylase, 0 and 1 h
Lane 14-15: Reaction mixture of 0.1% (w/v) MG_1 with α-glucosidase, 0 and 3 h
Lane 16-17: Reaction mixture of 0.1% (w/v) MG_1 with glucoamylase and α-glucosidase, 0 and 4 h

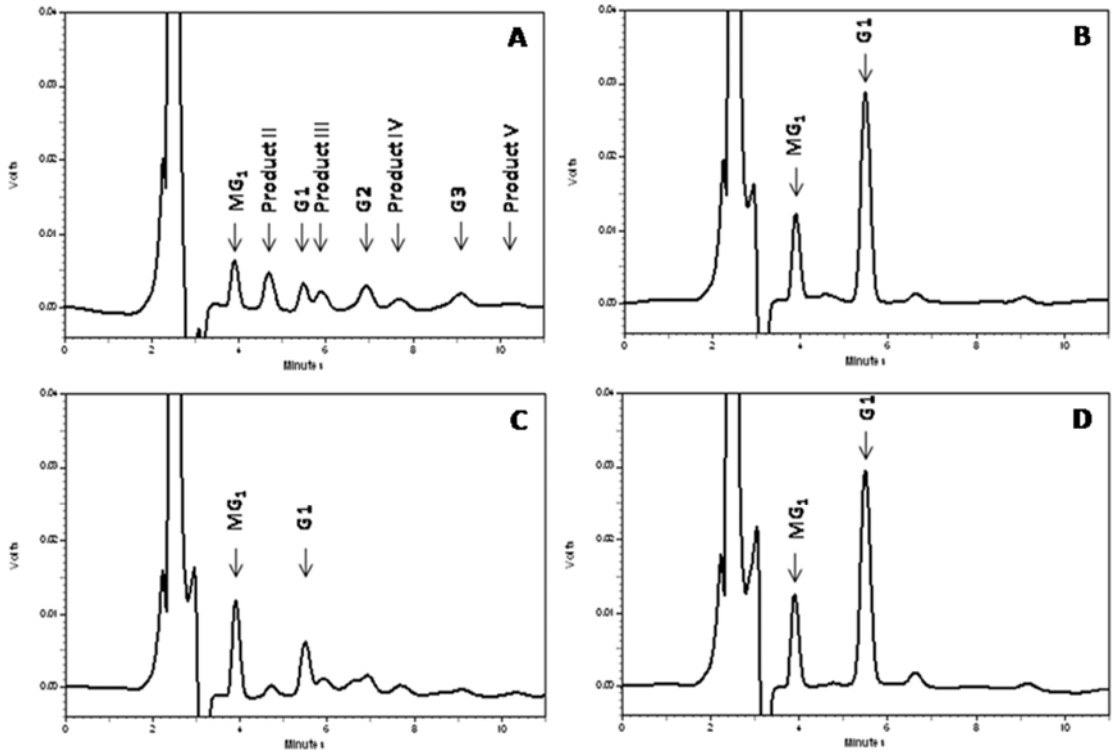


Figure 15 **HPLC chromatogram of amylolytic enzyme treatment of CGTase's reaction products.**

A: Reaction mixture (24 h)

B: Reaction mixture (24 h) with glucoamylase

C: Reaction mixture (24 h) with α-glucosidase

D: Reaction mixture (24 h) with glucoamylase and α-glucosidase

mixture was treated with α-glucosidase (final concentration of 20 U/mL) before TLC analysis. When the reaction mixture was treated with α-glucosidase (Figure 14, Lane 8), MG_1 and glucose were observed. These results were in accordance with HPLC profile (Figure 15C) where MG_1 and glucose peaks were detected. From these results, α-glucosidase did not seem to hydrolyze the α-linkage between methanol and glucose because MG_1 was still detected.

Moreover, after the reaction mixture was treated with glucoamylase and further hydrolyzed by α-glucosidase (Figure 14, Lane 9 and Figure 15D), the amount of MG_1 and glucose increased whereas other transfer products disappeared. The treatment of CGTase reaction products by α-glucosidase gave the similar results with glucoamylase treated reactions in which it could confirm the α-linkage of glucose residues of these glycosides. Nevertheless, the configuration between the methyl group from methanol and the glucose unit from β-CD could not be concluded because MG_1 in the reaction mixture of CGTase could not be degraded by α-glucosidase.

Glucoamylase and α-glucosidase were then checked to confirm that MG_1 could not be hydrolyzed by both enzymes. The reaction of glucoamylase (final concentration, 20 U/mL) with 0.1% (w/v) MG_1 in 50 mM acetate buffer pH 6.0 was incubated for 1 hour. After incubation, the intensity of MG_1 spot was remained in the same amount as at time zero (Lane 12-13). This result also confirmed that glucoamylase could not catalyze MG_1. After incubation of α-glucosidase (final concentration, 20 U/mL) with 0.1% (w/v) MG_1 in 50 mM acetate buffer pH 6.0 for 3 hours, a spot of MG_1 disappeared whereas the glucose spot increased (Lane 15). This phenomenon was also observed in glucoamylase and α-glucosidase treated reaction (Lane 17). These results suggested that MG_1 could be catalyzed into glucose and

methanol by α-glucosidase which contradictated the previous results (Figure 14, Lane 8-9). Yao *et al.* (2003) reported the inhibition of α-glucosidase from *Saccharomyces cerevisiae* by some D-glucose analogs including α-glucose which had K_i value of 0.93 mM. In order to confirm this experiment, glucose was added to MG_1-hydrolysis reaction by α-glucosidase. As can be seen in Figure 16 Lane 3-6, when the concentration of glucose increased, the intensity of MG_1 spot was also increased. This result showed that α-glucosidase was inhibited by glucose. Thus, certain amount of glucose produced in the CGTase catalyzed reaction (Figure 14, Lane 6) could inhibit α-glucosidase. In addition, as can be seen in HPLC profile (Figure 15C), there were small amounts of some transfer and oligosaccharide products still occurred which could be due to the inactivation of the enzyme by the presence of glucose.

Furthermore, CGTase was also checked whether it could hydrolyze the MG_1 besides transglycosylation reaction, CGTase-hydrolysis reaction which used MG_1 as a substrate was performed. After incubation of CGTase (final concentration, 200 U/mL) with 0.1% (w/v) MG_1 in 50 mM acetate buffer pH 6.0 for 24 hours, reaction products were analyzed by TLC. No hydrolysis product was observed (Figure 14, Lane 11). Thus, CGTase could not hydrolyze MG_1.

3.5.3 Mass spectrometry (MS)

The molecular weights of synthesized products were elucidated by mass spectrometer with the positive mode as described in section 2.13.3. From Figure 17, the pseudomolecular ion $[M+Na]^+$ of products displayed at m/z 217 (194 plus 23 of sodium molecule), m/z 379 (356 plus 23 of sodium molecule) and m/z 541 (518 plus 23 of sodium molecule). These corresponded to the size of methyl monoglucoside

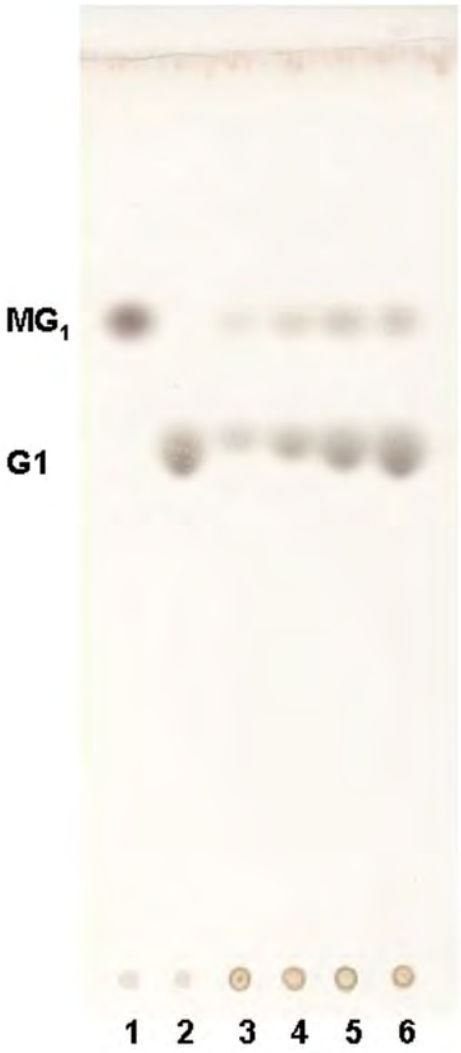


Figure 16 **TLC chromatogram of reaction product of α-glucosidase incubated with MG_1 and glucose for 3 hours.**

Lane 1: Standard methyl monoglucoside (20 μg)

Lane 2: Standard glucose (20 μg)

Lane 3: Reaction mixture of 0.1% (w/v) MG_1

Lane 4: Reaction mixture of 0.1% (w/v) MG_1 and 0.1% (w/v) G1

Lane 5: Reaction mixture of 0.1% (w/v) MG_1 and 0.2% (w/v) G1

Lane 6: Reaction mixture of 0.1% (w/v) MG_1 and 0.3% (w/v) G1

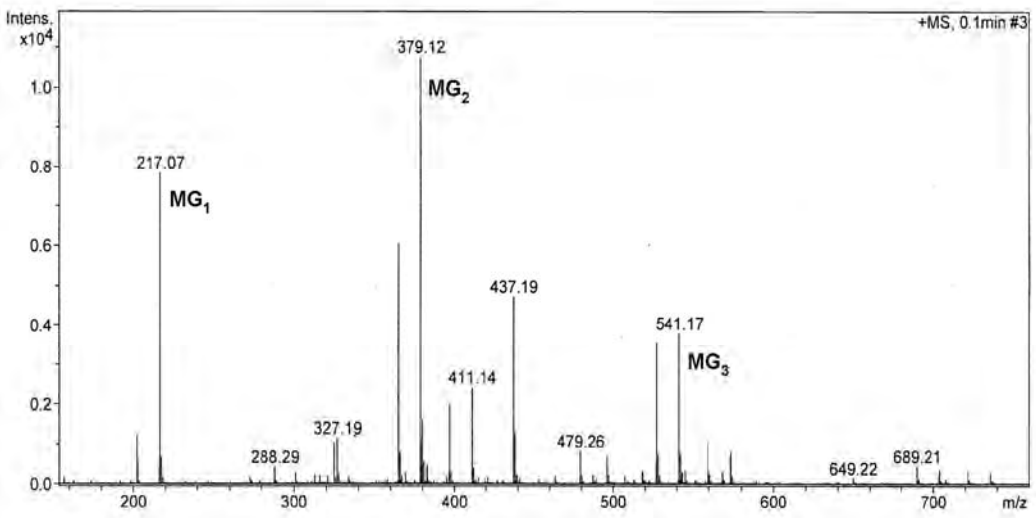


Figure 17 ESI-TOF mass spectra of reaction products.

(MG_1, $C_7H_{14}O_6$), methyl maltoside (MG_2, $C_{13}H_{24}O_{11}$) and methyl maltotrioside (MG_3, $C_{19}H_{34}O_{16}$), respectively.

3.6 Optimization of methyl glycoside synthesis

In order to find the appropriate conditions for the production of methyl glycosides, several parameters involved in the reaction including temperature, pH, incubation time, substrates and enzyme concentration were optimized. The reaction was performed as described in section 2.14 and reaction products were analyzed by HPLC. The optimum conditions were considered from the total amount of all transglycosylation products (MG_n) which were hydrolyzed into MG_1. The total amount of products in the form of MG_1 was calculated from MG_1 standard curve.

3.6.1 Effect of temperature

The effect of temperature on methyl glycoside synthesis was determined as described in section 2.14.1. The temperatures used were in the range of 20-60°C. The reactions were performed by incubating 0.6% (w/v) β-CD with 200 U/mL CGTase in 30% (v/v) methanol, pH 6.0 at various temperatures for 24 hours. The synthesized product was detected by HPLC. From Figure 18, it was found that CGTase could catalyze the transglycosylation to methanol from β-CD at the temperature ranging from 20-50°C with the highest amount of MG_1 formed at 40°C. At temperature higher than 40°C, the product formed dramatically decreased. Thus, the optimal temperature of 40°C was chosen for MG_1 production.

3.6.2 Effect of pH

The effect of pH on CGTase-catalyzed coupling reaction from β-CD to methanol for methyl glycoside production was investigated. The six conditions of different pH values in three kinds of buffer solution were employed. The three diverse buffers consisting of acetate buffer (pH 5.0-6.0), phosphate buffer (pH 6.0-7.0) and Tris-glycine buffer (pH 7.0-8.0) with the same concentration were used as the main environment of the reaction mixture. The protocol was done as shown in section 2.14.2 with optimum temperature of 40°C. The amount of MG_1 was analyzed by the same method as described in the previous section. As shown in Figure 19, the amount of MG_1 was maximum at the reaction pH of 6.0.

3.6.3 Effect of methanol concentration

To determine the effect of an acceptor on MG_1 production, various concentrations of methanol 10-50% (v/v) and 0.6% (w/v) β-CD donor in 50 mM acetate buffer pH 6.0 were incubated with 200 U/mL of CGTase at 40°C for 24 hours (as described in section 2.14.3). The amount of product increased when methanol concentration increased from 10-30% (v/v). However, when methanol concentration was more than 30% (v/v), MG_1 concentration decreased (Figure 20). Thus, 30% (v/v) methanol was chosen to be the optimum concentration.

3.6.4 Effect of β-CD concentration

In order to investigate the effect of the concentration of a glycosyl donor, the condition as mentioned in section 2.14.4 with various β-CD concentrations was used. It was found that product yield varied with β-CD concentration. From Figure 21, the

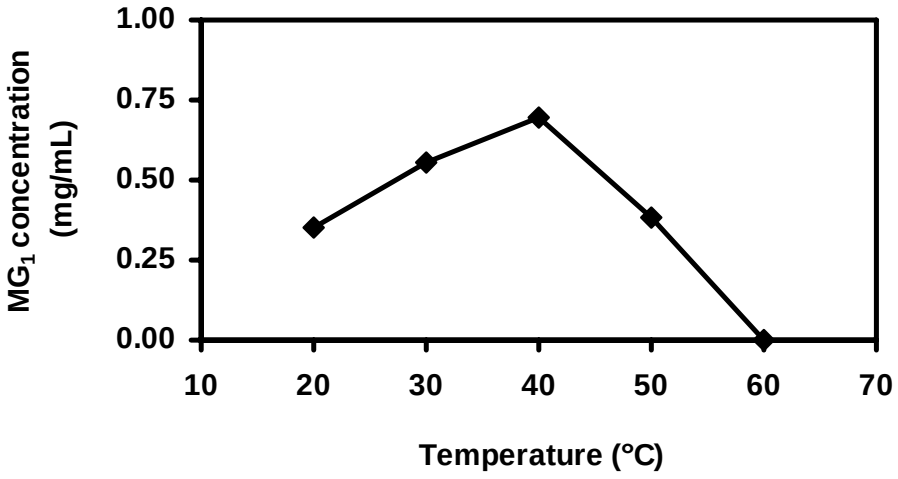


Figure 18 **Effect of temperature on MG_1 production by CGTase.** The CGTase (200 U/mL) was incubated with a mixture containing 30% (v/v) methanol and 0.6% (w/v) β-CD at indicated temperatures for 24 hours.

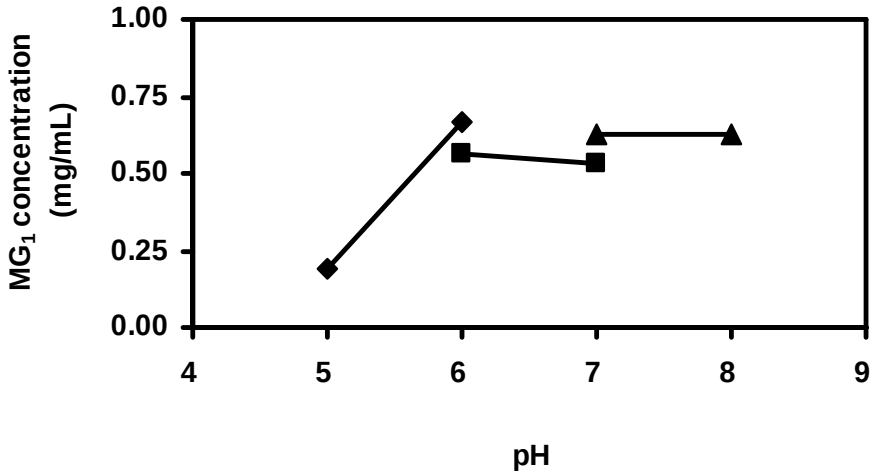


Figure 19 **Effect of pH on MG_1 production by CGTase.** The CGTase (200 U/mL) was incubated with a mixture containing 30% (v/v) and 0.6% (w/v) β-CD at different pH values at 40°C for 24 hours.

♦ Acetate buffer

■ Phosphate buffer

▲ Tris-glycine buffer

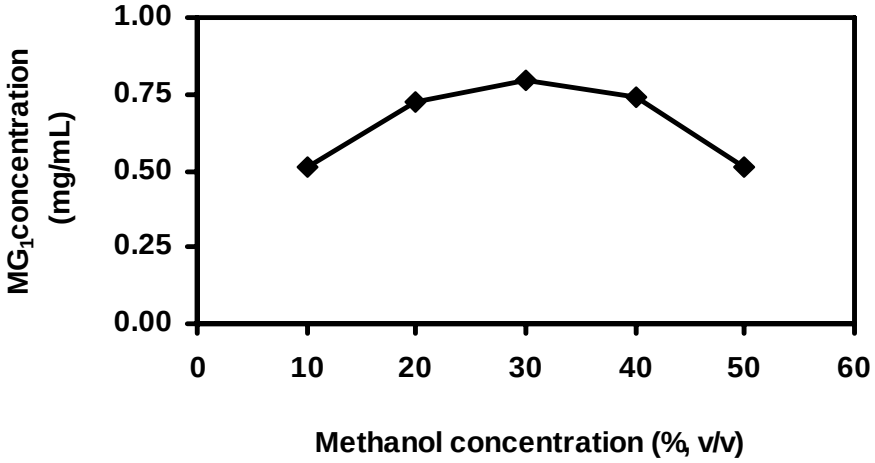


Figure 20 **Effect of methanol concentration on the synthesis of MG_1.** The reaction mixture containing 0.6% (w/v) β-CD and various amount of methanol concentration (10-50%, v/v) was incubated with 200 U/mL of CGTase at 40°C, pH 6.0 for 24 hours.

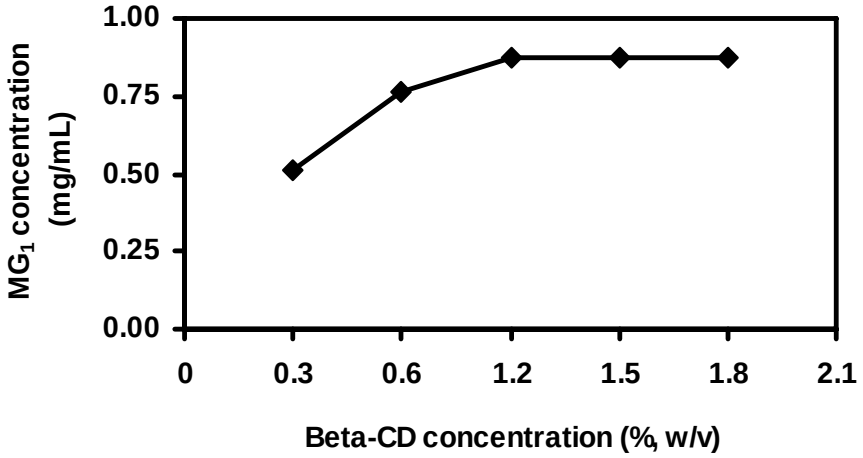


Figure 21 **Effect of beta-CD concentration on the synthesis of MG_1.** The reaction mixture containing 30% (v/v) methanol, various amount of β-CD (0.3-1.8%, w/v) was incubated with 200 U/mL of CGTase at 40°C, pH 6.0 for 24 hours.

yield of MG_1 increased when β-CD rose from 0.3 to 1.2% (w/v) and further increase in β-CD concentration (1.2-1.8% (w/v)) did not result in an increase of the product. The β-CD concentration of 1.2% (w/v) was then chosen to be the optimum concentration.

3.6.5 Effect of enzyme concentration

The optimization of the MG_1 production for a higher yield was performed by varying amount of CGTase. Various amounts of CGTase concentration (40-240 U/mL, determined with dextrinizing activity) were tested. The amount of MG_1 increased rapidly when enzyme concentration increased from 40-240 U/mL and reached maximum at 240 U/mL of CGTase (Figure 22). Thus, this enzyme concentration was chosen to be the optimum concentration.

3.6.6 Effect of incubation time

The incubation time was varied from 24-120 hours with optimum temperature and pH of 40°C and 6.0, respectively. After the experiment was carried out as described in section 2.14.6, the reaction mixtures were analyzed by HPLC. Figure 23 showed that the production of MG_1 was clearly affected by incubation time. The amount of product rapidly increased until it reached 96 hours. The highest amount of MG_1 formed was obtained at 96 hour-incubation time. The prolongation of the incubation time did not increase the MG_1 production.

Therefore, the optimum condition for methyl glycoside production by CGTase from *Paenibacillus* sp. RB01 was: to incubate 240 U/mL of CGTase with 1.2% (w/v) β-CD (donor) and 30% (v/v) methanol (acceptor) in 50 mM acetate buffer pH 6.0 at 40°C for 96 hours. After optimization, the total yield of MG_n in the form of methyl

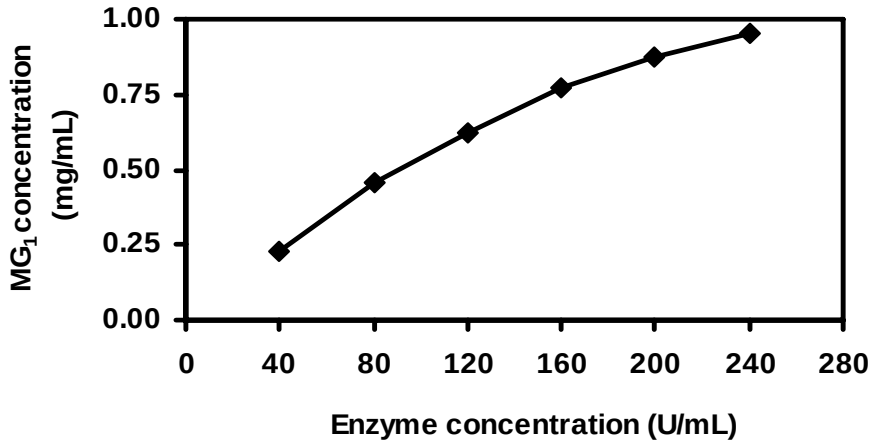


Figure 22 **Effect of enzyme concentration on MG_1 synthesis.** The reaction mixture containing 30% (w/v) of methanol and 1.2% (v/v) β-CD was incubated with CGTase (40-240 U/mL), at 40°C, pH 6.0 for 24 hours.

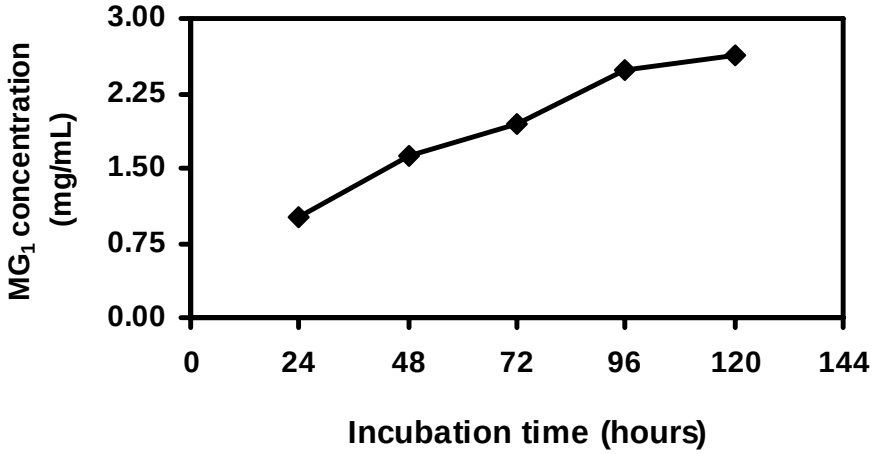


Figure 23 **Effect of incubation time on MG synthesis.** The CGTase 240 U/mL was incubated with a mixture containing 30% (v/v) methanol and 1.8% (w/v) β-CD at 40°C, pH 6.0 for 24-120 hours.

monoglucoside was 90% which was calculated from the mole ratio of product formed in comparison with β-CD used. The HPLC profiles of reaction products before and after optimization were compared in Figure 24.

3.7 Time course of methyl glycoside production

As shown in the result from section 3.5 (Figure 13B), CGTase produced a series of transglycosylation products of MG_n (Product I-V, see Section 3.5.1). In order to examine in detail of the formation of synthesized products, the reaction under several incubation times was performed. After MG_n production catalyzed by CGTase was done under optimized condition as described in section 2.16, the amounts of these products in the reaction mixture were determined by HPLC. From Figure 25, the yields of MG_n increased gradually during the incubation time and plateau after 96 hours. The increase of MG_n with long chain oligosaccharides (Product IV and Product V) was slower than that of short chain oligosaccharides (Product I,II and III). The degree of transfer products was in the order of Product II, Product I, Product III, Product IV and Product V. In further investigation, only MG_1 (Product I) was purified and characterized.

3.8 Large scale preparation and purification of MG_1

After the reaction conditions were optimized, the large scale of reaction mixture (10 mL) was then produced as described in section 2.17.1. After glucoamylase-hydrolysis reaction, the reaction mixture was concentrated to 2 mL with a rotary evaporator at 40°C and then applied on Amberlite IRA-900 column

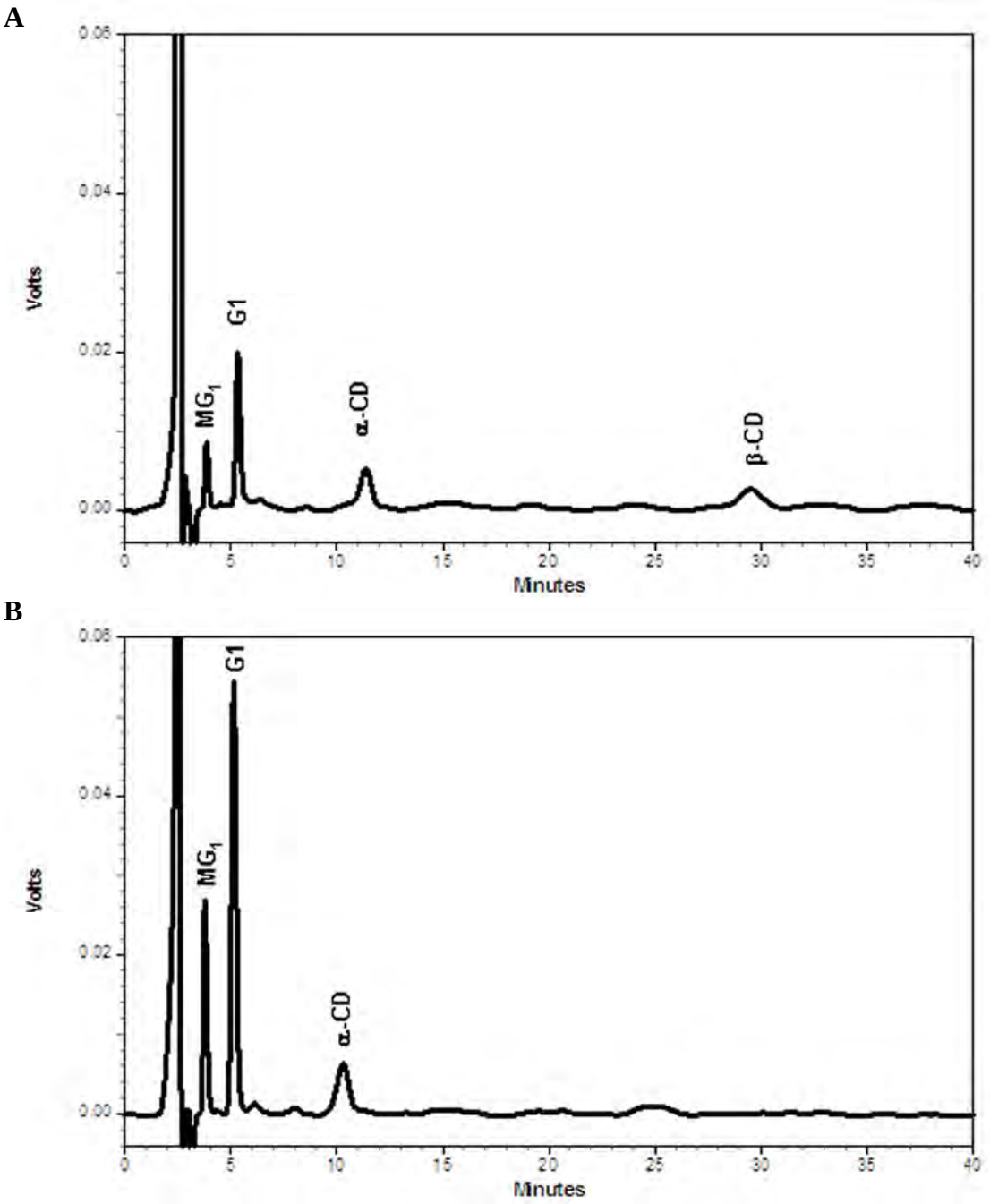


Figure 24 **HPLC chromatogram of CGTase's reaction mixture treated with glucoamylase.**

A: Before optimization

B: After optimization

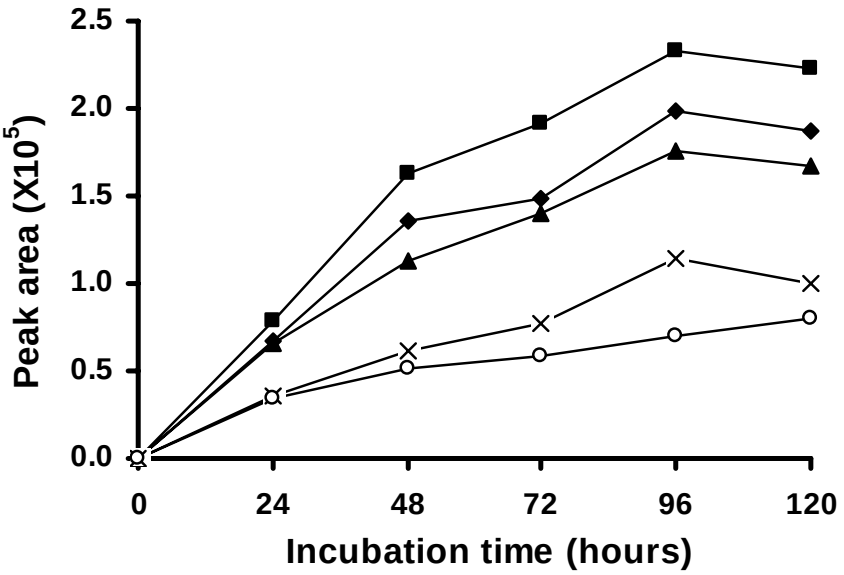


Figure 25 **Time course of MG_n production with CGTase.** The CGTase 240 U/mL was incubated with a mixture containing 30% (v/v) methanol and 1.8% (w/v) β-CD at 40°C, pH 6.0 for 24-120 hours.

♦ Product I

■ Product II

▲ Product III

× Product IV

o Product V

pretreated with 0.1 N NaOH. The column was eluted with water at a flow rate of 0.5 mL/min. The collected fractions were reacted with phenol-sulfuric reagent and measured spectrophotometrically for total sugar at a wavelength of 485 nm to construct the purification profile (Figure 26). One main product peak was observed and was further subjected to TLC analysis (System III) as described in section 2.13.1. It can clearly be seen that after purification by Amberlite IRA-900 column, the by-products (glucose and α-CD) were successfully removed and only MG_1 was separated through this column since only MG_1 spot was observed (Figure 27, Lane 8). Thereafter, the pooled-fraction product (fraction 12-27) was lyophilized to obtain the dried glycoside product of 20 mg which was further used for structure elucidation.

3.9 Structure elucidation of MG_1 by NMR

^{13}C NMR spectra of MG_1 were obtained after the experiment was performed as described in Section 2.17.4. The D_2O was used to dissolve this compound. ^{13}C NMR spectrum of MG_1 is shown in Figure 28. In order to identified the carbon framework of MG_1, its chemical shifts were compared with those of standard MG_1 (see ^{13}C NMR spectrum in Appendix I) and those of previous report (de Segura *et al.*, 2006) as shown in Table 8. de Segura *et al.* (2006) synthesized methyl α-D-glucooligosaccharides from sucrose as glucosyl donor and MG_1 as acceptor by entrapped dextransucrase from *Leuconostoc mesenteroides* B-1299. The α-configuration of the D-glucosyl residue in MG_1 was confirmed by the anomeric carbon of sugar signal at δ = 99.29 which was found to be similar to previous report (δ = 100.4). The carbon of methyl group which connects through glycosyl linkage (CH_3-O) has signal at δ = 55.04 which also corresponded to previous work (δ = 56.1). Other

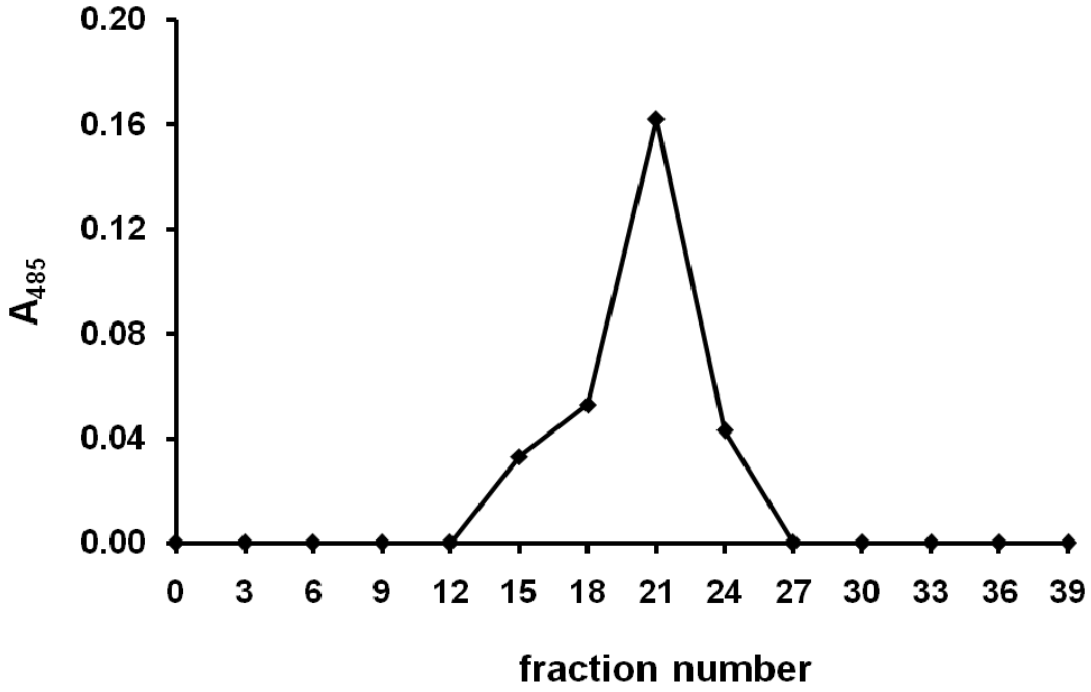


Figure 26 MG_1 purification profile by Amberlite IRA-900 (2.3 × 22 cm) eluted with water at a flow rate of 0.5 mL/min and collected fraction size of 4 mL.

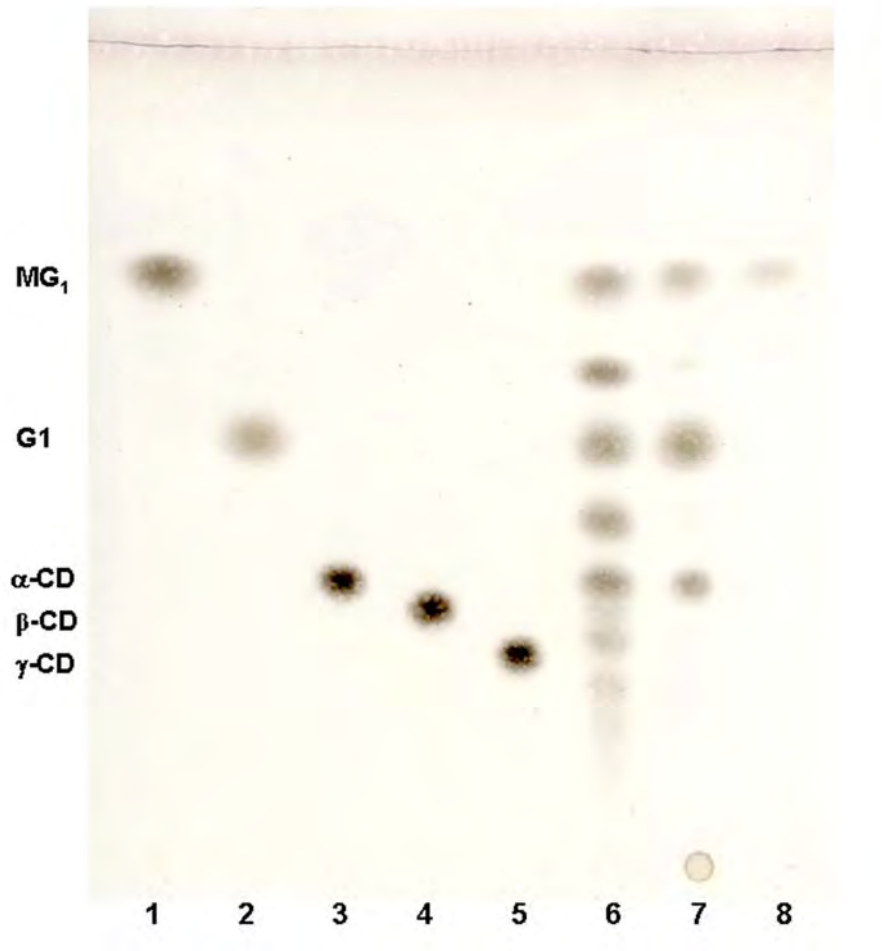


Figure 27 **TLC chromatogram of the fraction purity after purification.** TLC condition was System III, 1-propanol/ethyl acetate/water/25% ammonia solution (6:1:3:1, v/v).

Lane 1: Standard methyl monoglucoside (10μg)
Lane 2: Standard glucose (10 μg)
Lane 3: Standard α-CD (10 μg)
Lane 4: Standard β-CD (10 μg)
Lane 5: Standard γ-CD (10 μg)
Lane 6: 96-Hour incubation reaction mixture
Lane 7: 96-Hour incubation reaction mixture after glucoamylase treatment
Lane 8: Fraction number 21

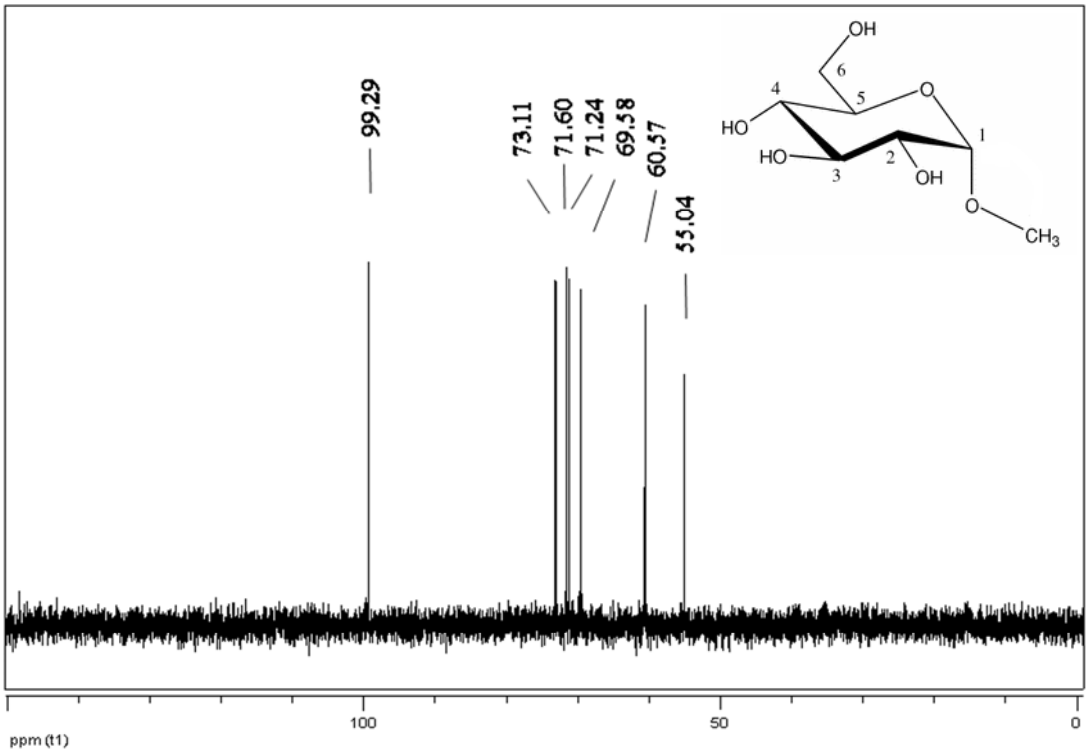


Figure 28 The 100 MHz ^{13}C-NMR of MG_1.

Table 8 **^{13}C NMR chemical shifts of standard MG_1, MG_1 from this experiment and MG_1 derivative from de Segura *et al.* (2006)**

Carbon position	MG_1 derivative from de Segura *et al.* (2006)	Standard MG_1	MG_1 from CGTase synthesis
C-1	100.4	99.29	99.29
C-2	72.4	71.59	71.60
C-3	74.5	73.11	73.11
C-4	70.5	69.59	69.58
C-5	71.0	71.24	71.24
C-6*	66.6	60.58	60.57
CH_3-O	56.1	55.04	55.04

* C-6 of MG_1 derivative was connected to glucose moiety.

chemical shifts of MG_1 in our experiment were the same as those of MG_1 derivatives reported by de Segura *et al.* (2006) except for the C-6 shift which was slightly shifted downfield due to conjugating group of glycosyl residues of methyl α-D-glucooligosaccharide in their study. From these data, the structure of MG_1 was identified as methyl α-D-glucopyranoside.

CHAPTER IV

DISCUSSION

The use of organic solvents as reaction media for enzymatic reactions provides numerous industrially attractive advantages, such as increased solubility of non-polar substrates, reversal of the thermodynamic equilibrium of hydrolysis reactions, suppression of water-dependent side reactions, alternation of substrate specificity and enantioselectivity, and elimination of microbial contamination (Doukyu and Ogino, 2010). In this research, organic solvents like miscible alcohols were utilized as glycosyl-acceptor substrate in the single phase synthesis of alkyl glycosides via transglycosylation reaction by enzymatic method.

Transglycosylation is considered to be an important method for the structural modification of compounds with useful biological activities. The conversion of lipophilic compounds into hydrophilic ones improves their physical properties while still retains their bioavailability. The important enzymes responsible for the synthesis of glycosidic linkage have been recognized as glycosyltransferases. Cyclodextrin glycosyltransferase (CGTase) is one of these enzymes that can catalyze the transglycosylation reaction from a glycosyl donor to an acceptor and has widely been utilized for the purpose of developing many modified compounds. For examples, the benzo[h]quinazoline derivatives having glucosyl moiety are water-soluble, which is important for their use in drug formulations (Markosyan *et al.*, 2009). Enzymatic synthesis of L-DOPA-α-glycosides increased stability against oxidative degradation that made it more preferable and beneficial than L-DOPA in treating Parkinson's

disease (Yoon *et al.*, 2009). Therefore, it is of great potential interest to synthesize alkyl glycosides, the valuable substances for surfactant utilization.

4.1 Purification of CGTase

The screening for thermotolerant bacteria producing extracellular CGTase, *Paenibacillus* sp. Strain RB01, was previously performed by Tesana (2001). Single common step in the partial purification of CGTase was the use of starch adsorption. This method considered the interaction between starch and enzyme not only adsorption but also substrate-enzyme affinity type binding. Consequently, CGTase was successfully separated from most of other non-binding proteins which were removed by centrifugation. The adsorbed enzyme was eluted with maltose solution that had higher affinity for binding with CGTase than starch. Thus, the enzyme-maltose can be easily separated and present in soluble fraction. After this step, the enzyme solution was dialyzed against buffer to get rid of maltose from enzyme. From partial purification table (Table 5), the enzyme with 38.1% yield and 46.3 fold increase in purity was obtained. From previous work on starch adsorbed CGTase from *Paenibacillus* sp. RB01 by Tesana (2001) and Thanadolsathien (2007),the yield was 57.3% and 83% with 26.8 and 17.4 fold increase in purity, respectively. These results suggested that the amount as well as the purity of enzyme depended on the quality of person-performing purification process. The purity of the enzyme was checked by native and SDS-PAGE. On native gel electrophoresis (Figure 6), the enzyme showed two bands (one major and one minor) with dextrinizing activity of isoform pattern which were in accordance with the work by Yenpetch (2002). When SDS gel electrophoresis was performed (Figure 7), the enzyme showed only one major one

band with two minor bands of lower molecular weight .The molecular weight of the major band was estimated to be 70 kDa with was approximate to CGTase from the previous study of Tesana (2001) and Yenpetch (2002). Thus, the partial purified enzyme was pure enough for further use in transglycosylation study (Aramsangtienchai, 2007 and Thanadolsathien, 2007).

4.2 Transglycosylation of β-cyclodextrin to alcohols

From the previous studies, CGTase has been used to elongate the carbohydrate part of shorter alkyl glycosides. Zhao *et al* (2008) reported for the first time that CGTase catalyzed transglycosylation reaction from dextrin to maltosides of butanol, octanol and lauryl alcohol affording novel maltosides with 3-4 glucose units. Later, Svensson *et al.* (2009) studied the CGTase's coupling of α-cyclodextrin to alkyl β-glycosides. The acceptor substrate dodecyl β-maltoside was thus converted to dodecyl β-D-maltooctaoside. So far, there have been no reports on the use of alcohol as a glycosyl acceptor. In this work, we demonstrated the formation of alkyl glycosides from β-CD and alcohols by the transglycosylation action of CGTase from *Paenibacillus* sp. RB01.

In this study, it was found that after incubation of CGTase with β-CD donor and various alcohols as glycosyl acceptor, CGTase from *Paenibacillus* sp. RB01 was able to transfer the glucose moiety of β-CD to short chain alkyl alcohols producing alkyl glycosides. The synthesized alkyl glycoside products were basically detected by TLC analysis. Thin layer chromatography is a separation method in which uniform thin layers of sorbent or selected media are used as a carrier medium. The TLC plate is composed of silica gel as the sorbent applied to an aluminum sheet backing. Via

adsorption chromatographic mechanism, the expected alkyl glycoside products with less polar due to the length of alkyl chains were migrated in larger R_f in the non-polar mobile phase (System I, ethyl acetate/acetic acid/water, 3:1:1). On the other hand, the oligosaccharides resulted from hydrolysis reaction are more polar and thus, strongly adsorbed on silica gel resulted in smaller R_f values.

On TLC plate, each alcohol acceptor gave a series of glycoside products (Rf = 0.26-0.62), except for methanol (see Figure 8). These observations demonstrated that the number of spots appeared was dependent on polarity of the products which correlated with the length as well as the conformation of alkyl chain and the number of glucose residues.

4.3 Influence of alcohol length and concentration on CGTase coupling activity

The determination of CGTase coupling activity has been related to the amounts of β-cyclodextrin used in coupling reaction. Since β-CD forms molecular inclusion complex with phenolphthalein (PHP) dye, a change of color of the complexed dye is measured (Vikmon, 1981). The decolorization of PHP due to the complexation with β-CD correlates with β-CD concentration. The advantages of the colorimetric method are suitable for small CD concentration and seldom require the removal of acyclic carbohydrates. Furthermore, the assay procedure is also easy to carry out and applicable to large sample series.

After 24-hour transglycosylation reaction of CGTase in buffer and in co-alcohol buffer solution with β-CD, the coupling activity of CGTase was then investigated. The results (Figure 9) demonstrated that the ratio of alcohol had

significant effect on coupling activity of CGTase. The higher the ratio, the lower the activity. The effect of alcohol on the activity of the enzyme has generally been observed. For example, the rate of starch hydrolysis by α-amylase from *Aspergillus oryzae* decreased with increasing concentration of methanol in the reaction mixture (Larsson *et al.*, 2005). The rate of sucrose consumption was also found to be lowered with the rising concentration of methanol in the reaction catalyzed by levansucrase from *Rahnella aquatilis* (Kim *et al.*, 2000). This behavior is a consequence of the antagonistic influence of the water activity reduction which resulted in the loss of the enzyme activity in the non-aqueous medium (Chahid *et al.*, 1992). Klibanov (1997) described that there are many causes of lower enzymatic activity in organic solvents compared with that in water such as active center blockage, conformational change and transition state destabilization. Organic solvents also affect CGTase activity differently depending on the source of enzymes as well as the type and concentration of solvents (Doukyu *et al.*, 2003). Moreover, the length of alcohol also had an important impact on the transglycosylation reaction. CGTase was more inactivated by the longer alkyl chain of alcohol and thus, could result in a lower yield of the product. This has been observed in many enzymes such as β-glucosidase (Lirdprapamongkol and Svasti, 2000; Svasti *et al.*, 2003) and α-rhamnosidase (Martearena *et al.*, 2003).

The formation of alkyl glycosides was also affected by the structure and concentration of alcohols. Alcohols as well as water were nucleophiles that can react with the enzyme via S_N2 nucleophilic substitution mechanism, thus alcohols competed with water as glycosyl acceptors. The nucleophilicity of alcohols is dependent on the size and arrangement of their substituent group. Therefore, it was reflected as the degree of intensity of alkyl glycosides: methanol gave the highest

amount of product, followed by ethanol and 1-propanol and primary alcohol (1-propanol) gave higher yield than secondary alcohol (2-propanol). Similarly, good yields of alkyl glucosides synthesized from Thai rosewood β-glucosidase were obtained with primary alcohol while little or no product was found with secondary and tertiary alcohols (Lirdprapamongkol and Svasti, 2000). Although the amount of alkyl glycosides formed increased with increasing concentrations of alcohol, it was found that when their concentrations exceeded 50%, the yield was dramatically decreased which could be due to the inactivation of the enzyme. The alcohol concentration for the production of alkyl glycosides has to be compromised between appropriate substrate concentration and enzyme activity for the transglycosylation reaction.

4.4 Characterization of methyl glycoside products

In order to elucidate the structure of alkyl glycosides synthesized by CGTase , the reaction with methanol as an acceptor was chosen here as a model. When β-CD, soluble starch, Fiberose® and Flomax® were used as donors in transglycosylation reaction with CGTase, the intensity of the product spots (MG_1) on TLC chromatogram were relatively similar although different types of substrates were used. Thus, these substrates were equally effective as glycosyl donor for this CGTase. This has been observed in the previous study of epicatechin glycosides synthesis by Aramsangtienchai (2007).

After incubation of the methanol-acceptor transglycosylation by CGTase with β-CD as glycosyl donor, the reaction products were detected by TLC and HPLC analysis. The solvent system for TLC development was optimized to effectively

separate the transglycosylation products from the hydrolysis products (see Figure 12). From Figure 12, TLC System II was able to separate the methyl glycoside products more effectively than System I. The glycosides with high polar will require a mobile phase of high interactivity if they are to migrate further on the TLC plate. Therefore, the composition of the mobile phase (System I) is altered by varying its water content to produce changes in separation. From TLC chromatogram (Figure 12, Lane 5), three spots of methyl glycoside products were detected. Product I at the R_f value of 0.74 corresponded to the standard MG_1, thus, it was believed to be methyl α-D-glucopyranoside. Product II and III were separated according to their polarities with the R_f values of 0.51 and 0.29, repectively (Table 7). However, when the reaction products were analyzed by HPLC (Figure 13), five product peaks at Rt 3.91, 4.70, 5.91, 7.69 and 10.33 minutes referred to as Product I to V, respectively were observed. The peak at Rt 3.91 minutes coincided with standard MG_1. Therefore, it was thought to be methyl. α-D-glucopyranoside. To examine product II-V, products collected from HPLC fraction collector were compared with products in the reaction mixture by TLC (Figure 12, Lane 6-12), it was found that at least 5 products were synthesized and the three major products from TLC analysis were corresponded to Product I to III from HPLC analysis. The analysis of saccharides used high performance liquid chromatography (HPLC) with amino column includes normal phase chromatography principle. The material base of Asahipak amino column is hydrophilic polymer gels conjugating with polyamine. As well as TLC, the separation of sugar compounds on HPLC amino column as stationary phase is based on the polarity and the size of their molecules.

The number of glycosyl units was preliminary characterized by hydrolysis reaction by glucoamylase. The function of glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) is to hydrolyze starch and other related poly- and oligosaccharides by inverting exo-acting mechanism to release β-glucose from the non-reducing ends of substrates (Chiba, 1997). From Figure 14 (Lane 7) and 15B, it can clearly be seen that glucoamylase can hydrolyze the reaction products into MG_1. Therefore, spots or peaks that disappeared after incubation with glucoamylase were presumed to be the transglycosylated products containing several glucose residues. The configuration of the products was also checked by α-glucosidase. α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) also known as maltase catalyzes the liberation of α-glucose from the non-reducing terminal of both homogenous and heterogenous substrates (Chiba, 1997). From Figure 14 (Lane 15 and 17), α-glucosidase, was found to be able to catalyze standard methyl monoglucoside and released glucose. However, it could not hydrolyze MG_1 in the reaction mixture (Figure14, Lane 8 and 15C). This observation was previously investigated and was found that it was due to product inhibition (Yao, *et al.*, 2003). The inhibition constant (K_i) of α-glucose was calculated to be 0.93 mM. Consequently, standard methyl monoglucoside was less hydrolyzed by α-glucosidase at high glucose-containing reaction mixture (Figure 16).

Using mass spectrometry, the three major methyl glycosides were identified as methyl monoglucoside, methyl maltoside and methyl maltotrioside. The transglycosylation characterization of CGTase was observed in many researches. For instance, CGTase from an alkalophilic *Bacillus* species produced hesperidin monoglucoside and a series of its oligoglucosides by the transglycosylation reaction

with hesperidin as an acceptor and soluble starch as a donor (Kometani *et al.*, 1994). Three transfer products of lactose acceptor were synthesized by CGTase from *Bacillus stearothermophilus* (Kitahata *et al.*, 1992). Yoon and Robyt (2006) described the CGTase mechanism of the formation of the glycosides via coupling reaction by using α-cyclodextrin and methyl α-D-glucopyranoside as donor and acceptor, respectively. In addition of coupling reaction, CGTase also catalyzes disproportion and cyclization reaction, simultaneously (Figure 29). Due to multi reactions of CGTase, there are many types of products, short chain and cyclic oligosaccharides synthesized in the reaction mixture (Figure 13B).

4.5 Optimization of methyl glycoside synthesis

To obtain high yield of alkyl glycosides, several parameters affecting the production such as concentration of donor, acceptor and enzyme and also the reaction pH and temperature which affected enzyme activity have yet to be optimized. In order to analyze the total yield of methyl glycosides, the reaction mixtures were treated with glucoamylase (final concentration, 200 U/mL) to remove all glucose residues sequentially from the non-reducing end of glucoside moiety conjugated to MG_1.

The factors that affected the activity of enzyme, for example; temperature and pH, were first optimized. Since CGTase obtained from thermotolerant *Paenibacillus* sp. RB01 was found to be most stable at 40-65°C and the pH optimum of 5-6 has previously been reported (Charoensakdi *et al.*, 2007). Therefore, the reaction mixture was incubated at various temperatures ranging from 20 to 60°C and pH in the range of 5 to 8. It was found that the temperature at 40°C was optimum for transglycosylation (Figure 18). At lower or higher temperature than 40°C, the amount of MG_1 formed

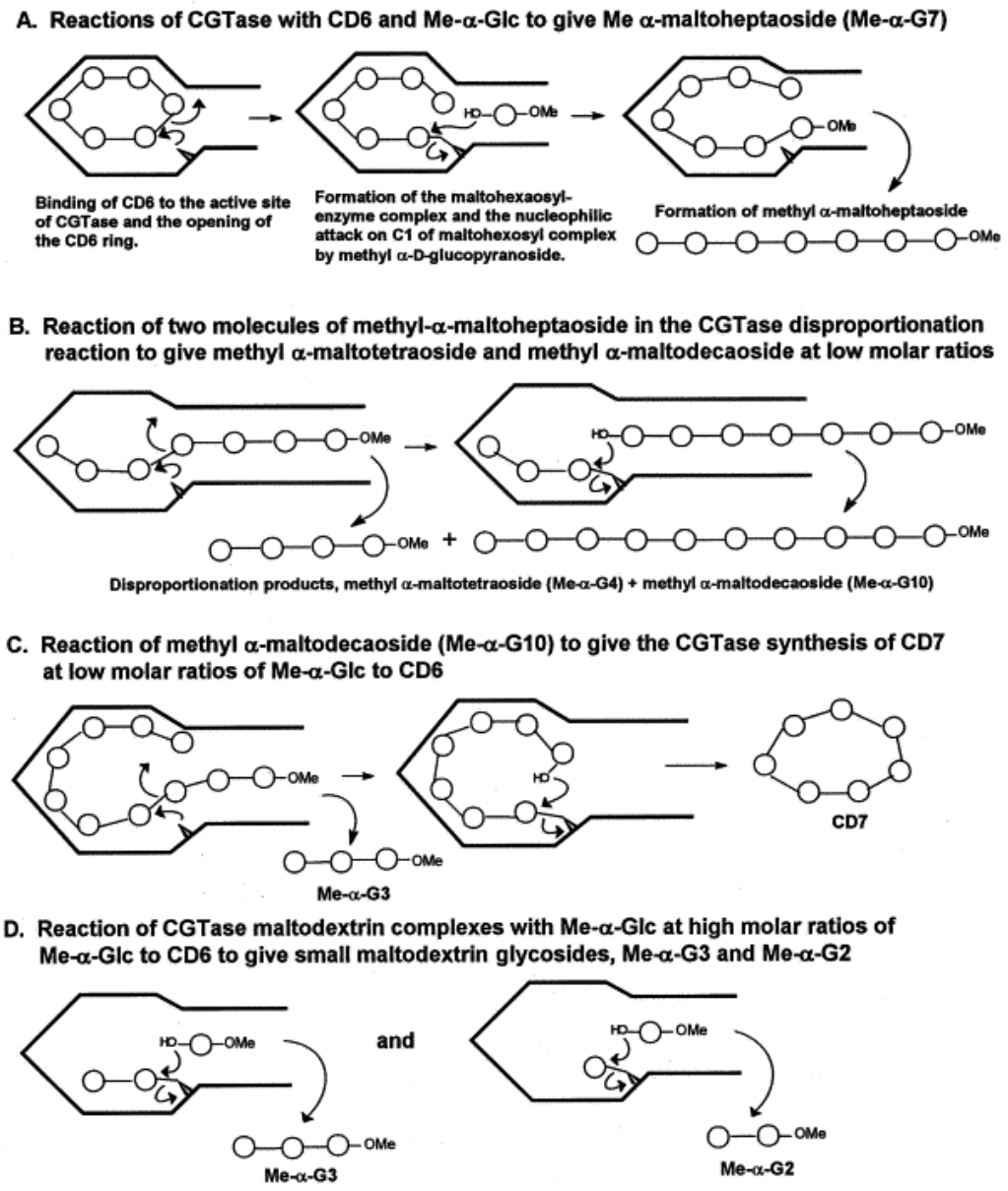


Figure 29 Reaction steps in the *B. macerans* CGTase coupling (acceptor) reactions (Yoon and Robyt, 2006).

was lower which could be explained by low enzyme activity. For the determination of its optimum pH, three kinds of buffer (acetate, phosphate and Tris-glycine buffer) were used to vary pH of the reaction mixture. CGTase was stable for a wide range of pH (6.0-8.0) in which the acetate buffer at pH 6.0 gave the best result (Figure 19). Likewise, the previous study of vitamin E glycoside synthesis by CGTase gave the similar result due to the broad range of pH for transglycosylation (Thanadolsathien, 2007).

Then, substrate and enzyme concentration as well as incubation time which promote the high production of methyl glycoside were optimized. To determine for the optimal methanol concentration, it was found that the yield of MG_1 was maximum at 30% (v/v) of methanol which had to be compromised with enzyme activity as discussed in section 4.3. The optimized donor concentration was obtained when 1.2-1.8% (w/v) of β-CD used which was twice higher than the reaction condition before optimization. However, the far increase of β-CD concentration did not affect the production yield and the final β-CD concentration of 1.2% (w/v) was then used for large-scale production (Figure 21). The amount of CGTase at 240 U/mL (dextrinizing activity) gave the highest production of MG_1. High degree of CGTase concentration used probably comes from the inactivation by solvent.

To determine for the optimal incubation time for MG_1 synthesis, the reaction was varied from 0-120 hours. For every 24-hour detection, it was found that the time used for the highest production of MG_1 was surprisingly long (96 hours) (Figure 23). This could be resulted from the acceptor specificity of CGTase since it can use different acceptor substrates. When it uses free sugar (typically maltose) as an acceptor in a transglycosylation, or disproportionation reaction, its activity is 100

times higher than hydrolysis reaction (Van der Veen *et al.*, 2000). Similar to water molecule, methanol which is nucleophile could react with CGTase in the same way. In addition, the yield obtained from the optimum incubation time was 2.5 times higher than other factors (substrate and enzyme). This result suggested that the time course was the most impact factor to maximize the product yield.

From Figure 25, the amounts of synthesized products (Product I-V) were varied according to the size of the oligosaccharide chain because CGTase exhibited glucoamylase-like activity which removed glucose units sequentially from the non-reducing end of oligosaccharide moiety conjugated to MG_1. This enzymatic property of CGTase for the MG_1 production were consistent with AA-2G synthesis of CGTase (Tanaka *et al.*, 1991 and Jun *et al.*, 2001). However, the quantity of Product I (MG_1) was lower than Product II (expected to be MG_2).

The optimal conditions for methyl glycoside production was to incubate 1.2% (w/v) of β-CD, 30% (v/v) methanol with 240 U/mL of CGTase, pH 6.0, 40°C, for 96 hours. After optimization, the yield of methyl monoglucoside product was 90% which was calculated from the mole ratio of product formed in comparison with β-CD used. Different enzyme catalyzing systems produced methyl glycoside products which differ in the type of linkages, the type of products formed and the production yield obtained. For example, Thai rosewood β-glucosidase (Lirdprapamongkol and Svasti, 2000) and levansucrase from *Rahnella aquatilis* (Kim *et al.*, 2000) gave yields of methyl β-glucoside (97%) and methyl β-D-fructoside (70%), respectively. However, only one monosaccharide residue was attached.

4.6 Large scale preparation, purification and identification of MG_1

To prepare large amounts of methyl monoglucoside for identification the production of MG_1 under optimimal conditions was scaled up 20 times. After the reaction mixture was incubated with CGTase, glucoamylase was added to hydrolyze the transglycosylated products containing many glucoside residues (MG_n) into monoglucoside product (MG_1). Then, the reaction mixture was concentrated and it was passed through the Amberlite IRA-900 column. Amberlite IRA-900 is strongly basic anion exchange resin with benzyltrialkylammonium functionality. This resin was used for decolorizing intermediate to light solutions, concentrating heparin at neutral pH and removing metal ions (Sigma-Aldrich, 2010). Roseman *et al.* (1952) investigated the behavior of carbohydrates toward strongly basic ion-exchange resins. They reported that reducing sugars were retained by the column, while nonreducing sugars including α-D-glucopyranoside were not appreciably retained. However, an exception was noted in the case of sucrose which although nonreducing was partially retained. Neuberger and Wilson (1971) explained that the separation of these sugars on the basic column is assumed to be due to an ion-exchange process involving ionization of one of the hydroxyl groups of the sugars. The acidity of sugars involves the anomeric effect and other forms of dipole interaction, since sugars are polybasic acid. From Amberlite IRA-900 column profile (Figure 26), the fractions eluted with water in the fraction range of 12-27 were MG_1 as examined by TLC (Figure 27). The yield of the product in the large scale preparation was 2.00 mg/mL which was similar to the yield (2.49 mg/mL) in the small scale reaction (0.5 mL).

The molecular structure of MG_1 was further identified by ^{13}C NMR (400 MHz). It can be seen that the ^{13}C NMR spectra of MG_1 from our experiment were similar to those of standard MG_1 (Figure 28 and Appendix I). To identify which signals corresponded to the position of carbons, the information of the previous study of de Segura *et al.* (2006) was also used. The anomeric carbon (C-1) and the carbon of methyl group (CH_3) were observed at the chemical shift (δ) of 99.29 and 55.04 ppm, respectively. Therefore, the transglycosylation product by CGTase was elucidated as methyl α-D-glucopyranoside.

In this study, a new characteristic of CGTase in synthesizing alkyl glycoside in alcohol-buffer system was discovered. CGTase can use different alcohols as acceptors in transglycosylation reaction in which β-cyclodextrin is a glycosyl donor to produce various alkyl glycosides having one to several monosaccharide units, which are difficult to prepare via other routes. Studies on the synthesis of alkyl glycosides with more hydrophobic alcohols could be further by controlling water activity and by the use of immobilized enzyme (Lirdprapamongkol and Svasti, 2000).

CHAPTER V

CONCLUSIONS

1. CGTase from thermotolerant *Paenibacillus* sp. RB01 was partially purified by starch adsorption. The specific activity was 8,377 units/mg with the purification fold of 46.3 and the obtained yield was 38%.
2. The enzyme was able to synthesize alkyl glycosides from various alcohols in which methanol was the best acceptor.
3. The molecular weights of MG_n were 194, 356, and 518 Daltons which corresponded to methyl monoglucoside, methyl maltoside and methyl maltotrioside, respectively.
4. The optimum conditions for CGTase transglycosylation were to incubate methanol at a final concentration of 30% (v/v) and 1.2% (w/v) of β-cyclodextrin with 240 U/mL of CGTase at pH 6.0 for 96 hours at 40°C.
5. Under optimized conditions, the transglycosylation yield of total MG_n was calculated to be 90% which was calculated from the mole ratio of product formed in comparison with β-CD used.
6. MG_1 was prepared in large scale, purified by Amberlite IRA-900 column chromatography and identified as methyl α-D-glucopyranoside.

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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.

50% (v/v) Glycerol

Glycerol 50 mL

Added 50 mL water and mixed well.

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.

Electrophoresis buffer

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g

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

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)

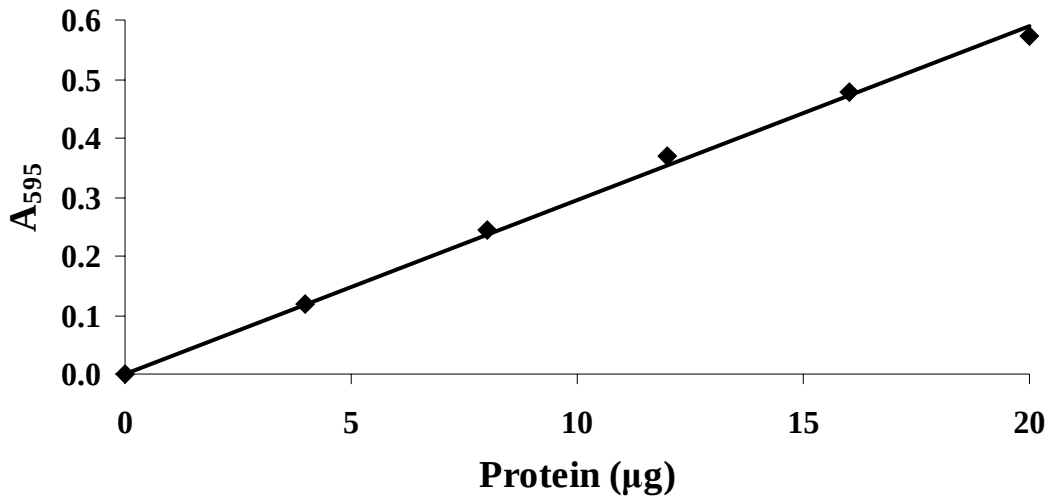
50 mM di-Potassium hydrogen phosphate (K_2HPO_4)	30.08 mL
50 mM Potassium dihydrogen phosphate (KH_2PO_4)	19.92 mL

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

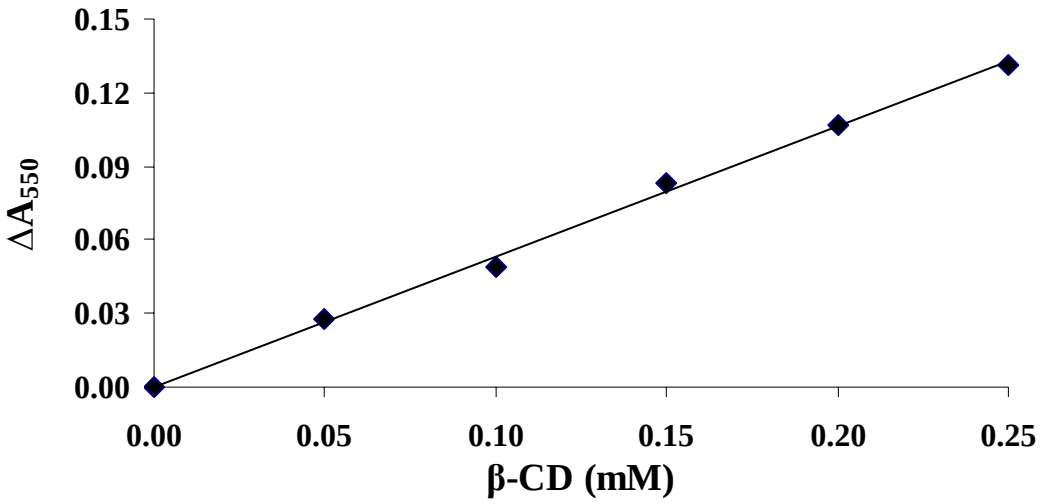
Tris (hydroxymethyl)-aminomethane	0.303 g
Glycine	0.188 g

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

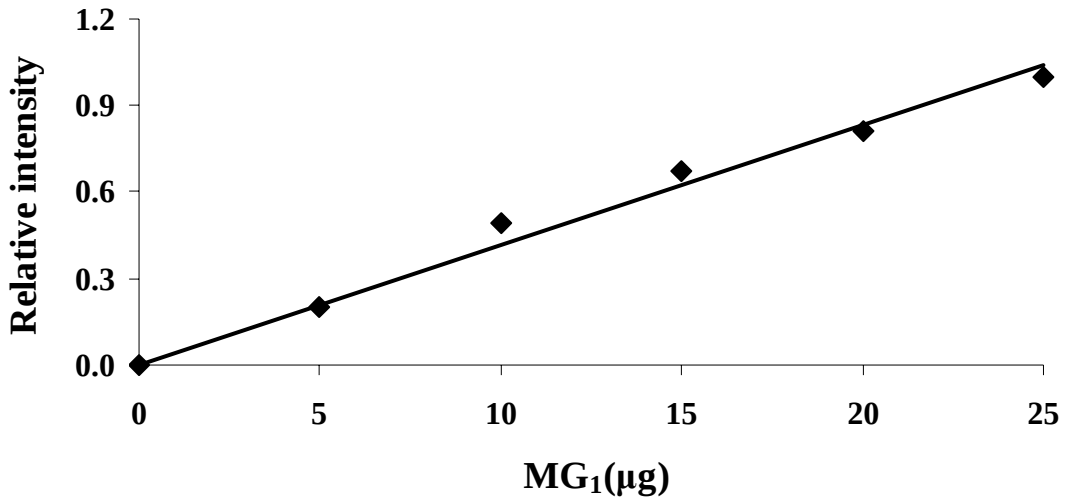
Appendix C Standard curve for protein determination by Bradford's method



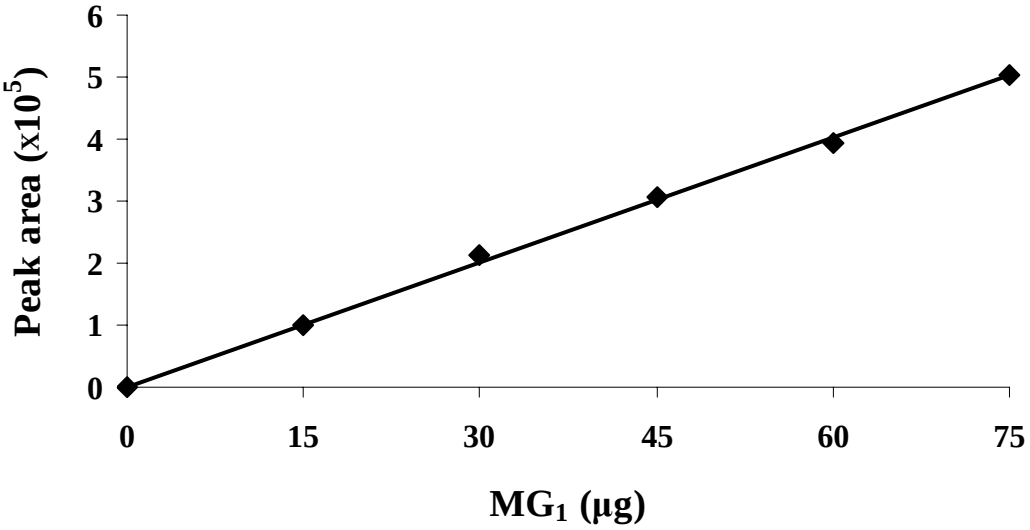
Appendix D Standard curve for β-CD determination by phenolphthalein method



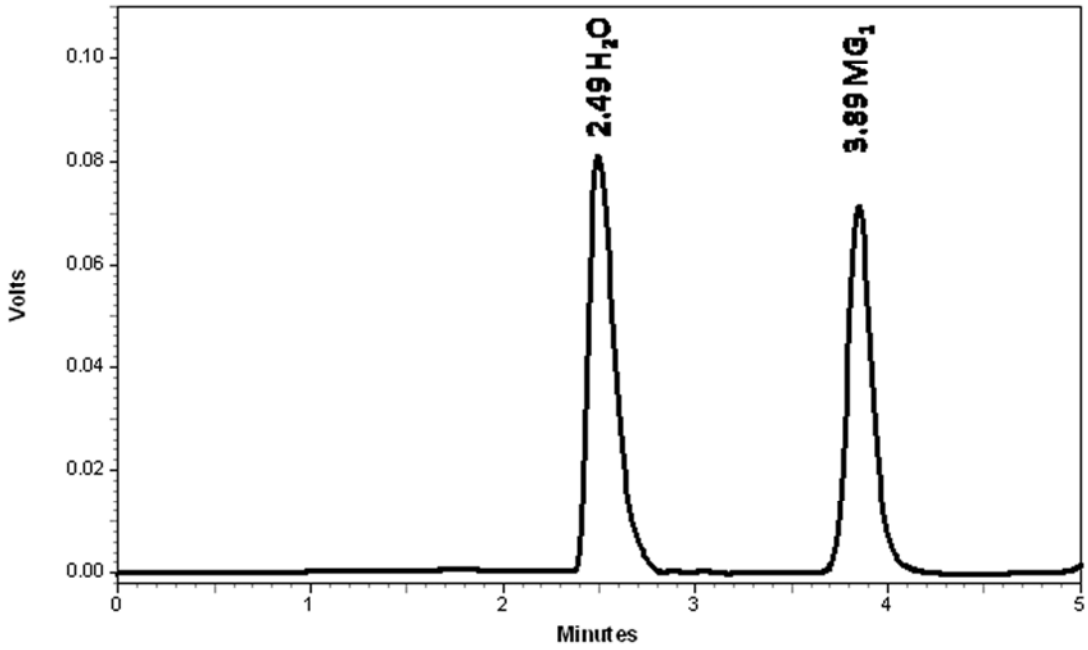
Appendix E Standard curve of MG_1 concentration by determination of spot intensity on TLC plate



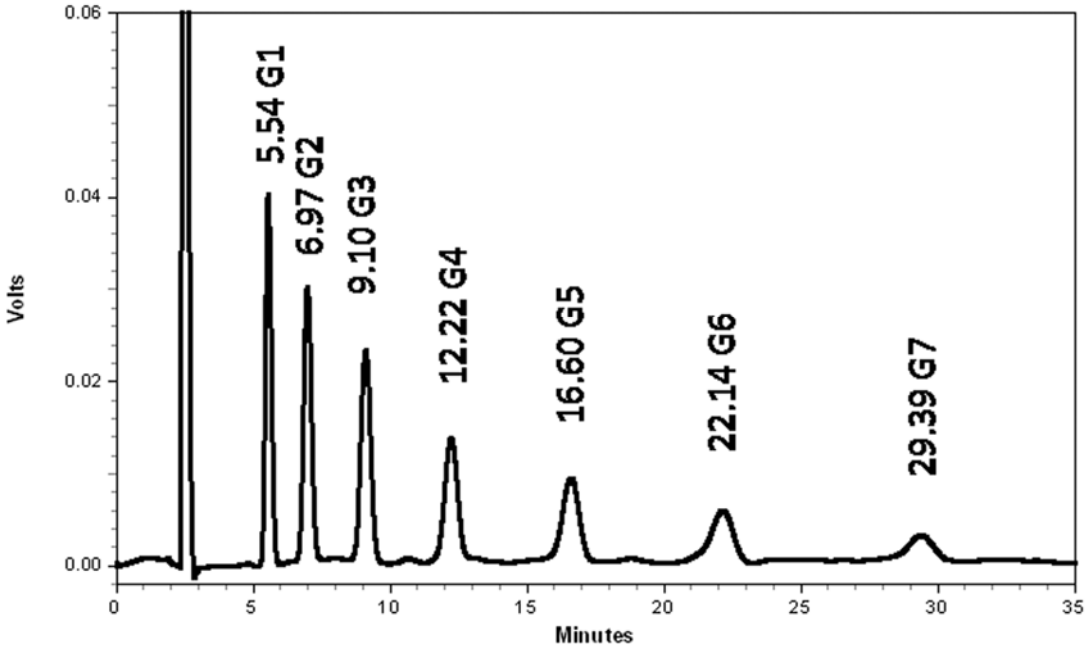
Appendix F Standard curve for MG_1 concentration by HPLC analysis



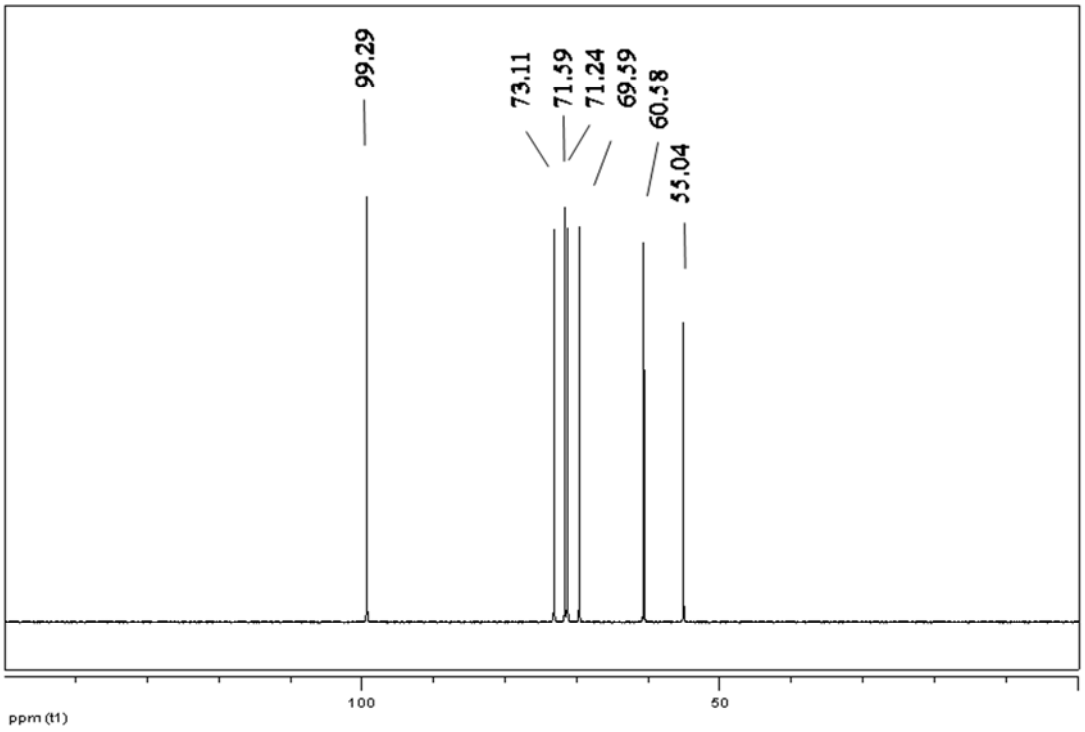
Appendix G HPLC chromatogram of standard MG_1



Appendix H HPLC chromatogram of standard G1-G7



Appendix I The 100 MHz ^{13}C-NMR of standard MG_1



BIOGRAPHY

Miss Kwanjai Chotipanang was born on July 3rd, 1980. After she had graduated with the Bachelor's degree of General science from Faculty of Science at Prince of Songkla University in 2003, she worked as laboratory technician at Excise Department until 2007. Hereafter, she has continued to study for the Master degree of Science in Biochemistry program, Faculty of Science at Chulalongkorn University.