ลักษณะสมบัติของผลิตภัณฑ์จากแทรนส์ไกลโคซิเลชันของรีคอมบิแนนต์ไซโคลเดกซ์ทริน ไกลโคซิลแทรนส์เฟอเรสจาก *Paenibacillus* sp. BT01

นางสาวประไพ หงษา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

CHARACTERIZATION OF TRANSGLYCOSYLATION PRODUCTS OF RECOMBINANT CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp.BT01

Miss Prapai Hongsa

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

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Thesis Title	CHARACTERIZATION OF TRANSGLYCOSYLATION PRODUCTS OF RECOMBINANT CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp.BT01
By	Miss Prapai Hongsa
Field of Study	Biochemistry
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ประไพ หงษา : ลักษณะสมบัติของผลิตภัณฑ์จากแทรนส์ไกลโคซิเลชันของรีคอมบิแนนต์ไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรสจาก *Paenibacillus* sp. BT 01 (CHARACTERIZATION OF TRANSGLYCOSYLATION PRODUCTS OF RECOMBINANT CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. BT01) อ.ที่ปรึกษาวิทยานิพนธ์หลัก ; รศ.ดร.ทิพาพร ลิมปเสนีย์ , 118 หน้า.

ในการทดลองนี้เป็นการนำ CGTase ที่ได้จากการโคลนยีนจาก *Paenibacillus* sp. BT01 มาเร่งปฏิกิริยาการโยกย้ายหมู่ไกลโคซิล โดยใช้บีตาไซโคลเดกซ์ทรินเป็นโมเลกุลตัวให้กลูโคไพราโนซิล และน้ำตาลชนิดต่างๆ เป็นตัวรับ เพื่อผลิตออลิโกแซ็กคาไรด์ใหม่ พบว่าซาลิซินเป็นโมเลกุลตัวรับที่ให้ผลิตภัณฑ์ซาลิซินไกลโคไซด์ที่น่าสนใจเมื่อทำการตรวจสอบโดยวิธี TLC พบผลิตภัณฑ์หลายตัวที่เคลื่อนที่ได้เร็ว และมีค่า Rf มากกว่ากลูโคส จากการวิเคราะห์ผลิตภัณฑ์ด้วยเทคนิค HPLC พบผลิตภัณฑ์ที่แยกได้ชัดเจน 3 ชนิด ซึ่งมีค่า Rt 6.1, 9.5 และ 15.7 นาที ตามลำดับ เรียกว่า ผลิตภัณฑ์ 1, 2 และ 3 พบว่าภาวะที่เหมาะสมต่อการสังเคราะห์ผลิตภัณฑ์ซาลิซินไกโคลไซด์คือ การบ่มเอนไซมรีคอมบิแนนต์ CGTase 100 ยูนิต/มล. กับ 3.0 (w/v) ซาลิซิน และ 1.8 %(w/v) บีตาไซโคลเดกซ์ทริน ใน 0.2 โมลาร์อะซีเตตบัฟเฟอร์ พีเอช 6 ที่ 50° ซ เป็นเวลา 3 ชั่วโมง ซึ่งได้ผลิตภัณฑ์รวมคิดเป็น 77.85 % โดยได้ผลิตภัณฑ์ 1, 2 และ 3 คิดเป็น 47.23%, 20.66% และ 9.96% ตามลำดับ เมื่อเพิ่มปริมาณการสังเคราะห์ซาลิซินไกลโคไซด์ และทำการแยกผลิตภัณฑ์ด้วย HPLC เมื่อทำการพิสูจน์โครงสร้างหลักด้วยเทคนิค MS และ NMR พบว่า ผลิตภัณฑ์ 1, 2 และ 3 มีขนาดโมเลกุล 417.2 , 633.2 และ 795.2 ดาลตัน โดยการวิเคราะห์ด้วย ESI-TOF MS และผลิตภัณฑ์ซาลิซินไกลโคไซด์สามารถจำแนกได้เป็น กลูโคซิลซาลิซิน (α-D-glucopyranosyl-1⟶4-salicin) มอลโทซิลซาลิซิน (α-D-glucopyranosyl-1⟶4-α-D-glucopyranosyl-1⟶4-salicin) และไตรโอซิลซาลิซิน (α-D-glucopyranosyl-1⟶4-α-D-glucopyranosyl-(1⟶4)-α-D-glucopyranosyl-(1⟶4)-salicin) โดยการวิเคราะห์ด้วย NMR ตามลำดับ ซึ่งสารทั้งสามมีความสามารถละลายน้ำได้ 72.23, 134.46 และ 207.76 มก.ต่อมล. ขณะที่ซาลิซินมีค่าการละลาย 23.31 มก.ต่อมล. ผลิตภัณฑ์ของซาลินไกลโคไซด์ยังมีความสามารถในการยับยั้งการแข็ง ตัวของเลือดด้วย การวัดระยะเวลาที่เลือดกลาย เป็นลิ่มเลือด Clotting time นี้บอกถึง intrinsic blood clotting process จึงน่าจะเป็นประโยชน์มากสำหรับใช้ในการทดสอบผู้ป่วยที่มีความบกพร่องในการแข็งตัวของเลือด (coagulation defect)

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : RECOMBINANT CYCLODEXTRIN GLYCOSYLTRANSFERASE / SALICIN / β-CD / SALICIN GLYCOSIDE / TRANSGLYCOSYLATION

PRAPAI HONGSA : CHARACTERIZATION OF TRANSGLYCOSYLATION PRODUCTS OF RECOMBINANT CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp BT01. ADVISOR : ASSOC.PROF.TIPAPORN LIMPASENI, Ph.D., 118 pp.

The aim of this study was to use cloned cylcodextrin glycosyltransferase from *Paenibacillus* sp.BT01 (pBT) in producing transglycosylation products. The enzyme was able to synthesize various products using beta cyclodextrin (β-CD) as glucosyl donor and various saccharides (glucose, mannose, fructose, xylose, maltose, etc.) as glucosyl acceptors. Salicin was found to be one of the efficient acceptor with interesting glucosylated derivatives which move faster than glucose standard (G1) when analyzed by TLC. Three kinds of products, with retention time of 6.1 minute, 9.5 minute and 15.7 minute were clearly separated on HPLC and named product 1, 2 and 3. The optimal condition for salicin glycoside production was 3.0 % (w/v) salicin, 1.8% (w/v) β-CD and 100 unit/ml recombinant CGTase , pH 6 at 50ºC for 3 hours. After optimization, product 1(Rt 6.1), product 2 (Rt 9.5) and product 3(Rt 15.7) were obtained with yield of 47.23 %, 20.66 % and 9.96 %, respectively. The molecular masses of transfer products 1, 2 and 3 were 417.2 dalton, 633.2 dalton and 795.2 dalton, respectively when analysed by ESI-TOF mass spectrophotometer. The results showed that recombinant CGTase transfer the glucose unit from β-CD to hydroxyl group of salicin. The transglycosylation products were identified as glucosyl salicin (α-D-glucopyranosyl-(1⟶4)-salicin), maltosyl salicin (α-D-glucopyranosyl-1⟶4-α-D-glucopyranosyl-1⟶4-salicin) and triosyl salicin (α-D-glucopyranosyl-1⟶4-α-D-glucopyranosyl-1⟶4-α-D-glucopyranosyl1⟶4-salicin) by NMR analysis. The water solubility of the glucosyl salicin maltosyl salicin and triosyl salicin were 72.23, 134.46 and 207.76 mg/ml respectively compared to 23.31 mg/ml for salicin. Salicin glycoside products showed higher anti-coagulation activity than salicin. These salicin glycosides may have useful application in patients with defects in blood coagulation.

Department : Biochemistry Student's Signature .....

Field of Study : Biochemistry Advisor's Signature .....

Academic Year : 2010

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LIST OF ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
CDs	cyclodextrin
CGTase	cyclodextrin glycosyltransferase
°C	degree celsius
Da	dalton
DEAE	diethylaminoethyl
et al.	Et.Alii (latin), and others
ESI-TOF-MS	electrospray ionization-time of flight mass spectrometry
g	gram
h	hour
HPLC	high performance liquid chromatography
IPTG	isopropylthiogalactoside
l	litre
µg	microgram
µl	microlitre
M	molar
min	minute
ml	millilitre
MW	molecular weight
ND-PAGE	non-denaturing polyacrylamide gel electrophoresis
ppm	part per million
Rf	relative mobility
rpm	revolution per minute
Rt	retention time
U	unit(s)
w/v	weight by volume

CHAPTER I

INTRODUCTION

1.1 Oligosaccharides

1.1.1 Chemical composition of oligosaccharides

Carbohydrates are an intrinsic part of the daily diet for mammals. In nature they exist in many forms, complex carbohydrates are formed by linkage of monosaccharides. In most monosaccharides there are two or more hydroxyl groups. Glycosidic bonds can join one monosaccharide to another via the many hydroxyl groupson the monosaccharides (Berg *et al.*, 2002). Glycosides are basically molecules, wherein a sugar is attached to a non-carbohydrate element, forming minor natural molecules. Glycosides have several important functions in all living organisms. A number of plants stock up compounds in the form of dormant glycosides. These chemicals may be set in motion by certain enzymes by means of hydrolysis that results in the sugar portion to be detached. The activation of these compounds by the enzymes make them accessible for utilization. A number of such glycosides enclosed by the plants are of therapeutic value. However, in the case of animals includes humans, often venoms are attached to sugar molecules as a portion of their abolition from the body.

Oligosaccharides are composed of 2 to 20 monosaccharide units joined together by glycosidic bonds and exist as a linear or branched arrangement. The production of oligosaccharides for use in food or as part of the diet (table 1) is either by direct extraction from plants, controlled hydrolysis of polysaccharides, or by enzymatic synthesis using enzymes which possess hydrolytic or transglycosylation activity. Three abundant disaccharides are sucrose, lactose, and maltose. Sucrose, a common table sugar, is obtained commercially from cane or beet. The anomeric carbon atoms of a glucose unit and a fructose unit are joined in this disaccharide; the configuration of this glycosidic linkage is α for glucose and β for fructose. Sucrose

Table 1. Dietary oligosaccharides available in food products on the market and the type of source available (Delzenne, 2003).

Type of oligosaccharides	**Natural occurrence**	**Industrial production process***
Fructo-oligosaccharides	Fruits and vegetables	Synthesis from saccharose
	onions, banana, garlic, etc.	Hydrolysis from chicory-root inulin
Galacto-oligosaccharides	Human milk	Enzymic synthesis from lactose
Lactulose		Synthesis from saccharose and lactose
Lactosucrose, glycosylsucrose		Synthesis from lactose
(Iso)malto-oligosaccharides		Hydrolysis or glycosyl transfer from starch
Xylo-oligosaccharides		Hydrolysis from polyxylans
Stacchyose, raffinose	Soyabean	
Palatinose-oligosaccharides		
Gentio-oligosaccharides		
Cyclodextrin		Synthesis from starch

*Most synthetic products are obtained through enzymic reactions.

can be cleaved into its component monosaccharides by the enzyme sucrase. Lactose, the disaccharide of milk, consists of galactose joined to glucose by a β-1, 4-glycosidic linkage. Lactose is hydrolyzed to these monosaccharides by lactase in human being and by β-galactosidase in bacteria. In maltose, two glucose units are joined by an α-1,4 glycosidic linkage, as stated earlier. Maltose comes from the hydrolysis of starch and is in turn hydrolyzed to glucose by maltase. Sucrase, lactase, and maltase are located on the outer surfaces of epithelial cells lining the small intestine (Berg *et al.*, 2002).

1.1.2 Properties of oligosaccharides

Oligosaccharides are water soluble and mildly sweet, typically 0.3-0.6 times as sweet as sucrose. The sweetness of the oligosaccharide product is dependent on the chemical structure, molecular mass of the oligosaccharides and the levels of mono- and disaccharides in the mixture (Crittenden and Playne, 1996).This sweetness decrease with the longer oligosaccharide chain length. This low sweetness intensity is quite useful in various kind of foods where the use of sucrose is resticted by high sweetness property (Roberfroid and Slavin, 2000). They may be used as bulking agents in conjunction with intense artificial sweeteners such as aspartame, phenylalanine or sucralose. Oligosaccharides can be used to mask the aftertastes produced by some of these intense sweeteners. When compared with mono- and disaccharides, the higher molecular weight of oligosaccharides provides increased viscosity, leading to improved body and mouthfeel. The oligosaccharide can also be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to Maillard reactions in heat-processed foods. They provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination. In addition they are presently used as low-cariogenic sugar substitutes in confectionery, chewing gums, yoghurts and drinks (Crittenden and Playne, 1996).

Some oligosaccharides are used as functional food ingredients that have a great potential to improve the quality of many food. In addition to providing useful modification to physicochemical properties of food, it has been reported that these oligosaccharides have various physicological function such as the improvement of

intestinal microflora base on the selective proliferation of biofidobacteria, stiumulation of mineral absorption, anti-cariogenicity, and the improvement of both plasma cholesterol and blood glucose level (Nakakuki, 2002). Effective bifidogenic doses appear to vary among the different oligosaccharide types. These bacteria are believed to be beneficial to health, and together with other health promoting microorganisms are termed probiotics (Crittenden and Playne, 1996). The functional properties evaluated until now are summarized in table 2.

1.2 Oligosaccharide production

1.2.1 The chemical and enzymatic synthesis of oligosaccharides

For the preparation of structurally well-defined oligosaccharides, the stereo- and regiospecificities of enzymes are very attractive properties compared to chemical processes that require complex protection and deprotection steps. At present, enzymatic processes are preferred in industry for the production of most commercial oligosaccharides.

Chemical method of oligosaccharide or glycoconjugate synthesis are well developed, often slow and regiospecifically nonspecific. These synthesis also generally require multi-step processes under harsh conditions. Due to carbohydrate contain multiple hydroxyl group of similar reactivity (figure 1), the total yield are often low and large-scale synthesis is not practical. In addition, stereospecific reactions giving the correct anomer (α or β) are often difficult (Nelson, 1988). On the contrary, enzymatic biotransformation has been employed as an alternative method for manufacturing pharmaceuticals, fine chemicals, and food ingredients, due to the high selective catalytic reactions and the use of environmentally friendly, mild reaction conditions (Figure 2) (Barreteau *et al.*, 2006).Two types of enzyme have been used for preparation of complex oligosaccharides, the glycosyltransferases (EC 2.4) and glycosidases (EC 3.2) (Nelson , 1988).

Table 2. Properties of oligosaccharides (Nakakuki, 2002).

Physicochemical property	Sweetness, bitterness, hygroscopicity, water activity, reinforcement agent for drinks, stabilization of active substances (protein, flavor, color, etc.), inclusion capability, etc.
Biological property	Digestibility, nondigestibility, noncariogenicity, anticariogenicity, bacteriostatic action, selective proliferation of bifidobacteria, improvement of serum lipids, and blood glucose, etc.
Other properties	Specific substrate for enzymes, enzyme inhibitors, elicitors, etc.

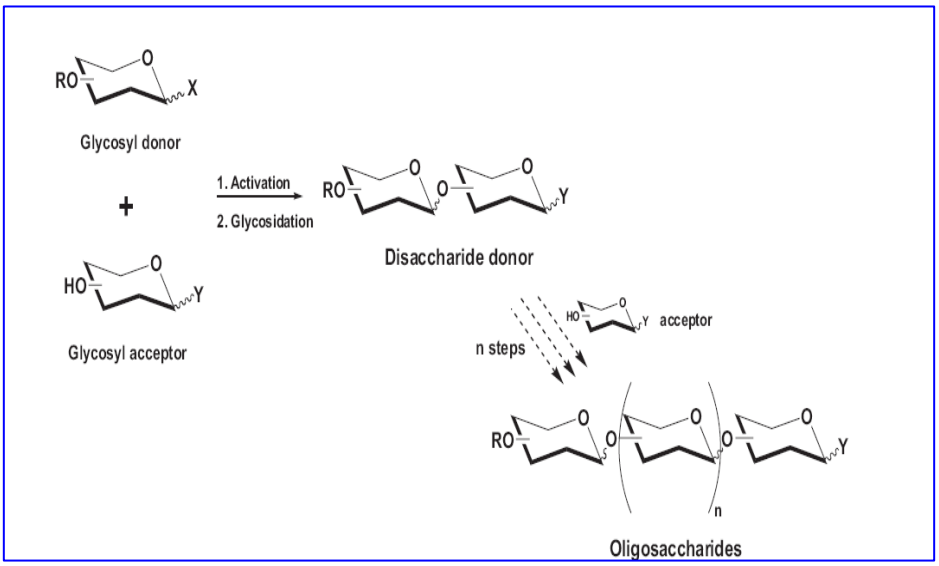


Figure 1. Current steps of the chemical synthesis of oligosaccharides (R: H or saccharides). (Barreteau *et al.*, 2006).

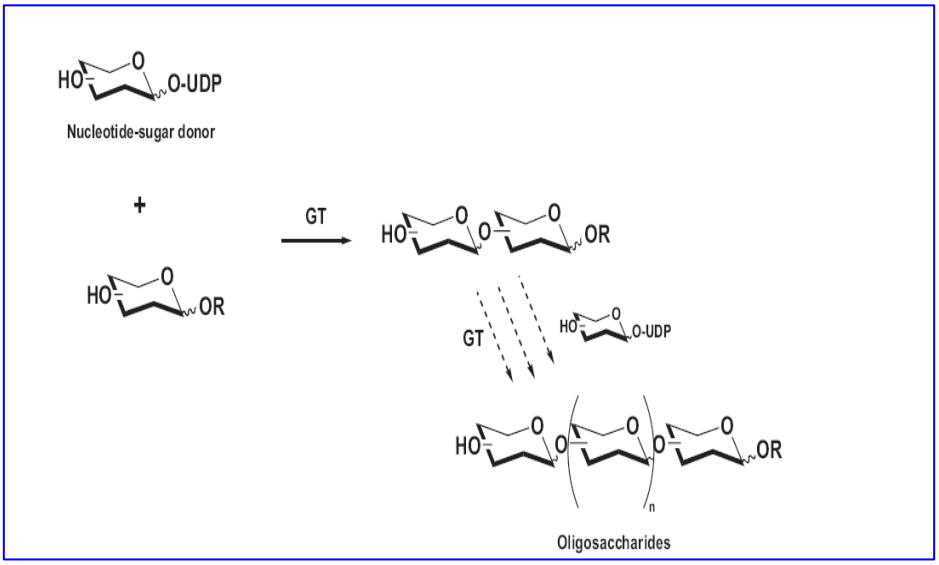


Figure 2. Current step of the enzymatic synthesis of oligosaccharides using glycosyltransferase (GT) (R: H or saccharides). (Barreteau *et al.*, 2006).

The knowledge of new oligosaccharide structures, associated with the advances of tranglycosylation research, indicated that there is the tendency of these compounds being employed as food ingredients in the future. The increment of this market implies procedure for their low cost manufacturing. The advances in synthesis of oligosaccharides by enzymes in the microorganisms are applied to generate some oligosaccharides in large scale, the improved glycosylation procedure (chemical or biochemical) seems to be much safer than the manufacturing of oligosaccharides and derivatives performed in present. Many di-, tri- and tetrasaccharides can be now synthesized routinely involving a minimum number of glycosylation steps oligosaccharide synthesis is developed (Barreteau *et al.,* 2006).

1.2.2. Enzymatic production of oligosaccharides

These enzymes catalyse the transfer of a glycosyl donor to an acceptor molecule forming a new glycosidic bond regio- and stereospecifically according to the nature of the sugar residue being transferred and the nature of the donor molecule. Glucansucrases (EC 2.4.1.5) and cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) are the most representative enzymes of the transglucosidase family, the natural substrates of which are sucrose and starch, respectively.

1.3 Glucansucrases

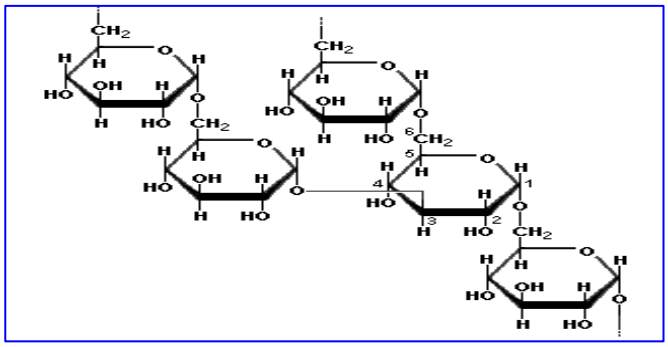
Several bacteria excrete a range of transglucosidases called glucansucrases that utilize sucrose to synthesize glucose polymers. Glucansucrase consists of subfamilies dextransucrase, mutansucrase, alternansucrase and reteranesucrase (Hijun *et al*., 2006). The enzyme in this family catalyzes three kinds of reaction; (a) polymerization of the glucose moiety of sucrose (b) glucose transfer to acceptors, and (c) sucrose hydrolysis. However, other short carbohydrates may also act as acceptors yielding the so-called acceptor products (Robyt and Walseth, 1978). The acceptor reaction corresponds to the transfer of glycosyl units from the sucrose donor onto various acceptor molecules. Some acceptors, such as mono- or disaccharide (*e.g.* maltose) present an increasing number of glucose moieties in their structure; others form a unique acceptor-product containing one glucose residue more than the

acceptor (Plou *et al*., 2007). Within glucansucrase family, the member enzymes show different linkage specifity of glucosyltransfrease. For example, dextransucrases (sucrose:1,6-α-D-glucan 6-α-D-glucosyltransferase) are glucansucrases produced by different *Leuconostoc mesenteroides* strains that convert sucrose into α-(1-6)-linked glucose polymers (dextrans), releasing fructose (Monchois *et al.*, 1999). The glucansucrase enzyme catalyzes the following reactions:

(a) Polymerzation reaction

glucansucrase

$(sucrose)_n \longrightarrow$ Glucan or dextran (polyglucose chain) + (Fructose)

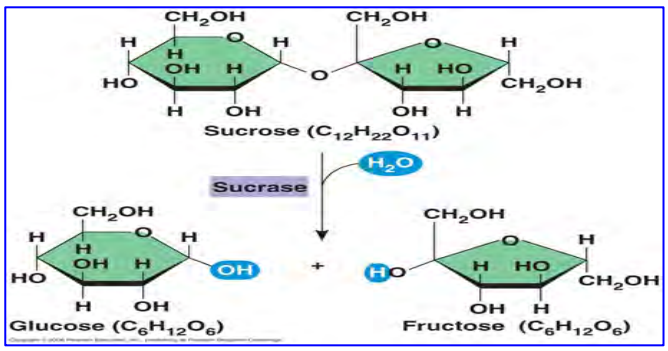


(b) Hydrolysis reaction

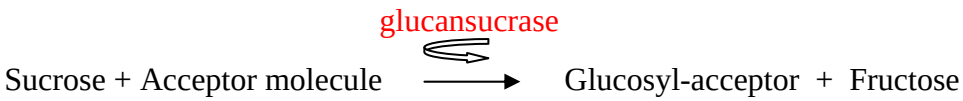
glucansucrase

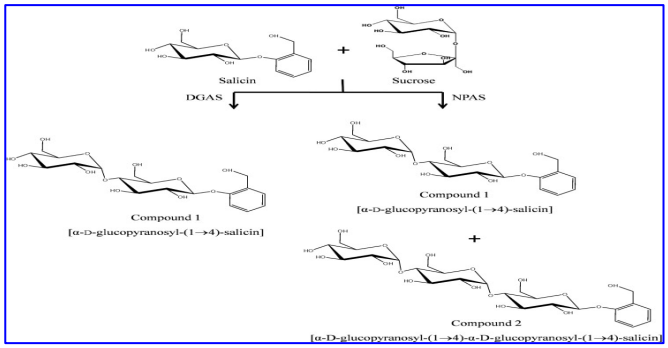
$Sucrose + H_2O \longrightarrow Glucose + Fructose$

$(Glucan)_n + H_2O \longrightarrow (Glucan)_n + Fructose$



(c) Acceptor reaction

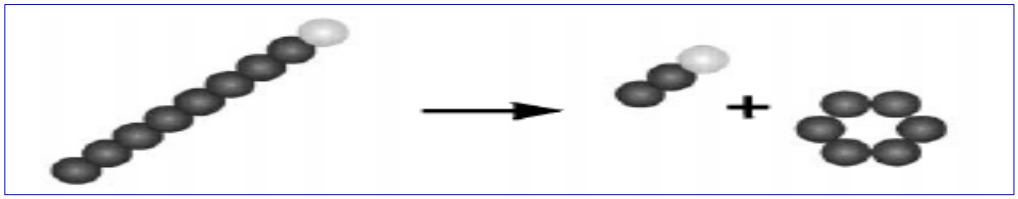




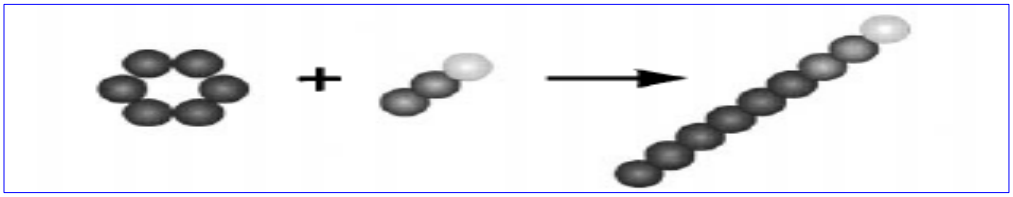
1.4 Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (E.C.2.4.1.19), CGTase catalyzed the production of cyclodextrin from starch and related α-(1,4)-linked glucose polymers via a transglycosylation reaction.CGTase catalyzes four related reactions; cyclization, coupling, disproportionation and hydrolysis (figure 3). Cyclization reaction is a reaction which cyclodextrins were formed from starch and related α-1,4-glucans through the intramolecular transglycosylation reaction. In the coupling reaction, cyclodextrin ring is cleaved and glycosyl unit transferred to an acceptor maltooligosaccharide substrate. A linear maltooligosaccharide is cleaved and the new reducing end is transfered to an acceptor maltooligosaccharide substrate in disproportionation. CGTase also has a weak hydrolytic activity (Van der Veen *et al.*, 2000). When reducing end is not transferred to carbohydrate acceptor but rather to a water molecule, the result is hydrolysis of amylase or the linearization of cyclodextrin (Kitahata et al., 1974). The major products of cyclization reaction are α-, β- and γ-cyclodextrins. CGTase is a member of the α- amylase family of glucosyl hydrolases. The enzyme in this group show a wide diversity in reaction specificity and many of them are active on starch. Whereas amylases generally hydrolysis glycosidic bonds in the starch molecules, CGTase mainly catalyzes transglycosylation reaction, which hydrolysis being a minor activity (Van der Veen *et al.*, 2000).

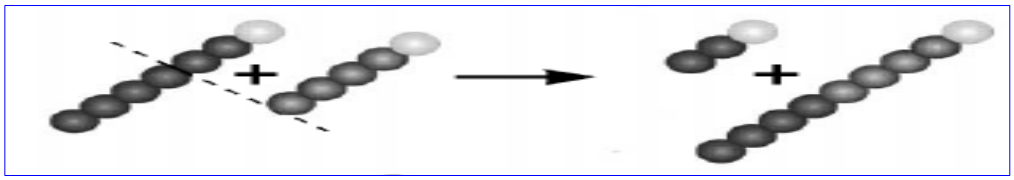
(a)



(b)



(c)



(e)

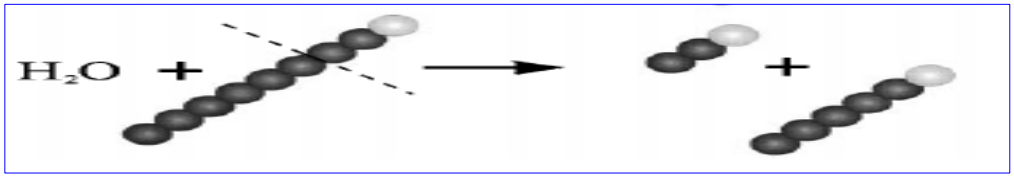


Figure 3. Schematic representation of the CGTase catalyzed reactions. The circles represent glucose residues;the white circles indicate the reducing end sugars. (a) Cyclization, (b) coupling, (c) disproportionation and (d) hydrolysis (Van der Veen *et al*., 2000).

Cyclodextrin glycosyltransferase (CGTase) is an extracellular enzyme.Since *Bicillus mercerans* was discovered as the first source capable of producing CGTase, a large number of microorganism have been identified as CGTase producers. These included aerobic mesophilic bacteria (*Bacillus macerans, Bacillus megaterium, Klebsiella oxytoca, Klebsiella Pseudomonas* and *Micrococcus luteus*), aerobic themophilic (*Bacillus stearothermophilus*),anaerobic thermophilic (*Thermoanaero bacterium thermosulfurigenes*), aerobic alkalophilic bacteria (*Bacillus circulans, Bacillus* sp.AL-6) and aerobic halophilic (*Bacillus halophilus)* (Tonkova , 1998). CGTase is classified into three types, α-CGTase, β-CGTase and γ-CGTase according to major CDs produced. The properties of bacterial CGTase are shown in table 3.

The acceptor specificity of CGTase is rather broad. A number of hydroxyl-containing compounds such as glycosides, sugar alcohols, vitamins, flavonoids, *etc*. may act as CGTase acceptors, in many cases with high efficiency (Aga *et al.*, 1991; Kim *et al.*, 1997). The transglycosylation activity of CGTase seems to be very dependent on enzyme source (Park *et al.*, 1998). Glycosylation often confers new stability/solubility properties to an aglycon (Kometani *et al.*, 1994). However, the best acceptors are carbohydrates with an α-D-glucopyranose structure in the chair form and equatorial hydroxyl groups at C-2, C-3 and C-4 (Tonkova, 1998). With maltose or glucose as acceptors and starch as donor, a series of maltooligosaccharides is produced (Martin *et al.*, 2001). The degree of polymerization of the oligosaccharides formed can be modulated by varying the starch to acceptor ratio. CGTase has a higher affinity for disaccharides compared to monosaccharides which suggests that the acceptor binding site can accommodate at least two glucopyranose moieties (Park *et al.*, 1998). For example, disaccharides such as isomaltose, gentiobiose, turanose, maltulose, isomaltulose, cellobiose and sucrose are good CGTase acceptors. A steric factor possibly plays a major role in diminishing the acceptor capacity of trisaccharides.

Table 3. Some properties of bacterial CGTase (Tonkova , 1998)

Producer	Optimum pH	Optimum Temp.(°C)	Molecular mass	Main CD produced	Reference
Bacillus macerans ATCC 8514	6.1-6.2	60°C	139,000	-	De Pinto, 1968
Bacillus macerans IFO 3490	5.0-5.7	55°C	-	α-CD	Kitahata, 1974
Bacillus macerans IAM1243	-	-	74,000	α-CD	Takano, 1986
Bacillus megaterrium No5	5.0-5.7	55°C	-	β-CD	Kitahata, 1974
Bacillus circutans var.*alkalophilus* ATCC21783	4.5-4.7	45°C	88,000	β-CD	Nakamura, 1990
Bacillus sp.AL-6 (alkalophilic strain)	7.0-10.0	60°C	74,000	γ-CD	Fujita, 1993
Bacillus cereus NCIMB	5.0	40°C	-	α-CD	Abelian, 1994
Bacillus sp.INMIA T6 (thermophilic strain)	6.5	55°C	38,000	α-CD	Abelian, 1994
Bacillus sp.INMIA T42(thermophilic strain)	6.5	55°C	35,000	β-CD	Abelian, 1994
Bacillus sp.INMIA A7/1(alkalophilic strain)	6.0	50°C	44,000	β-CD	Abelian, 1994
Bacillus sp.INMIA 1919	4.0	50°C	42,000	α-CD	Abelian, 1994
Bacillus hylophilus INMIA 3839	7.0	60-62°C	71,000	β-CD	Abelian, 1995
Thermoanaerobacterium thermosulferigene EM1	4.5-7.0	80-85°C	68,000	β-CD	Wind, 1995

1.5 Cyclodextrins

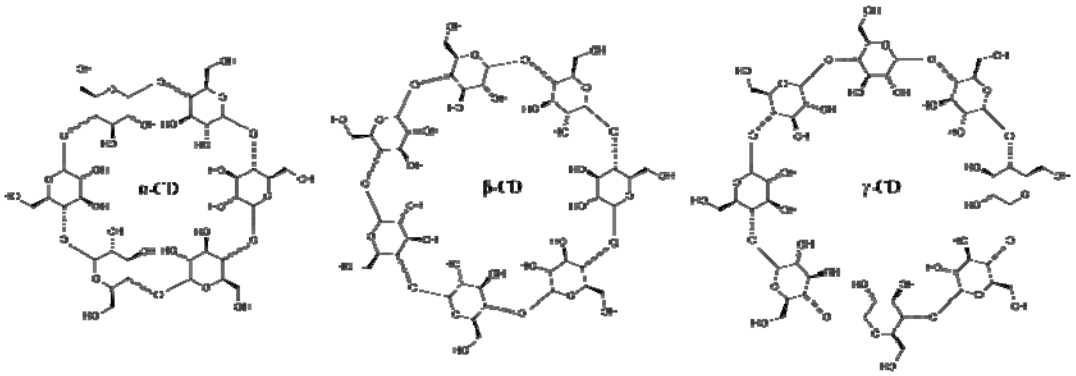
1.5.1 Structures and characteristics

Cyclodextrins are the oligomers of anhydroglucose units join to form a ring structure with α-1,4 glycosidic bonds. Cyclodextrins are of three types: containing a number of glucose monomer of 6, 7 and 8 glucose units call α- (alpha), β- (beta) and γ- (gamma) cyclodextrin, respectively as shown in figure 4 (a). β- cyclodextrin is most accessible, the lowest-priced and generally the most useful. The main properties of those cyclodextrins are given in table 4. The crystal structures show each of the glucose units is in the rigid $^4C_1$ chair conformation, giving the molecule the shape of a hollow truncated cone. The cone is formed by the carbon skeletons of the glucose units and the glycosidic oxygen atom between them, with the primary hydroxyl groups on the outer part of the narrow side of the cone and the secondary hydroxyls at the wide face as shown in figure 4 (b). The C-2-OH group of one glucopyranoside unit can from a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the β-cyclodextrin molecule, a complete secondary belt is formed by these H bonds, as a consequence, the β-cyclodextrin is rather rigid structure. This intramolecular H-bone formation is probable the explanation for the observation that β-cyclodextrin has the lowest solubility of all cyclodextrins. The H-bone belt is incomplete in the α–cyclodextrin molecule, because one glucopyranose unit is in a distorted position,thus only four H-bonds can be established simultaneously instead of six possible bonds. γ-cycodextrin is a non-coplanar and more flexible structure. So,it is the most soluble of all cyclodextrins. Hydrolysis rate of cyclodextrins increases in the order α–cyclodextrin< β-cyclodextrin < γ-cycodextrin (Szejtli,1998).

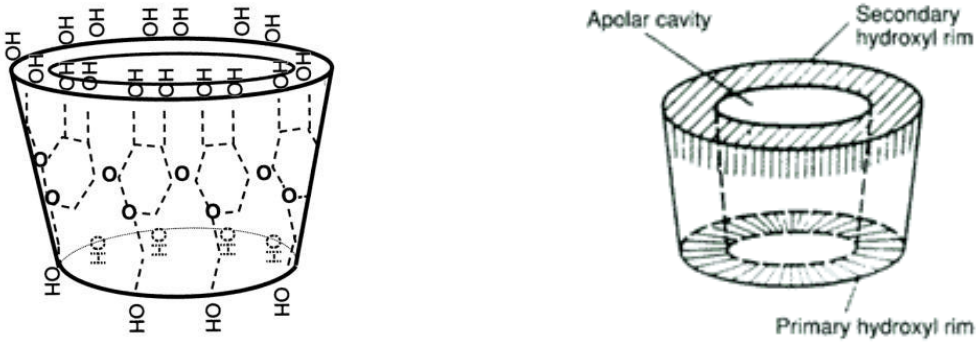
1.5.2 Cyclodextrin inclusion complexes

In an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecule that are energetically unfavorable (polar-apolar interaction) and therefore can be readily substituted by appropriate “guest molecule”, which are less polar than water (figure 5). The dissolved cyclodextrin is the “host” molecule, and part of the driving force of the complex formation is an appropriate “guest” molecule.

One, two, or three CD molecules contain one or more entrapped “guest” molecules. Most frequently, the host:guest ratio is 1:1. This is the essence of “molecular encapsulation”. This is the simplest and most frequent case. However, 2:1, 1:2, 2:2, or even more complicated associations, and higher-order equilibria exist, almost always simultaneously. The inclusion complexes formed can be isolated as stable amorphous, or microcrystalline substances. Upon dissolving these complexes, an equilibrium is established very rapidly between dissociated and associated species, and this is expressed by the complex stability constant *Ka*. The association of the CD and guest molecules, and the dissociation of the CD/guest complex formed is governed by a thermodynamic equilibrium.



(a)



(b)

Figure 4. Chemical structure of α- (alpha), β- (beta) and γ- (gamma) cyclodextrin (CD), consisting of 6, 7, and 8 glucose units (a) (Szejtli ,2004).
Structure of cyclodextrin showing glucose molecule arrangement (b).

Table 4. Cyclodextrin properties (Szejtli, 1998)

Property	**α- CD**	**β- CD**	**γ- CD**
Nunber of glucose unit	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100ml)	14.5	1.85	23.2
pKa	12.33	12.2	12.08
Inner diameter (nm)	0.45-0.57	0.62-0.78	0.79-0.95
Outer diameter (nm)	1.37	1.53	1.69
Height (nm)	0.79	0.79	0.79
Cavity volume $(nm)^3$	0.174	0.262	0.472

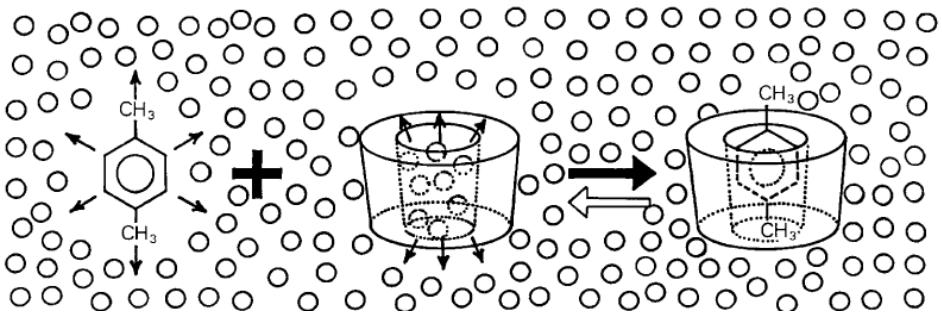


Figure 5 Schematic representation of the association - dissolation of the host (cyclodextrin) and guest (*p*-xylene). The formed guest/host inclusion complex can be isolated as a microcrystalline powder. (Szejtli , 2004)

1.5.3 Applications of cyclodextrins

Cyclodextrins are used to obtain certain benefits that result from complexation with the cyclodextrins. These include alteration of the solubility of the guest compound, stabilization against the effects of light, heat, and oxidation, masking of unwanted physiological effects, reduction of volatility, and others. In some applications, more than one benefit is obtained by complexation with cyclodextrins. These are discussed below with examples of uses for the complexes. Table 5 lists selected products containing cyclodextrins (Hedges, 1998).

These characteriticss of cyclodextrins or their derivatives make them suitable for applications in analytical chemistry, agriculture, the pharmaceutical field, in food and toilet articles. In addition to the common use of CDs, they are used in separation science because they allow discriminating between positional isomers, functional groups, homologues and enantiomers. This property makes them a useful agent for a wide variety of separations.

Less than 10% of all produced CDs and CD derivatives are consumed by the pharmaindustry. Though minute amounts amounts of cyclodextrins are used in drug formulations, the field of application has been most thoroughly studied. Cyclodextrins are used to increase stability, solubility, and bioavailability of drugs. The controlled released property is very useful both in drug and cosmetic fields.

In texile industry, CDs have various possibilities, but the blockbuster will be the chemical bindind of CDs onto the surface of nature and synthetic fibers. The immobilized CDs can bind volatile molecules like the unpleasant components of sweat or cigarette smoke. When previously charged, the slow release of fragrances, insect repellents, or even drugs (for transdermal delivery) will be possible by wearing boned CD-containing garments.

In chemical industry, the number of examples for application of CDs rapidly increases. For example, for convervation of wood products, water-insoluble fungicides have to be impregnated into the wood structures (door and window frames, etc.) Earlier, this was possible only by dissolving these water-insoluble fungicides in

organic solvents, now it is possible to use simply aqueous cyclodextrin solutions for this purpose. To reduce the high viscosity of polyurethane thickening agent containing emulsion-type coatings (to facilitate the spraying), CDs are very appropriate, and by adding a small amount of very stable complex-forming detergents just before the spraying the high viscosity can be restored within minutes. CDs can be used in hydrocarbon-polluted soil remediation: the CD mobilizes the insoluble polyaromatic hydrocarbons, bringing them into the aqueous phase, where the soil microorganisms can rapidly metabolize them. CDs, incorporated into packaging plastic films, strongly reduce the loss of the aroma substances by pervaporation, or incorporating CD-complex fungicides into the packaging flims can elongate shelf-life of food products. CDs have wide fields of utilization in sensors, in diagnostic kits, and in analytical chemistry, particularly in the chromatographic techniques (Szejtli, 2004)

1.5.4 β-cyclodextrin safety status

The Joint FAO/WHO Expert Committee on food Additive (JECFA) considers β-cyclodextrin as approved encapsulating agent for food additive, flavoring and vitamins; thickening agent with an ADI (acceptable dairy intake) of 0-5 mg/kg body weight (bw), based on the no-observed-effect level (NOEL) of 1.25% in the diet (equal to 470 mg/kg bw/day) in the one year study in dogs and savety factor of 100 (JECFA, 2005). In present, β-cyclodextrin has been recognized as GRAS (Generally Considered As Safety) for use as a flavor modifier in the food categories at use levels specified in GRAS application submitted to the United States Food and Drug Administration, FDA (Food Navigator, 2001). Table 6 showns summary of regulatory status of the natural cyclodextrins in parts of the world (Loftsson, 2007)

Table 5. Selected applications of cyclodextrins (Hedges, 1998)

Industrial	Use	
Food		
cinnamon-flavored	apples stabilize flavor	Hungary
flavored tea	stabilize flavor	Hungary
peppermint-flavored	chewing gum flavor delivery	Hungary
mustard oil steak sauce	improve solubility	Japan
acetic acid	convert to a powder	Japan
cinnamon-flavored	chewing gum flavor delivery	Japan
aloe-containing beverage	mask bitterness	Japan
lemon and grapefruit candies	flavor delivery	Japan
mint and green tea mints	flavor delivery	Japan
vitamin B fruit juice beverage	mask vitamin odor	Japan
water purifier	absorb odor	Japan
lemon-flavored sugar	flavor stabilization	Hungary
processed cheese	cholesterol removal	Belgium
Pharmaceutical		
itraconazole	increased solubility	U.S., U.K.
piroxicam	reduce irritation	Europe
PGE1	increased stability	Germany,Italy, Japan
PGE2	solubility, stability	Japan
garlic oil	mask odor	Germany
hydrocortisone	increased solubility	
Cosmetics and Personal Care Items		
artificial tanning lotion	stability, mask odor	U.S.
powdered hair bleach	stability	U.K., Belgium, U.S.
perfume	prolonged release	Japan
cold cream	solubility	U.S.
skin cleanser	tocopherol carrier	Italy
Miscellaneous		
laundry drier sheet	fragrance control	U.S.
chromatography column	separations	U.S.

Table 6 Regulatory status of the natural cyclodextrins (January 2004) (Loftsson, 2007)

	Food approval			Pharmacopoeia Monographs		
	US	Europr	Japan	USP/NF*	Ph.Eur.**	JP***
α- CD	In preparation	Planned	Yes	No	No	No
β- CD	GRAS	Food additive	Yes	Yes	Yes	Yes
γ-CD	GRAS	Pending	No	No	In progress	Yes

* United States Pharmacopoeia-National Formulary.

** European Pharmacopoeia

*** Japanese Pharmacopoeia

1.6 Application of transglycosylation activity of CGTase

The transglycosylation activity of CGTase is current utilized for industrial application in saccharide and glycoside products such as coupling sugar and transglycosylated stevide. Coupling sugar is commercial name of the maltooligosyl-fructose, has about half sweetness of sucrose, induces less Maillard reaction with proteins and amino acids, prevents starch ingredients from retrogradation, and retains moisture of foods (Nakano and Kitahata, 2005), using the intermoleculecular transglycosylation reaction of CGTase, the coupling sugar is produced from the mixture of starch hydrolyzates as substrate and sucrose as an acceptor (Kitahata, 2000). The maltosyl fructose is initially formed and also acts as acceptor, yielding maltotriosyl fructose. A series of maltooligosyl fructoside is subsequently formed (Martyín *el al*., 2004). The industrial processes for the CGTase catalyzed synthesis of coupling sugar have been patented using the enzyme from *Bacillus macerans* and *Thermoanaerobacter* sp. The sweetness of coupling is between 55 and 65 percent of that of sucrose with the low cariogenicity property. Beside, the transglycosylation of stevioside, a plant flavonoid, using starch as a donor is also catalysed by CGTase. The enzyme from *Bacillus stearothermophilus* FERM-P No 2222 (U.S.Pat.No.4,219,517) (Magomet *et al*., 2005) is used in industrial production of glucosyl stevioside. The glucosyl stevioside as the nature sweetener, with a high-yielding and low cost production, is now sold competitively on commercial scale. Rutin is a quersetin glycoside spreading widely in plants. Hesperidin obtained from mandarin orange peel also decreases capillary permeability and fragility. These flavonoids are transglycosylated successfully by the CGTases from *B. stearothermophilus* and alkalophilic *Bacillus* sp., respectively. α-1,4-Glycosylation specifically occurs to the glucose residue in rutinose (6-*O*-α-L-rhamnosyl-D-glucose) moiety of the flavonoids. The aqueous solubility of rutin and hesperidin increased 4,000 and 300 times, respectively, and the products show stabilizing activity for natural pigments against oxidation or ultraviolet radiation.

At present, several works on transglycosylation of other interesting compounds to make useful glycosides or oligosaccharides by the action of CGTase have been conducted. A novel glycoside, 4^{G}-α-D-glucopyranosyl rutin, is readily

water-soluble, substantially tasteless and odorless and readily hydrolyzable in vivo. It exhibits the same molecular absorption coefficient and physiological activities inherent to rutin. It is very useful as a highly-safe, natural yellow coloring agent, antioxidant, stabilizer, quality-improving agent, preventive, remedy, uv-absorbent and deterioration-preventing agent in foods, beverages, tobaccos, cigarets, feeds, pet foods, pharmaceuticals, cosmetics and plastics. Transglycosylation with CGTase from *Bacillus macerans* (Suzuki, 1991) suggested that 4.3% of catechin was converted to (+)-catechin 3'-O-α-D-glucopyranoside. This glucoside inhibited the activity of tyrosinase from mushroom which can be applied to prevent discoloring of perishable food or to suppress melanogenesis in animal cells (Funayama et al., 1993). 4^{G}-α-D-glucopyranosyl hesperidin was also synthesized by transglucosylation with CGTase from alkalophilic *Bacillus* sp. The solubility of mono- and diglucoside in water was about 300 times higher than that of hesperidin. The products also had a stabilizing effect on yellow pigment crocin, from fruits of *Gardenia jasminosides*, against ultraviolet irradiation (Kometani *et al*., 1994). 3^{G}-α-D-glucopyranosyl neohesperidin was also synthesized using transglycosylation activity of CGTase to reduce bitterness of neohesperidin (one of the nature flavonoids in lime and lemon juice). This neohesperidin glycoside was approximately 1500 times more soluble in water than neohesperidin and 10 times less bitter (Kometani *et al*., 1996). 2-O-α-glucopyranosyl-L-ascorbic acid (2G-AA) is utilized as a substrate to synthesize 6-O-acyl-2G-AA that is used as components in cosmetics with high stability and activity in vivo and can probable be used as food supplements in the future (Yamoto, *et al*., 2002).

This research thus aims to use recombinant CGTase from *Paenibacillus* sp. BT01, isolated from soil in Thai starch factory in Nakhon Pathom province of Thailand for enzymatic synthesis of potentially useful glycosides or oligosaccharides. Futhermore, optimization of oligosaccharide synthesis for high product yield will also be perfomed in the hope to obtain products with improved properties beneficial for industrial used.

Objectives of this research.

1. To prepare purified recombinant CGTase from *Paenibacillus* sp.BT01
2. To synthesize and determine oligosaccharide or glycoside products
3. To optimize transglycosylation reaction
4. To prepare large scale, isolate and characterise oligosaccharides or glycosides
5. To determine some physical and biological properties of the products

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments.

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan.

Autopipette: Multipette plus, Eppendorf, Germany.

Autopipette: Nichipet EX, Nichiryo, Japan.

Balance: AB204-S, Mettler Toledo, Switzerland.

Balance: PB303-S, Mettler Toledo, Switzerland.

Bench Top Refrigerated Centrifuge: Model HARRIER 18/80, Sanyo Gellenkamp PLC, UK.

Centrifuge, refrigerated: Model J-21C, Beckman Instrument Inc, USA.

Electrophoresis unit: Model Mini-protein II Cell, Bio-Rad, USA.

Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS): micrOTOF, Bruker, Germany.

Evaporator: BUCHI Rotavapor R-200, Switzerland.

Fourier Transform Nuclear Magnetic Resonance (FT-NMR) : INOVA-500, Varian, USA.

Fraction collector: Frac-100, Pharmacia Biotech, Sweden.

High Performance Liquid Chromatography: Model LC-3A Shimadzu, Japan.

High Performance Liquid Chromatography: Model LC-1100 Algilant, USA.

Incubator: Memmert, Germany.

Incubator shaker: Innova 4000, New Brunswick Scientific, USA.

Laminar flow: Model BVT-124, International Sciencetific Supply Co., USA.

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA.

NH_2 (Amino) Column: Shodex Asahipak NH_2 P-50 4E column (4.6x250 mm), Japan.

Peristaltic pump: pump p-1, Pharmacia Biotech, Sweden.

Power supply: Model EC 135-90-LVD CE, EC Apparatus Inc., USA.

pH meter: pH900, Precisa, Switzerland.

Syringe: Holder 13 mm SST Swinney Syringe, Millipore, USA.

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

UV-VIS Spectrophotometer: DU650 Spectrophotometer, Beckman, USA.
Vortex: Model K-550-GE, Scientific Industries, USA.
Water bath: Memmert, Germany.

2.2 Chemicals.

Acetonitrile (HPLC grade): LAB-SCAN Analytical Science, Ireland.
Acrylamide: Merck, USA.
Ampicillin, Sigma, USA.
Agar: Merck, Germany.
Agarose, FMC Bioproduct, USA.
D(+)-Arabinose: Sigma, USA.
Bacto-peptone: Difco Laboratories, USA.
Beef extract: Difco Laoratories, USA.
Bovine serum albumin (BSA): Sigma, USA.
n-Buthanol : Carlo Erba reagent, Germany.
D(+)-Cellobiose: Fluka, Slovakia.
Coomassie Brilliant blue G-250, R-250: Sigma, USA.
α,β and γ-cyclodextrin , Sigma, USA.
DEAE-Celluose resin: DE 32, Whatman Biosystems Ltd., England.
Dialysis tubing: Sigma, USA.
Egg albumin soluble: Difco Laboratories, USA.
Ethylenediamine tetraacetic acid (EDTA): Fluka, Switzerland.
Ethanol:MECK, German.
Ethyl acetate: Scharlau, Spain.
D(-)-Fructose: Sigma, USA.
Glacial acetic acid: BDH, England.
D(+)-Glucose: Sigma, USA.
2-deoxy-glucose: Sigma, USA.
2-deoxy-galactose: Sigma, USA.
D(+)-fucose: Sigma, USA.
Glycerol: Scharlau, Spain.
Glycine: Sigma, USA.

Hydrochloric acid: Merck, Germany.

Iodine, Baker chemical, USA.

Lactose : Sucrose: Bio Basic Inc., Canada.

Lactulose: Fluka, Italy.

Magnesium sulphate 7-hydrate: BDH, England.

Maltose: Sigma, USA.

Maltotriose, Maltotetraose, Maltohexaose, Maltoheptaose: Sigma, USA.

Maltopentaose: Wako, Japan.

D(+)-mannose: Sigma, USA.

Melibiose: Sigma, USA.

Methanol: Merck, Germany.

N, N' –Methylene-bis-acrylamide: Sigma, USA.

Palatinose: Sigma, USA.

Peptone from meat: Merck, USA.

Potassium dihydrogen phosphate: Merck, Germany.

D(+)-Raffinose pentahydrate: Nacalaitesque Inc., Japan.

Rhamnose : Sigma, USA.

Salicin: Sigma, USA.

Sodium acetate: BDH, England.

Sodium chloride: USB, USA.

Di-Sodium hydrogenphosphate: Fluka, Switzerland.

Sodium hydroxide: Carlo Erba, Italy.

Soluble starch, potato: Sigma, USA.

Sorbitol: Sigma, USA.

Sucrose: Bio Basic Inc., Canada.

Sulphuric acid: BDH, England.

TEMED (*N, N, N', N'*-tetramethylene-ethylenediamine): Fluka, Germany.

Tri-Ammonium Citrate: BDH, England.

2, 3, 5 Triphenyltetrazolium chloride (TTC): Kanto Chemical Co Inc., Japan.

Tris (hydroxymethyl)-aminomethane: USB, USA.

Xylitol: Sigma, USA.

Xylose: Sigma, USA.

Yeast extract: Scharlau, Spain

2.3 Bacteria.

Recombinant CGTase was purified from CGTase expressed from pBT transformant. This transformant was *Escherichia coli* BL21(DE3) obtained by cloning CGTase gene from *Paenibacillus* sp. BT01 into pET-17b vector and expressed (Tantanarat, 2006).

2.4 Media Preparation

2.4.1 Luria-Bertani broth (LB medium)

LB-medium consisted of 1.0 % bacto tryptone from meat, 0.5 % yeast extract and 0.5 % NaCl, supplemented with 100 μg/ml ampicillin when needed.

2.4.2 LB-starch agar.

LB-starch agar consisted of 1.0 % bacto tryptone from meat, 0.5% yeast extract and 0.5 % NaCl, 1.5 % agar and 1.0 % soluble starch, supplemented with 100 μg/ml ampicillin when needed.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

The *E.coli* BL21(DE3) transformant colony (pBT) was transferred into LB medium containing 100 μg/ml ampicillin at 37 ℃ with rotary shaking overnight.

2.5.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured at 37°C with shaking. When the turbidity of the culture at 660 nm reached OD. 0.6, IPTG was added to final concentration of 0.2 mM to induce CGTase gene expression and cultivation was continued at 37°C for 24 hours. After

cultivation, bacterial cell mass was removed by centrifugation at 3,500 rpm for minutes at 4°C. Culture broth and bacterial cell mass with crude enzyme were collected.

2.6 Purification of CGTase

CGTase was purified from crude extract from pBT cloned cell (section 2.5.2) by two steps of purification; starch adsorption and DEAE-cellulose column chromatography.

2.6.1 Starch adsorption

Corn starch was oven dried at 120 °C for 30 minutes and cooled to room temperature (Kato and Horikoshi, 1984) according to the starch adsorption method modified by (Kuttiarcheewa 1994), It was then gradually sprinkled into stirring crude CGTase broth to make the concentration of 5 % (w/v) concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 5,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCI containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake with 62.5 ml TB1 buffer containing 0.2 M maltose, by stirring for 10 minutes. The process was repeated once. The combined CGTase eluted was recovered by centrifugation at 5000 rpm for 30 minutes. The solution was dialyzed against water at 4° C with 3 changes of 10 mM Tris-HCI pH 8.0 (TB2).

2.6.2 DEAE-cellulose column chromatography

DEAE-cellulose was activated by washing sequentially with excess volume of 0.5 M NaOH followed by distilled water until pH was about 7.0. The activated cellulose was equilibrated with 10 mM Tris-HCI pH 8.0 (TB2). The prepared DEAE-cellulose was packed into the column (15 X 28 cm) and was equilibrated with TB2. The dialyzed protein solution from starch adsorption was applied to DEAE-cellulose column. Unbound proteins were eluted from the column with the elution buffer; washing was continued until the absorbance at 280 nm of eluant decreased to almost

zero. After the column was washed thoroughly with the TB2 buffer, the bound proteins were eluted from the column with linear salt gradient of 0 to 0.2 M sodium chloride in the same buffer. Fractions of 4.0 ml were continuously collected. The protein and activity profile of the eluted fractions were monitored by measuring the absorbance at 280 nm and assay of dextrinizing activity (Section 2.8.1). Fractions with enzyme activity were pooled for further determination.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was employed for analysis of enzyme purification according to Bollag *et al.*, (1996), using the non-denaturing slab gels. The gels were visualized by coomassie blue staining and dextrinizing acitivity staining.

2.7.1 Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE)

Discontinuous non-denaturing PAGE was performed on 7.5 % (w/v) stacking gels. Tris-glycine buffer pH 8.3 was used as electrode buffer (see Appendix 1). The electrophoresis was run from cathode towards anode at constant current of 16 mA per slab at room temperature in a Mini-Gel electrophoresis unit (Bio-RAD).

2.7.2 Detection of proteins and CGTase

2.7.2.1 Coomassie blue staining

Gels from ND-PAGE 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 at least 2 hours. The slab gels were destained with a solution of 10 % methanol and 10 % acetic acid for 1-2 hours, followed by several changes of destaining solution (10 % methanol, 10 % glacial acetic acid and 50 % distilled water) until gel background was clear.

2.7.2.2 Dextrinizing activity staining

CGTase activity was detected on ND-PAGE using the method slightly modified from Kobayashe *et al.* (1978). The running gel was soaked in 10 ml of substrate solution, containing 0.2%(w/v) potato 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% I_2 in 2 % KI) was added for color development at room temperature. The clear zone on the blue background indicated starch degrading activity of the enzyme.

2.8 Enzyme assay

CGTase activity was determined by assay of starch degrading (dextrinizing) activity and cyclization (CD-forming) activity.

2.8.1 Dextrinizing activity

Dextrinizing activity of CGTase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (10-100 µl) was incubated with 0.3 ml starch substrate (0.2g % soluble potato starch in 0.2 M phosphate buffer, pH 6.0) at 40 °C for 10 minutes. The reaction was stopped with 4 ml of 0.2 M HCI. Then 0.5 ml of iodine reagent (0.02 % I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, HCI 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 minute under the described conditions.

2.8.2 Cyclization activity assay

Cyclization activity was determined by the phenolphthalein method (modified from Goel and Nene, (1995)). Purified CGTase was added to 1.0 ml of 1.0% acceptor and 1.0% donor in 0.2 M acetate buffer, pH 6.0. The reaction mixture was incubated for 30 minutes at 60°C. Reaction was stopped by boiling for 10 minutes. Fifty microliters of the reaction mixture was incubated with 2.0 ml of phenolphthalein solution. Absorption was measured at 550 nm and β-CD-phenolphthalein complex (see Appendix D). One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the corresponding condition.

Phenolphthalein solution was prepared with 1 ml of 4 mM phenolphthalein solution in absolute ethanol, 100 ml of 125 mM Na_2CO_3 solution in distilled water and 4 ml ethanol. Solution was prepared freshly before starting the experiment. β-CD standard 0 to 2.5 mM was prepared.

One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the described condition.

2.9 Protein determination

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard protein (see Appendix 3)**.**

One hundred microlitres of sample was mixed with 1 ml of coomassie blue reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of coomassie blue reagent contained 100 mg coomassie blue G-250, 50 ml of 95 % ethanol, 100 ml of 85% H_3PO_4 and distilled water.

2.10 Transglycosylation of saccharides and detection of products

2.10.1 Acceptor specificity

Purified recombinant CGTase (70 units/ml) in acetate buffer, pH 6.0 was incubated with β-CD as a glucosyl donor and various acceptors : 1% of glucose, mannose, fructose, xylose, rhamnose, arabinose, xylitol, sorbitol, 2-deoxy-glucose, 2-deoxy-galactose, salicin, fucose, maltose, cellobiose, palatinose, sucrose, lactose, lactulose, melibiose, maltose, maltotetraose, raffinose and acarbose. Th reaction mixture was incubated at 60 °C for 24 hours. The products were analyzed by thin – layer chromatography (TLC). The saccharide with best acceptor activity was selected.

2.10.2 Donor specificity

Transglycosylation efficiency of the acceptor from 2.10.1 was investigated by incubation with recombinant CGTase (70 units/ml) in acetate buffer, pH 6.0 and 1% different glucosyl donor :maltohexaose(G6), maltoheptaose (G7), alpha-cyclohexaose (α-CD), glucosyl-alpha- cyclohexaose (G1-α-CD), maltosyl-alpha- cyclohexaose (G2-α-CD), beta-cycloheptaose(β-CD), glucosyl-beta-cycloheptaose (G1-β-CD), maltosyl-beta-cycloheptaose (G2- β-CD), 2-hydroxypropyl-beta-cycloheptaose (OH-β-CD) and gamma-cyclooctaose (γ-CD) at 60 °C for 24 hours. The products were analyzed by high performance liquid chromatography (HPLC).

2.10.3 Detection of products

2.10.3.1 Thin – layer chromatography (TLC)

The products were analyzed by thin – layer chromatography (TLC) using silica gel 60. Solvent system was n-buthanol : ethanol : water (5:5:3, v/v/v). Spots were detected by spraying with concentrated sulfuric acid H_2SO_4: methanol (1:2, v/v) (Funayama *et al.*,1993) followed by heating at 120°C for 20 minutes.

2.10.3.2 High performance liquid chromatography (HPLC)

The products were separated on HPLC using Shodex Asahipak NH_2P-50 4E column (4.6x250 mm) and detected with UV detector at 256 nm. The reaction mixture of glycoside synthesis was filtered through Nylon 0.45 μm disc filter before injection and eluted with acetonitrile: water (75:25, v/v) using a flow rate of 1 ml/min at 40 °C. Product yield was calculated from peak area.

2.12 Determination of transglycosylation efficiency

Efficiency was judged by transglycosylation yield, which could be determined from the product yield directly. The yield of transglycosylated products was calculated from the ratio of peak area of product to the initial peak area of acceptor in HPLC profile, using the equation:

$$\text{Product yield (\%)} = \frac{\text{Peak area of product}}{\text{Peak area of acceptor at } t_o} \times 100$$

2.13 Optimization of transglycosylation reaction

2.13.1 Effect of acceptor concentration

The reaction was performed by incubation of 70 unit/ml of recombinant CGTase with various acceptor (salicin) concentrations (0.0, 0.10, 0.125, 0.25, 0.50, 1.0, 2.0 and 3.0%, w/v) and 1% (w/v) β-CD in the 0.20 M acetate pH 6.0 at 60 °C for 24 hours. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC. The optimum salicin concentration was judged from the determination of peak area of products.

2.13.2 Effect of donor concentration

The reaction was performed by incubation of 70 unit/ml of recombinant CGTase with various donor (β-CD) concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.8

and 2.0%, w/v) and optimum salicin concentration (3%, w/v) in the 0.2 M acetate pH 6.0 at 60 ºC for 24 hours. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC. The optimum donor concentration was judged from the determination of peak area of products.

2.13.3 Effect of enzyme concentration

The reaction was performed by incubation of optimum concentration of salicin acceptor and optimum concentration of β-CD donor at various enzyme concentrations (0, 10, 20, 40, 50, 70, 100, 150 and 200 unit/ml) in the 0.2 M acetate pH 6.0 at 60 °C for 24 hours. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC. The optimum enzyme concentration was judged from the determination of peak area of products.

2.13.4 Effect of pH

The reaction was performed by incubation of appropriate concentration of recombinant CGTase with optimum concentration of salicin acceptor and β-CD donor in 0.2 M buffer in the pH range of 4.0 to 10.0 at 60 ° C for 24 hours. The buffers used were sodium acetate (pH 4.0 to 6.0) , potassium phosphate (pH 6.0 to 8.0) and Tris-HCl (pH 8.0 to 10.0) (see Appendix 2). The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC. The optimum pH was judged from the determination of peak area of products.

2.13.5 Effect of temperature

The reaction was performed by incubation of appropriate concentration of recombinant CGTase with optimum concentration of salicin acceptor and β-CD donor at optimum pH at various temperatures (30, 40, 50, 60 and 70° C) for 24 hours. The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC. The optimum temperature was judged from the determination of peak area of products.

2.13.6 Effect of incubation time

The reaction was performed by incubation of appropriate concentration of recombinant CGTase with optimum concentration of salicin acceptor and β-CD donor at optimum pH and temperature for various times (0, 0.5, 3, 6, 12, 18, 24, and 36 hours.) The reaction was stopped by boiling for 10 minutes, and then analyzed by HPLC. The optimum incubation time was judged from the determination of peak area of products.

2.14 Large scale preparation and isolation of salicin glycoside products

In the initial experiments : to investigate salicin glycoside products, optimization of the transglycosylation reaction and determine transglycosylation efficiency, small scale reaction mixture of 1.0 ml was used. To prepare higher amount of products for characterization, larger scale preparation (20 ml) of reaction mixture using optimum condition for transglycosylation as obtained from section 2.13 was performed. After the reaction was stopped, the reaction mixture of each transfer product was fractionated and purified by HPLC. The salicin glycoside products were separated from sugars and enzyme by elution of the column with 75:25 acetonenitrile: water (v/v) (1ml/min, fraction size 0.5 ml). The fractions containing products were collected and re-chromatographed on HPLC as described in section 2.10.3.2. The purified product peak was collected for further characterization.

2.15 Charaterization of glycoside products

2.15.1 Mass Spectrometry

Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was recorded on a microTOF at the Service Unit of the National Center for Genetic Engineering and Biotechnology. The mixture of methanol : H_2O (1:1 w/v) was used as solvent of sample for analysys.

2.15.2 Nuclear Magnetic Resonance

For structural elucidation, ^{1}H and ^{13}C NMR spectrum were determined using Varian Gemini 400 MHz spectrometer at the department of Chemistry, Chulalongkorn University. The operation was at 400 MHz for protons and 100 MHz for carbons. The chemical shifts were expressed in ppm downfield from the signal of tetramethylsilane (TMS), which was used as internal standard.

2.16 Determination of properties of salicin glycoside products

2.16.1 Water solubility analysis

Excess salicin and salicin glycosides were mixed with 200 μl of water in an eppendorf tubes at room temperature. After 20 min of mixing with vortex mixer at 20°C, each of the samples was diluted and filtered through a 0.45 μm membrane for HPLC analysis, in order to determine the concentrations.

2.16.2 Anticoagulant activity

Blood was drawn from healthy rabbit. Salicin and salicin glycoside products 1,2 and 3 were separately dissolved in PBS buffer (pH 7.4) to make 10% solution. Then 1.4 ml blood was mixed with 0.1 ml (10%, w/v) of each solution and incubated from 10 to 30 min. The time needed for clotting was recorded after the addition of activator. If there was still no clotting observed after 30 min, the sample was defined as no blood clot formation (NC). Heparin was used as a positive control.

CHAPTER III

RESULTS

3.1 Purification of recombinant CGTase

Crude enzyme from pBT transformant (section 2.5) was purified by starch adsorption followed by DEAE-cellulose column chromatography.

3.1.1 Preparation of crude enzyme

After cultivation of *E. coli* BL21(DE3) transformant (pBT) in enzyme production medium, the cells were removed by centrifugation at 3,500 rpm for 15 minutes at 4 °C. The crude enzyme in the supernatant fraction was collected. Crude enzyme solution contained 62 mg protein and 1.22 x 10^5 units of dextrinizing activity.

3.1.2 Enzyme purification steps

3.1.2.1 Starch adsorption

Crude enzyme from pBT prepared in section 3.1.1 was subjected to starch adsorption as described in section 2.6.1. The recovered proteins and enzyme activity were 7.0 mg protein and 6.0 x 10^4 units of dextrinizing activity, respectively. Thus, the specific activity of the enzyme from this step was 8.6 x 10^3 unit/mg protein. The enzyme was purified 4 fold with 49 % yield.

3.1.2.2 DEAE-cellulose column chromatography

The enzyme from starch adsorption was dialyzed to get rid of maltose and loaded to DEAE-cellose column (section 2.6.2). The chromatographic profiles was shown in Figure 1. Unbound proteins were eluted from the column by the elution buffer. The bound proteins were eluted by linear salt gradient of 0 to 0.2 M sodium chloride in the same buffer. The enzyme were eluted at approximately 0.1 M. The fractions with dextrinizing activity were pooled, dialyzed against distilled water. This step yielded 4.5 mg protein and 4.5 x 10^4 units of dextrinizing activity, respectively.

The specific activity of the enzyme was 1.0 x 10^4 unit/mg protein. The enzyme was purified 5 fold with 37 % yield. The enzyme from this step was kept at 4°C for future experiments. Table 7 summarized the results of overall purification process.

3.1.3 Determination of enzyme purity

Purified CGTase from recombinant pBT was electrophoresed on 7.5 % non-denaturing polyacrylamide gel electrophoresis and stained for both protein and dextrinizing activity as described in section 2.7. Lanes 1 to 3 in figure 7 showed protein staining of sample from each step of purification of recombinant CGTase. The protein band from DEAE-cellulose column in lane 3 indicated that the enzyme was highly purified. The dextrinizing activity stains in lanes 4-6 of figure 7 showed typical characteristics of CGTase activity, 2-3 bands on iodine staining. Both gels confirmed that the protein band corresponded to recombinant CGTase activity.

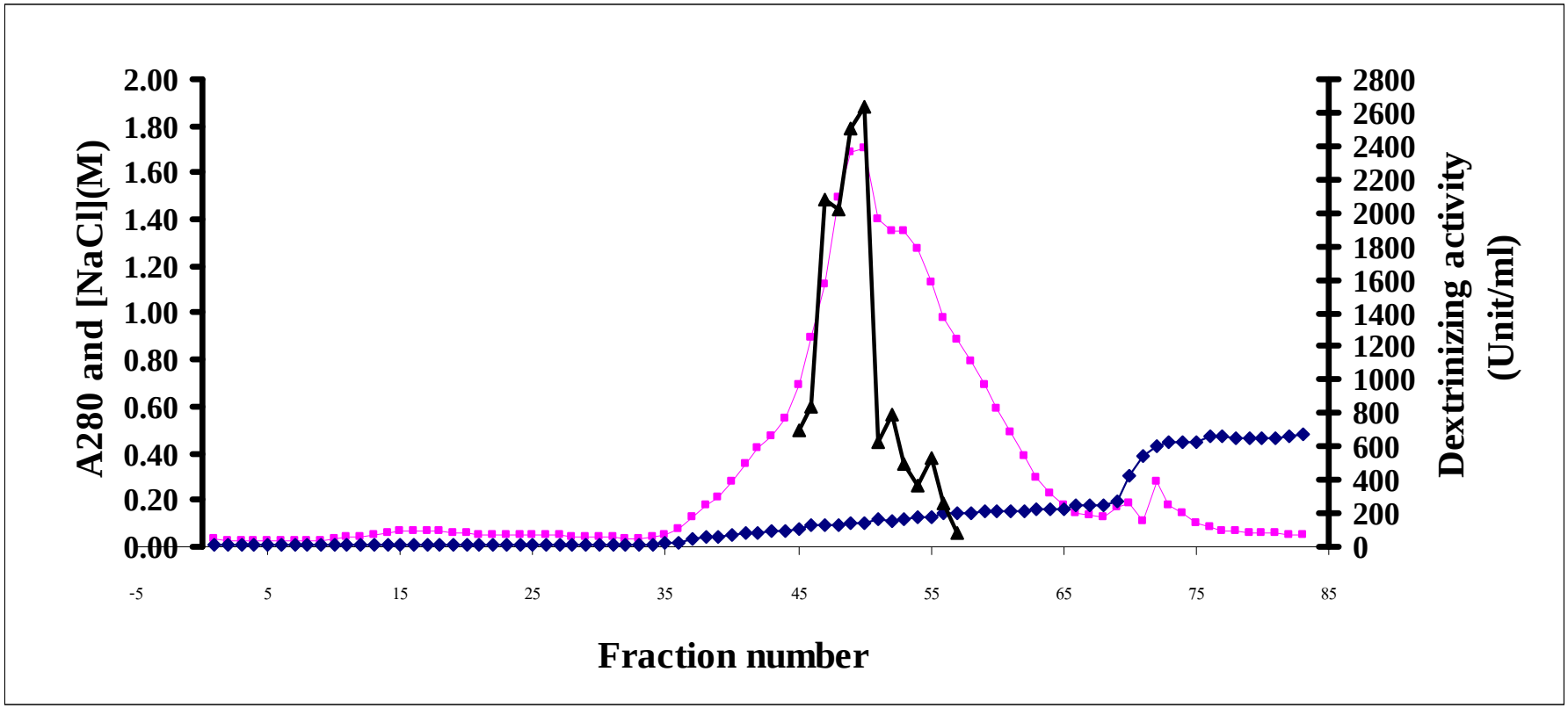


Figure 6 DEAE-cellulose column profile of CGTase from recombinant pBT separation at pH 8.0. Column, elution was by 0-0.2 M NaCl in TB 2 buffer. Fractions of 6 ml were collected.

A280 Dextrinizing activity Conductivity

Table 7 Purification of CGTase from pBT transformant

Fraction	**Total volumn (ml)**	**Activity (U/ml)**	**Total activity (U)**	**Protein (mg/ml)**	**Total protein (mg)**	**Specific activity (U/mg protein)**	**Purification fold**	**yield (%)**
Crude CGTase	567	216	12.3×10^4	0.11	62	1.96×10^3	1	100
5% strach adsorbtion	50	1208	6.0×10^4	0.14	7.0	8.63×10^3	4	49
DEAE-cellulose	45	1006	4.5×10^4	0.10	4.5	10.10×10^3	5	37

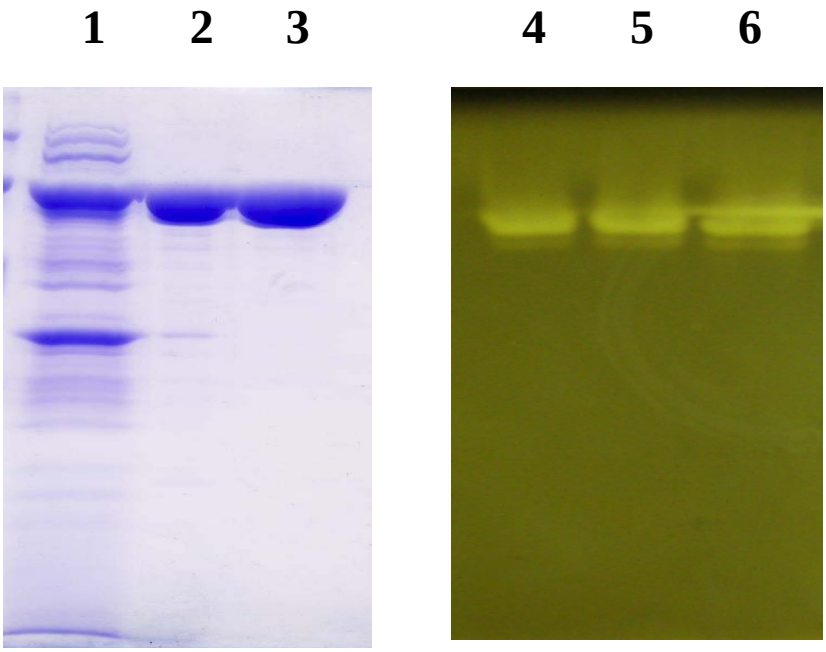


Figure 7 Non-denaturing PAGE (7.5%gel) of recombinant CGTase from different step of purification

Coomassie blue staining

Lane 1 Crude enzyme (100 µg proein)
Lane 2 Concentrated starch adsorbed enzyme (50 µg proein)
Lane 3 Concentrated DEAE cellulose column (20 µg proein)

Dextrinizing activity staining

Lane 4 Crude enzyme
Lane 5 Starch adsorbed enzyme
Lane 6 DEAE cellulose column

0.2 Units of dextrinizing activity were loaded to each well

3.2 Transglycosylation products catalyzed by the purified recombinant CGTase

3.2.1 Acceptor specificity

Various saccharides were used to investigate transglycosylation reaction of recombinant CGTase with β-CD as donor. The products were analyzed by TLC and residual amounts of β-CD were determined by measuring the decrease in β-CD. TLC chromatograms (figures 8 to 11) showed that xylose, glucose, salicin, cellobiose, palatinose, maltose, D-arabinose, fucose, rhamnose, 2-deoxy-glucose, sucrose, maltotriose and maltotetraose yielded prominent products with utilization of β-CD at 4.21% (xylose), 12.18% (glucose), 21.79% (salicin), 23.89% (cellobiose), 25.89% (palatinose) and 50.82% (maltose) (figure 12 and table 8). This result suggested that recombinant CGTase can use these saccharides as acceptors. The other saccharides: mannose, fructose, arabinose, xylitol, sorbital, 2-deoxy-galactose, fucose, melibiose, lactose, lactulose, laffinose and acarbose, yielded little or no products. Salicin was one of the best acceptors judging from clearly distinct and resolvable spots, suggesting these may be products with different structures from others. Thus, we decided to further investigate the transglycosylation reaction by recombinant CGTase from *Paenibacillus* sp. BT01, using salicin as acceptor.

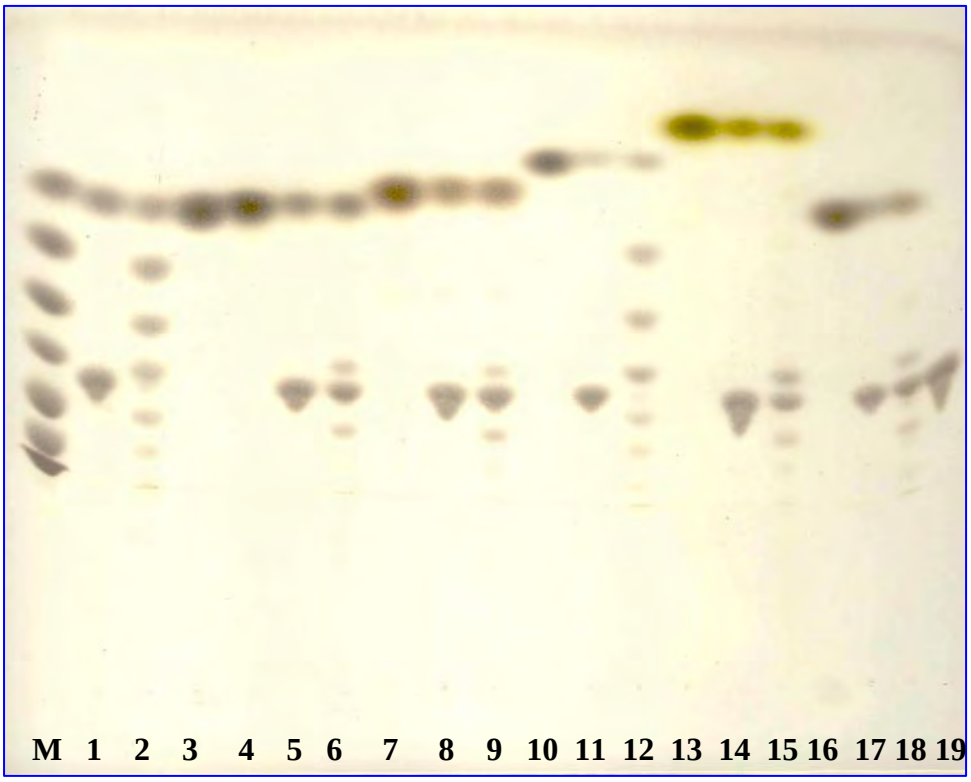


Figure 8 TLC chromagram of reaction products of recombinant CGTase incubated with β - CD and various acceptors.

(M)	standard maltooligosaccharides (G1-G7).
(1)- (2)	glucose+β-CD+ recombinant CGTase 0 and 24 hour.
(3)	standard glucose.
(4)	standard fructose.
(5)- (6)	fructose+β-CD+ recombinant CGTase 0 and 24 hour.
(7)	standard mannose.
(8)-(9)	mannose+β-CD+ recombinant CGTase 0 and 24 hour.
(10)	standard xylose.
(11)- (12)	xylose+β-CD+ recombinant CGTase 0 and 24 hour.
(13)	standard rhamnose.
(14)- (15)	rhamnose+β-CD+recombinant CGTase 0 and 24 hour.
(16)	standard D-arabinose.
(17)- (18)	arabinose+β-CD+recombinant CGTase 0 and 24 hour.
(19)	standard β-CD

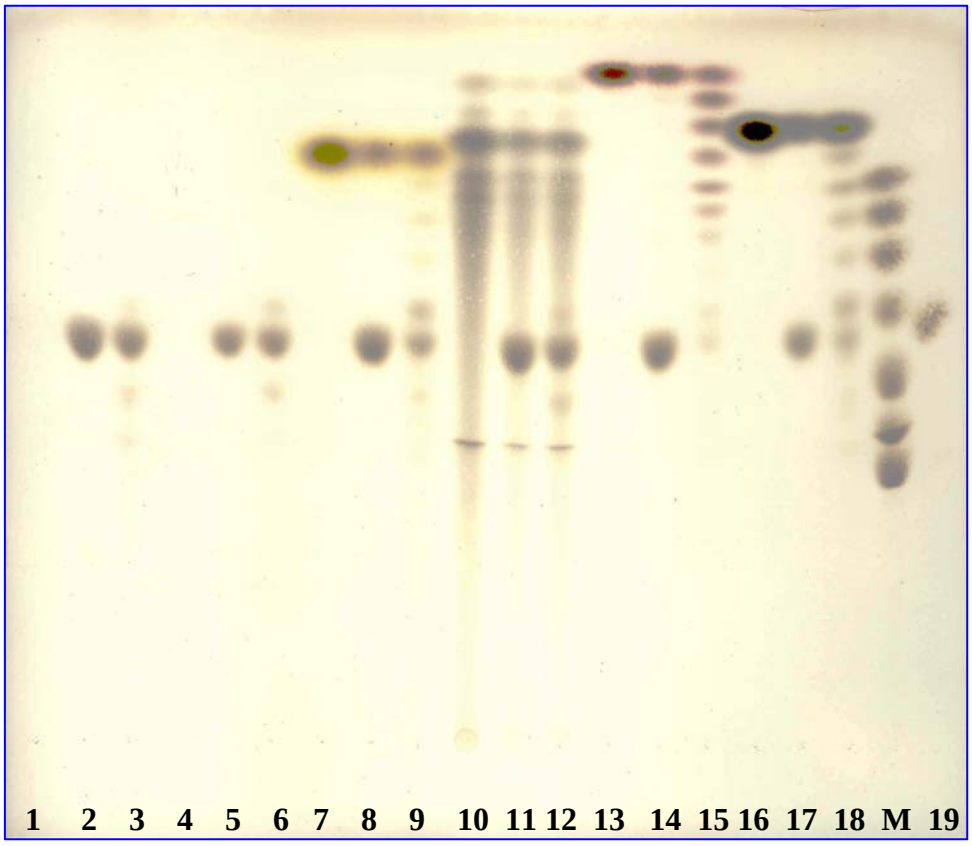


Figure 9 TLC chromagram of reaction products of recombinant CGTase incubated with β-CD and various acceptors.

(1) standard xylitol

(2)-(3) xylitol+β-CD+ recombinant CGTase 0 and 24 hour.

(4) standard sorbitol

(5)-(6) sorbitol+β-CD+ recombinant CGTase 0 and 24 hour.

(7) standard fucose

(8)-(9) fucose+β-CD+ recombinant CGTase 0 and 24 hour.

(10) standard 2-deoxy-galactose

(11)-(12) 2-deoxy-galactose+β-CD+recombinant CGTase 0 and 24 hour.

(13) standard salicin

(14)-(15) salicin+β-CD+ recombinant CGTase 0 and 24 hour.

(16) standard 2-deoxy-glucose

(17)-(18) 2-deoxy-glucose+β-CD+ recombinant CGTase 0 and 24 hour.

(M) maltooligosaccharide (G1-G7)

(19) standard β-CD

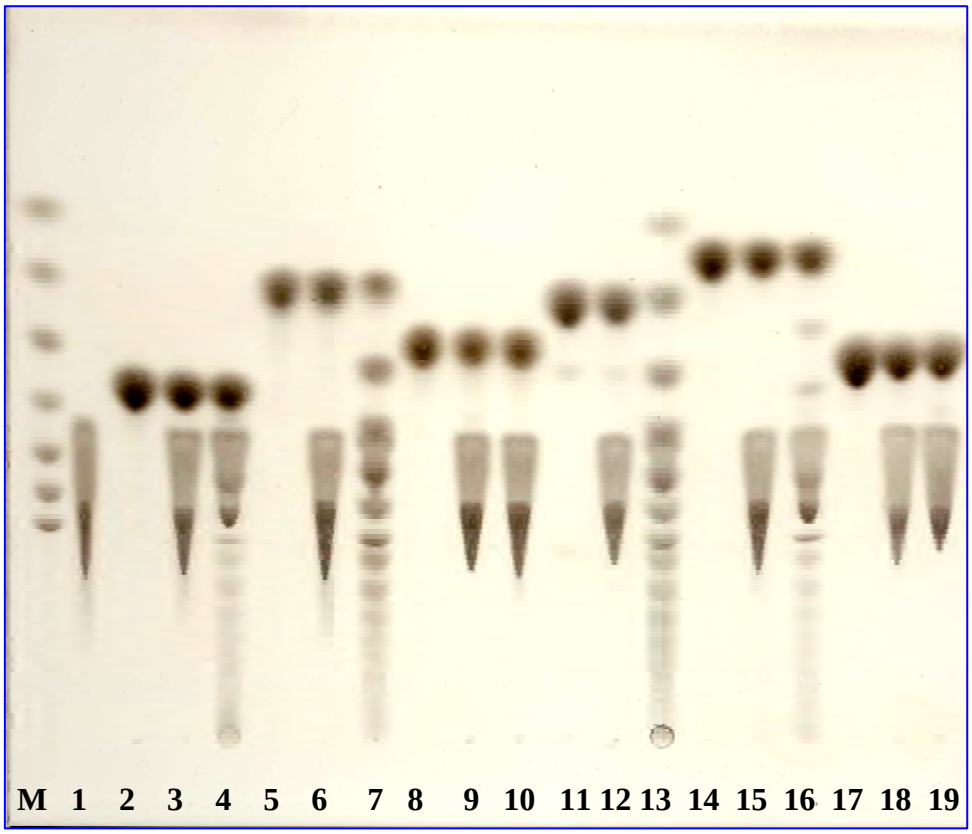


Figure 10 TLC chromagram of reaction products of recombinant CGTase incubated with β-CD and various acceptors.

(M)	maltooligosaccharide (G1-G7).
(1)	standard β-CD.
(2)	standard melibiose.
(3)-(4)	melibiose +β-CD +recombinant CGTase 0 and 24 hours.
(5)	standard palatinose.
(6)-(7)	palatinose + β-CD+ recombinant CGTase 0 and 24 hours.
(8)	standard lactulose.
(9)-(10)	lactulose + β-CD+ recombinant CGTase 0 and 24 hours.
(11)	standard maltose.
(12)-(13)	maltose + β-CD+recombinant CGTase 0 and 24 hours.
(14)	standard sucrose.
(15)-(16)	sucrose + β-CD+ recombinant CGTase 0 and 24 hours.
(17)	standard lactose.
(18)-(19)	lactose + β-CD+recombinant CGTase 0 and 24 hours.

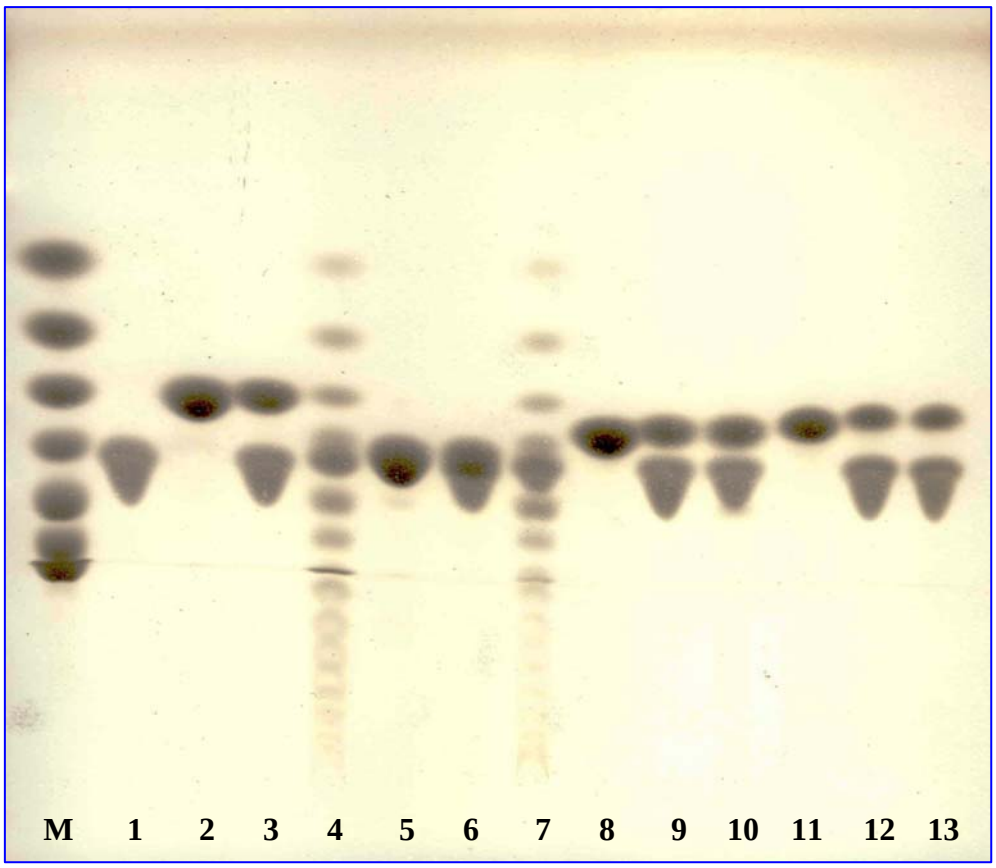


Figure 11 TLC chromagram of reaction products of recombinant CGTase incubated with β - CD and various acceptors.

(M)	maltooligosaccharide (G1-G7)
(1)	standard β-CD
(2)	standard G3
(3)-(4)	G3+β-CD+ recombinant CGTase 0 and 24 hour.
(5)	standard G4
(6)-(7)	G4+β-CD+ recombinant CGTase 0 and 24 hour.
(8)	standard raffinose
(9)-(10)	raffinose+β-CD+ recombinant CGTase 0 and 24 hour.
(11)	standard acarbose
(12)-(13)	acarbose+β-CD+ recombinant CGTase 0 and 24 hour.

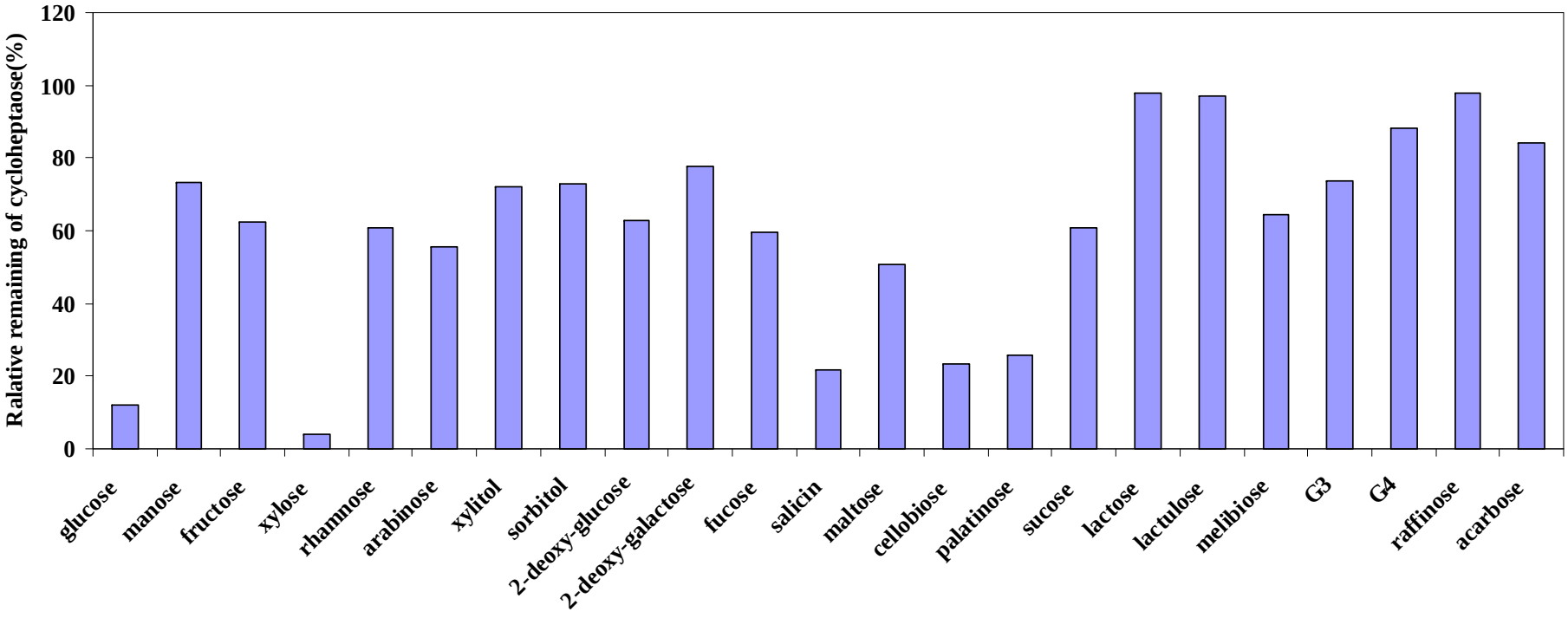


Figure 12 Remaining of β-CD in transglycosylation reaction of the recombinant CGTase using 1% β-CD as a glycosyl donor with different types of acceptors.

Table 8 Effect of substrates (acceptors) on the percentage remaining of β-CD in transglycosylation reaction .

Substrate(acceptors)	product	Percentage remaining of β-CD (%)
Glucose	+++	12.18
Mannose	+	73.29
Fructose	+	62.27
Xylose	+++	4.21
Rhamnose	++	60.77
Arabinose	+	55.49
Xylitol	+	72.22
Sorbitol	+	72.90
2-deoxy-glucose	+++	62.81
2-deoxy-galactose	+	77.55
Fucose	+	59.49
Salicin	+++	21.79
Maltose	+++	50.82
Cellobiose	+++	23.89
Palatinose	+++	25.89
Sucrose	+++	63.81
Lactose	-	97.85
Lactulose	-	97.12
Melibiose	+	64.29
Maltotriose	+++	73.69
Maltotetraose	+++	88.30
Raffinose	-	97.90
Acarbose	-	84.16

\+ = amount of the products
\- = no the products

3.2.2 Donor specificity

In order to determine the potency of different glycosyl donor for salicin, 3% salicin was used as acceptor with 1% maltohexaose (G6), maltoheptaose (G7), alpha-cyclohexaose (α-CD), glucosyl-alpha-cyclohexaose(G1-α-CD),maltosyl-alpha-cyclohexaose(G2-α-CD), beta-cycloheptaose β-CD), glucosyl-beta-cycloheptaose (G1- β-CD), maltosyl-beta- cycloheptaose(G2-β-CD), 2-hydroxypropyl-beta-cycloheptaose (OH-β-CD) and gamma-cyclooctaose (γ-CD) as donors in transglycosylation reaction with recombinant CGTase (70 units/ml) in acetate buffer, pH 6.0 at 60 °C for 24 hours. The products were analyzed by HPLC using the conditions described in section 2.10.2. The transglycosylation yields were determined from the ratio of peak area of products to the initial peak area of acceptor. The transglycosylation yields of G6, G7, α-CD, G1-α-CD,G2-α-CD, β-CD, G1-β-CD, G2-β-CD ,OH-β-CD and γ-CD were 42.77, 43.10, 44.92, 23.31, 20.73, 45.42, 33.54, 27.15, 0.00 and 44.26% respectively using salicin as acceptor (table 9 and figure 13). The transglycosylation yield of G6, G7, α-CD, β-CD and γ-CD were significantly higher than G1-α-CD, G2-α-CD, G1-β-CD and G2-β-CD whereas no product was detected for OH-β-CD. From this experiment, it can be concluded that β-CD was the donor for transglycosylation reaction with salicin.

Table 9 Yields of transglycosylated products expressed as peak areas and product yield (%) at different donors

Donor	Product yields								
	Peak area x 10^4				Yield (%)				Ratio of product 1:2:3
	Product 1	Product 2	Product 3	Total peak area x 10^3	Product 1	Product 2	Product 3	Total	
	Rt 6.1	Rt 9.4	Rt 15.7		Rt 6.1	Rt 9.4	Rt 15.7		
G6	0.86	0.31	0.13	13.00	28.29	10.20	4.28	42.77	1.00:0.36:0.15
G7	0.86	0.32	0.13	13.10	28.29	10.53	4.28	43.10	1.00:0.37:0.15
α -CD	0.79	0.39	0.19	13.70	25.90	12.79	6.23	44.92	1.00:0.49:0.24
G1- α -CD	0.54	0.13	0.02	6.90	18.24	4.39	0.68	23.31	1.00:0.24:0.04
G2- α -CD	0.5	0.13	0.00	6.30	16.45	4.28	0.00	20.73	1.00:0.26:0.00
β -CD	0.78	0.38	0.18	13.40	26.44	12.88	6.1	45.42	1.00:0.49:0.23
G1- β -CD	0.58	0.17	0.04	7.90	19.33	12.88	1.33	33.54	1.00:0.29:0.07
G2- β -CD	0.61	0.17	0.04	8.20	20.2	5.63	1.32	27.15	1.00:0.28:0.07
OH- β -CD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00:0.00:0.00
γ -CD	0.79	0.38	0.18	13.50	25.9	12.46	5.9	44.26	1.00:0.48:0.23

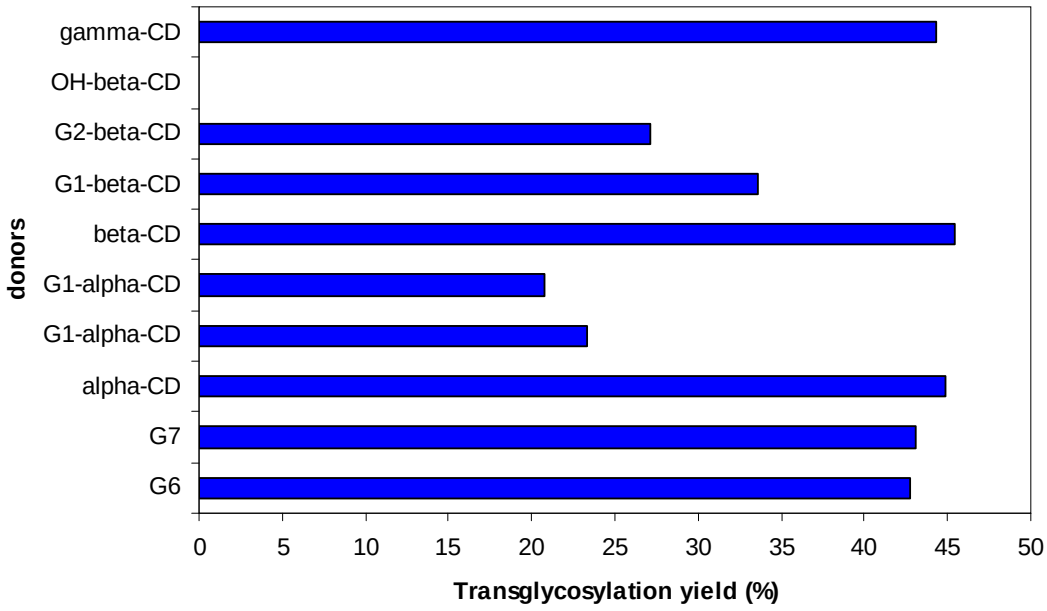


Figure 13 Transglycosylation yield of recombinant CGTase when using salicin as an acceptor with different types of donor

3.3 Optimization of transglycosylation reaction

The suitable condition for the production of salicin glycosides were determined using β-CD as a glucosyl donor. The reaction was performed as described in section 2.10 and reaction products were analyzed by HPLC. During optimization, the yield of products were determined from peak areas of product at different conditions. After that, the optimum condition was judged from the maximum percent yield of transglycosylated product, calculated from ratio of peak area of product to that of initial concentration of salicin (see section 2.12).

3.3.1 Effect of concentration of salicin acceptor

When incubating 70 units/ml of recombinant CGTase with 1% (w/v) β-CD donor and various concentration of salicin 0-3.0 % (w/v) in 0.2 M acetate buffer pH 6.0 at 60 °C for 24 hours (section 2.13.1), it was found that number of products and product yields varied with salicin concentration (table 10 and figure 14). The yield of product 1 at retention time 6.1 minutes was increased when increased salicin concentration from 0 to 3.0 % (w/v), and yield of the other two products were decreased when increases salicin concentration from 2.0 to 3.0 % (w/v). The maximum total yield was obtained around 61.85 to 68.32 %, when the reaction mixture was incubated with salicin concentration in the range of 1.0 to 3.0 % (w/v). In contrast, the yield of products 2 and 3 were significantly decreased when salicin concentration was increased. The 3% (w/v) salicin concentration gave the maximum yield of the major product 1 (retention time 6.1 minute). Thus, it was chosen to be the optimum concentration.

3.3.2 Effect of β-CD concentrations

Using the condition as mentioned in 3.3.1, 3% (w/v) salicin were incubated with various β-CD concentrations. The yield of each product was shown in table 11 and figure 15. In the range 0.1 to 0.3 % (w/v) of β-CD , two products at retention time 6.1 minutes (product 1) and retention time 9.5 minutes (product 2) were found, and yield of these two products increased with increased β-CD concentration. The yield

of product 3 at retention time 15.7 minutes was observed when β-CD concentration in the range 0.40 to 2.0 % (w/v). Maximum total product yield was obtained at β-CD concentration in the range of 1.8 to 2.0 % (w/v). In order to save the used of β-CD, 1.8 % (w/v) β-CD was chosen to be the optimum concentration.

Table 10 Yields of transglycosylated products expressed as peak areas and product yield (%) at different salicin concentrations

Salicin concentrations (%,w/v)	Product yields								
	Peak area x 10^4				Yield (%)				Ratio of products 1:2:3
	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00:0.00:0.00
0.10	0.08	0.04	0.02	0.14	29.63	14.81	7.41	51.85	1.00:0.50:0.25
0.125	0.11	0.06	0.03	0.20	28.95	15.79	7.90	52.64	1.00:0.55:0.27
0.25	0.21	0.10	0.06	0.37	28.00	16.00	8.00	52.00	1.00:0.57:0.29
0.50	0.44	0.23	0.13	0.80	28.21	14.74	8.33	51.28	1.00:0.52:0.30
1.00	0.87	0.44	0.23	1.54	34.94	17.67	9.24	61.85	1.00:0.51:0.26
2.00	1.57	0.69	0.30	2.56	38.01	16.71	7.26	61.98	1.00:0.44:0.19
3.00	2.33	0.83	0.29	3.45	46.14	16.44	5.74	68.32	1.00:0.36:0.12

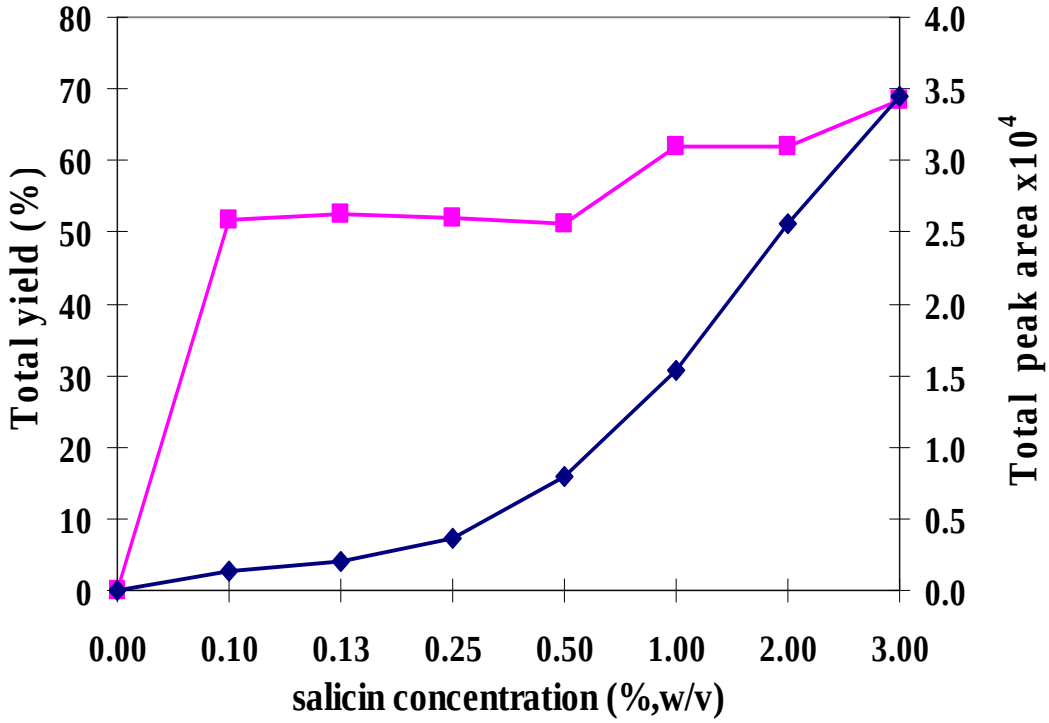


Figure 14 Effect of salicin concentration on transglycosylation yield

Total yield (%)

Total peak area x 10^4

Table 11 Yields of transglycosylated products expressed as peak areas and product yield (%) at different β-CD concentrations

β -CD concentrations (%,w/v)	Product yields								
	Peak area x 10^4				Yield (%)				Ratio of product 1:2:3
	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00:0.00:0.00
0.10	0.31	0.06	-	0.37	5.37	1.11	-	6.84	1.00:0.20:0.00
0.20	0.74	0.10	-	0.84	13.48	1.82	-	15.30	1.00:0.14:0.00
0.30	1.10	0.14	-	1.24	19.71	2.51	-	22.22	1.00:0.13:0.00
0.40	1.39	0.21	0.02	1.62	24.91	3.76	0.38	29.05	1.00:0.15:0.02
0.50	1.60	0.29	0.05	1.94	28.47	5.16	0.89	34.52	1.00:0.18:0.03
1.00	2.19	0.67	0.20	3.06	39.04	11.94	3.57	54.55	1.00:0.31:0.09
1.80	2.62	1.11	0.46	4.19	47.12	19.96	8.27	75.35	1.00:0.42:0.18
2.00	2.65	1.19	0.53	4.37	47.66	21.40	9.53	78.59	1.00:0.45:0.20

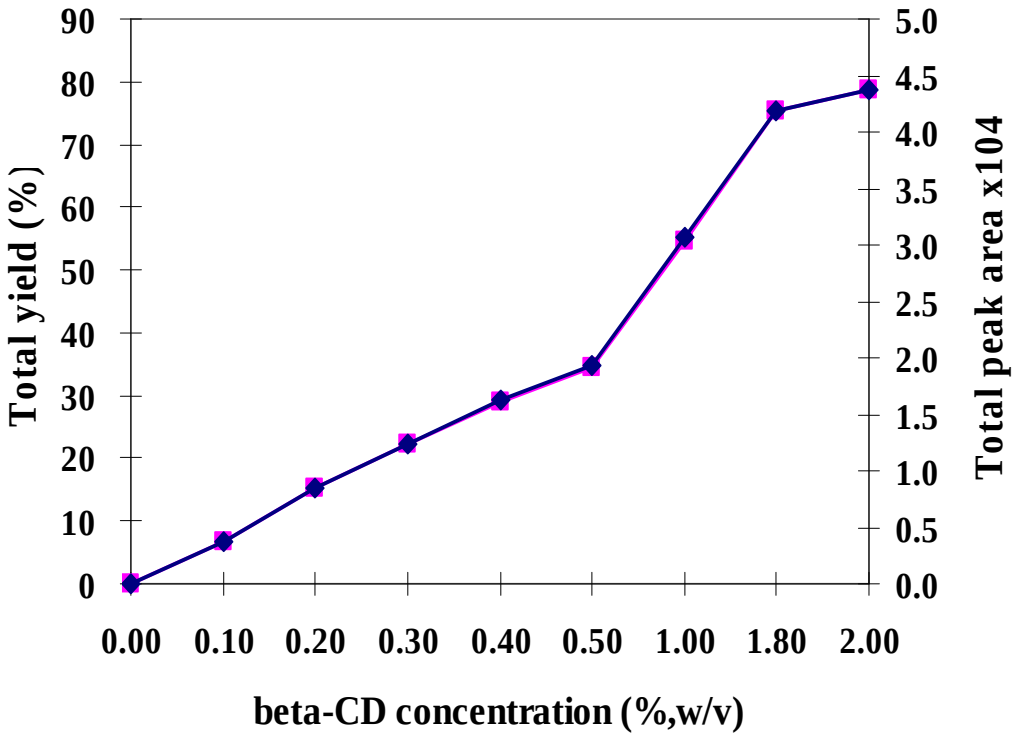


Figure 15 Effect of β-CD concentration on transglycosylation yield

Total yield (%)

Total peak area x 10^4

3.3.3 Effect of enzyme concentration

Using the condition as mentioned in section 3.3.2, 3% (w/v) salicin and 1.8 % (w/v) β-CD were incubated with various concentrations of recombinant CGTase. It was found that the yields of products significantly increased at high concentration of recombinant CGTase. The yield of each transglycosylated products and total product were shown in table 12 and figure 16. The maximum yield of 83.35% was obtained when incubated with 100 unit/ml of enzyme while the raio of products were not much different at any enzyme concentration. The yield of each product was also maximized at this recombinant CGTase concentration. Further increase in enzyme concentration did not result in increase in product yield. Yields significantly increased in the enzyme concentration range 40 to 100 unit/ml. The amount of products observed were in decreasing order of products 1,2 and 3 respectively. Thus, 100 unit/ml recombinant CGTase was chosen to be optimum concentration.

3.3.4 Effect of pH

Using the condition as mentioned in section 3.3.3, the reactions were performed at various pHs. It was found that product yields varied with pH. The yield of transglycosylated products were shown in table 13 and figure 17, the maximum yield of product 1,2 and 3 were obtained when the reaction mixture was performed in acetate buffer pH 6.0. It was also observed that the yields of products 1, 2 and 3 were also maximum at pH 6.0 to pH 7 (phosphate buffer). However, the percent yields or ratio of products at pH 6.0 (acetate buffer) or phosphate buffer pH 6-7 were not significantly different. Acetate buffer at pH 6.0 was chosen to be the optimum pH.

Table 12 Yields of transglycosylated products expressed as peak areas and product yield (%) at different recombinant CGTase concentrations

CGTase concentrations (%,w/v)	Product yields								
	Peak area x 10^4				Yield (%)				Ratio of product 1:2:3
	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00:0.00:0.00
10	2.01	1.03	0.52	3.56	36.28	18.59	9.39	64.26	1.00:0.52:0.26
20	2.06	1.08	0.56	3.70	33.48	20.97	10.87	65.32	1.00:0.62:0.32
40	2.12	1.10	0.57	3.79	40.46	21.00	10.88	72.34	1.00:0.52:0.27
50	2.23	1.11	0.54	3.88	42.23	20.83	10.23	73.29	1.00:0.49:0.24
70	2.21	1.11	0.54	3.86	41.70	20.94	10.19	72.83	1.00:0.50:0.24
100	2.21	1.11	0.55	3.87	46.19	24.18	11.98	83.35	1.00:0.52:0.26
150	1.97	0.94	0.45	3.36	40.97	18.25	8.73	67.95	1.00:0.45:0.21
200	2.11	1.06	0.52	3.69	40.97	20.58	10.10	71.65	1.00:0.50:0.25

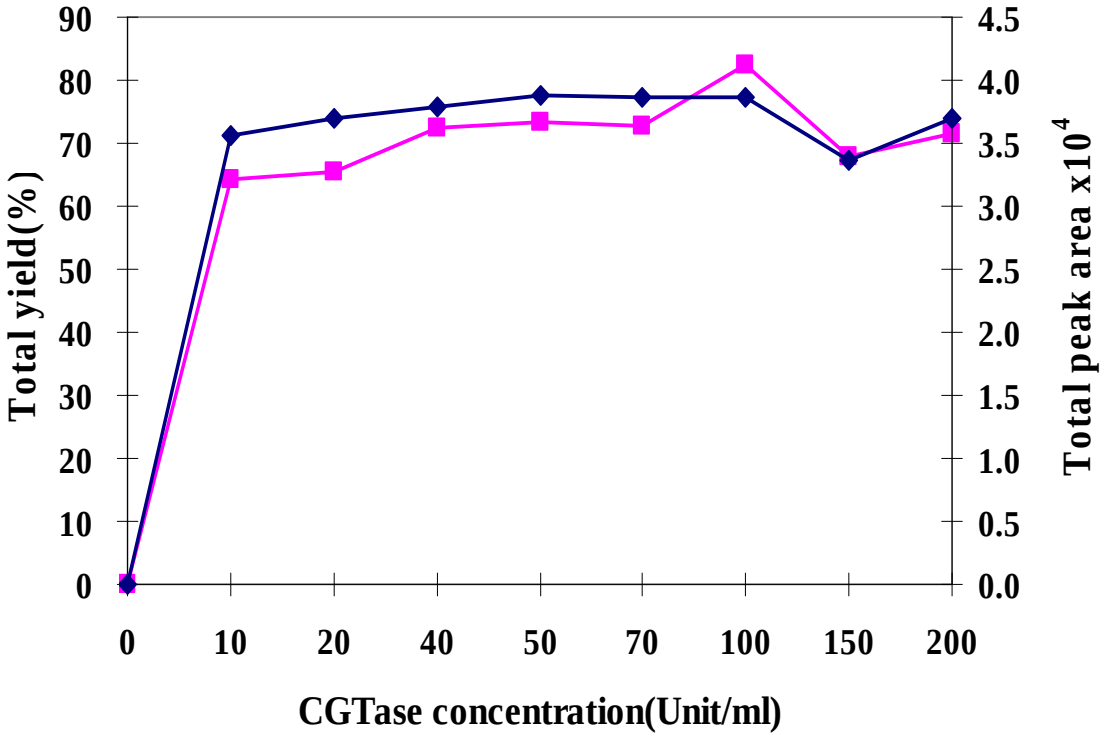


Figure 16 Effect of recombinant CGTase concentration on transglycosylation yield

Total yield (%)

Total peak area x 10^4

Table 13 Yields of transglycosylated products expressed as peak areas and product yield (%) at different pH

Buffer	pH	Product yields								
		Peak area x 10^4				Yield (%)				Ratio of product 1:2:3
		Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	
Acetate	4.0	0.04	-	-	0.04	0.76	-	-	0.76	1.00:0.00:0.00
	5.0	2.37	0.83	0.52	3.72	40.35	16.15	10.18	66.68	1.00:0.40:0.25
	6.0	2.59	1.22	0.57	4.38	49.95	23.63	10.89	84.47	1.00:0.47:0.22
Phosphate	6.0	2.62	1.27	0.61	4.50	49.93	24.19	11.53	85.65	1.00:0.48:0.23
	7.0	2.57	1.26	0.61	4.44	48.77	23.86	11.47	84.10	1.00:0.49:0.24
	8.0	2.46	1.22	0.60	4.28	47.46	23.56	11.57	82.59	1.00:0.50:0.24
Tris-HCl	8.0	2.55	1.22	0.56	4.33	47.81	22.78	10.58	81.17	1.00:0.48:0.22
	9.0	2.72	1.23	0.58	4.53	42.60	19.86	11.99	74.45	1.00:0.47:0.28
	10.0	2.55	1.22	0.57	4.34	37.03	17.73	12.80	67.56	1.00:0.48:0.35

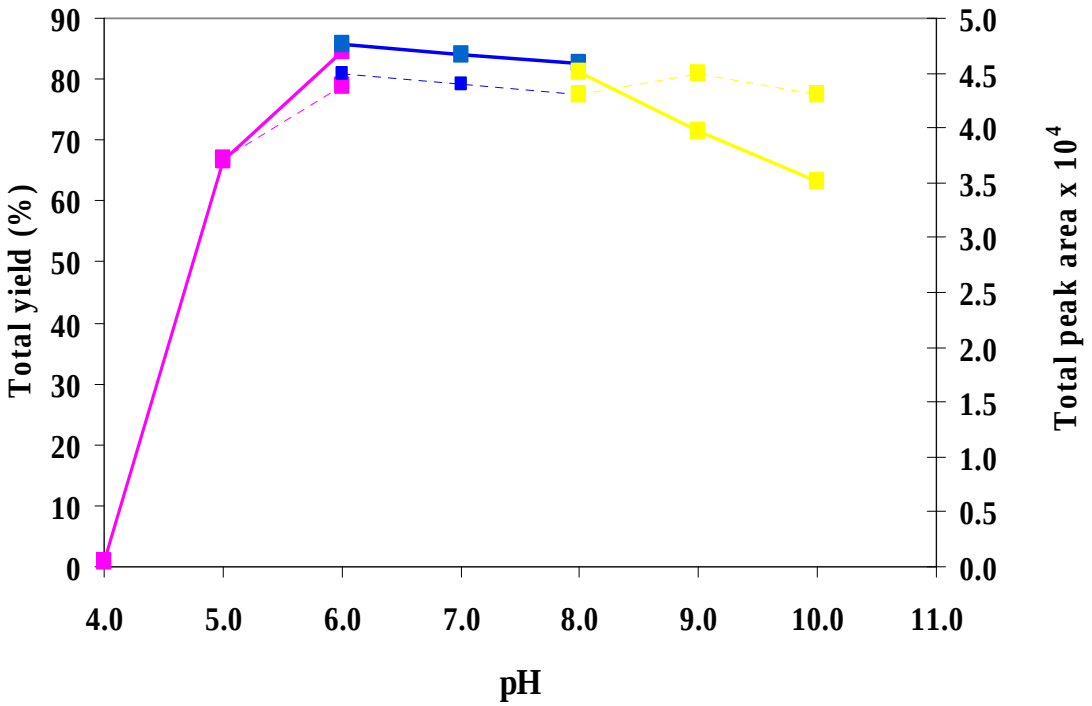


Figure 17 Effect of pH on transglycosylation yield

Sodium acetate buffer (pH 4.0-6.0)

Phosphate buffer (pH 6.0-8.0)

Tris-HCl buffer (pH8.0-10.0)

Total yield (%)

Total peak area x 10^4

3.3.5 Effect of temperature

Using the condition as mention in section 3.3.4, 3% salicin and 1.8% (w/v) β-CD were incubated at acetate buffer pH6.0 at various temperatures, the product yield of transglycosylated products were shown in table 14 and figure 18 .It was found that the product yields significantly varied with temperatures. The maximum total yield was obtained at 87.55 % when the reaction mixture was incubated at 50°C. It was observed that the maximum yield of product 1, product 2 and product 3 were 51.00 %, 24.86 % and 11.69% yields respectively. No significant difference was observed on product ratio at the varied temperatures. Thus, 50°C was chosen to be the optimum temperature.

3.3.6 Effect of incubation time

Using the condition as mentioned in 3.3.5, the reactions were performed at various incubation times. Product yield of transglycosylated products were shown in table 15 and figure 19 . The total product yield was obtained at around 69.19 % to 77.85 % when incubation time was in the range of 30 minute to 36 hours with the maximum yield observed at 3 hours. Again, incubation time did not affect product ratio. Therefore, it was chosen for the optimum incubation time.

Table 14 Yields of transglycosylated products expressed as peak areas and product yield (%) at different incubation temperature

Temperature (°C)	Product yields								
	Peak area x 10^4				Yield (%)				Ratio of product 1:2:3
	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	
30	2.49	1.24	0.59	4.32	42.83	21.36	10.21	74.40	1.00:0.50:0.24
40	2.32	1.14	0.54	4.00	43.76	21.46	10.24	75.46	1.00:0.49:0.23
50	2.84	1.39	0.65	4.88	51.00	24.86	11.69	87.55	1.00:0.49:0.23
60	2.59	1.29	0.67	4.45	47.57	22.56	10.47	80.63	1.00:0.47:0.22
70	2.51	1.16	0.63	4.30	46.33	21.45	11.67	79.45	1.00:0.46:0.25

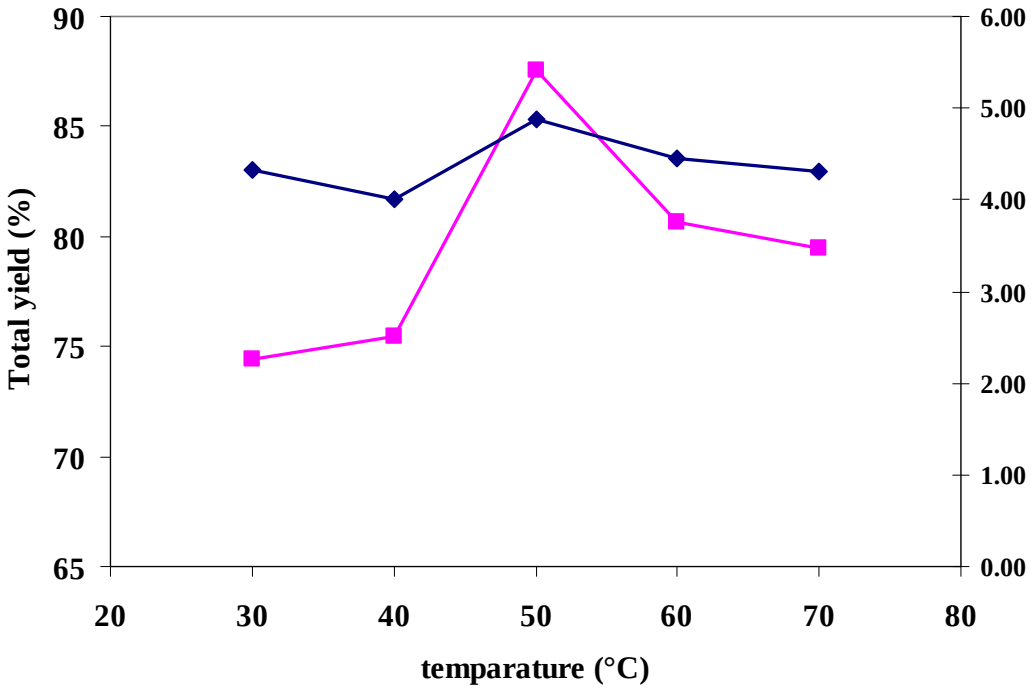


Figure 18 Effect of temperature on transglycosylation yield

Total yield (%)

Total peak area x 10^4

Table 15 Yields of transglycosylated products expressed as peak areas and product yield (%) at different incubation time.

Incubation Time (hours)	Product yields								
	Peak area x 10^4				Yield (%)				
	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Product 1 Rt 6.1	Product 2 Rt 9.4	Product 3 Rt 15.7	Total	Ratio of product 1:2:3
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00:0.00:0.00
0.5	1.98	0.81	0.49	3.28	36.53	14.95	9.04	60.52	1.00:0.41:0.25
1.0	2.17	1.05	0.53	3.75	40.04	19.37	9.78	69.19	1.00:0.48:0.24
2.0	2.22	1.10	0.54	3.86	40.96	20.30	9.96	71.22	1.00:0.50:0.24
3.0	2.56	1.12	0.54	4.22	47.23	20.66	9.96	77.85	1.00:0.44:0.21
6.0	2.23	1.09	0.53	3.85	41.14	20.11	9.78	71.03	1.00:0.49:0.24
12.0	2.24	1.08	0.52	3.84	41.33	19.92	9.59	70.84	1.00:0.48:0.23
18.0	2.27	1.08	0.51	3.86	41.88	19.92	9.41	71.21	1.00:0.48:0.23
24.0	2.26	1.07	0.50	3.83	41.70	19.74	9.23	70.67	1.00:0.47:0.22
36.0	2.27	1.06	0.49	3.82	41.88	19.56	9.04	70.48	1.00:0.47:0.22

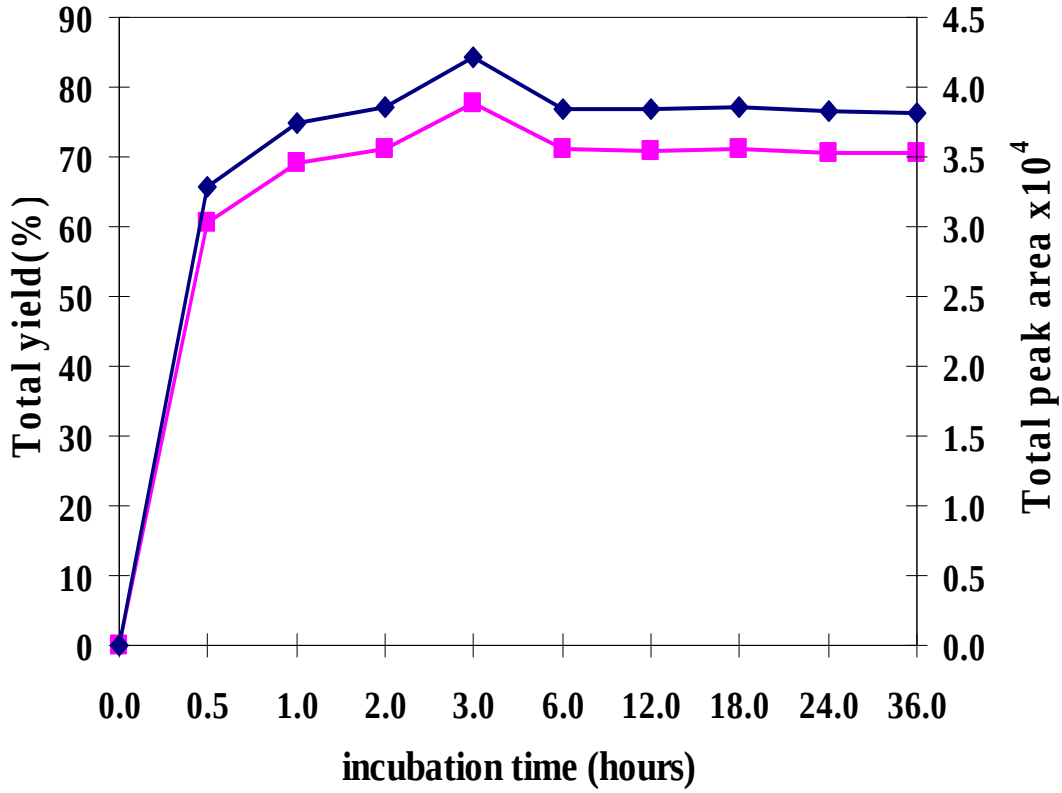


Figure 19 Effect of incubation time on transglycosylation yield

Total yield (%)

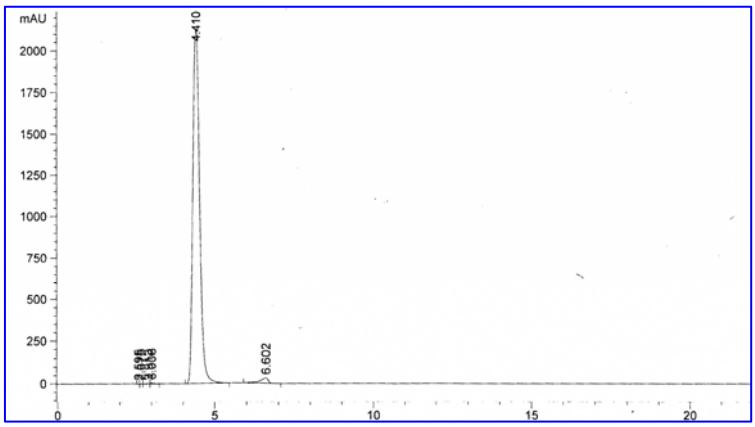
Total peak area x 10^4

3.4 Isolation of salicin glycoside products.

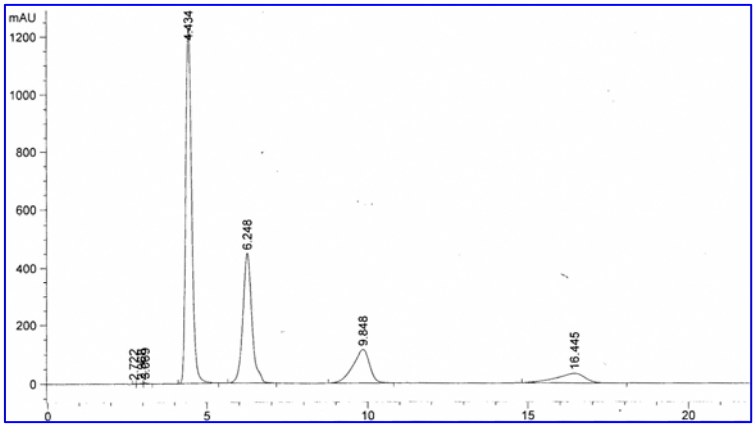
To purify each reaction products for further studies, larger scale of reaction mixture was performed.For production of higher amounts of glycoside products, the larger scale of reaction mixture (20 ml) was prepared as described in section 2.14, using the optimum conditions for transglycosylation obtained in section 3.3, the reaction mixture of 20 ml was applied to HPLC on the Shodex Asahipak NH_2P-50 4E column (4.6x250 mm). The column was eluted with 75:25 acetonitrile: water at a flow rate of 1 ml/min, fraction size of 1 ml were collected. Four main peaks (I, II, III and IV) were observed (figure 20), they was identified as salicin, product 1, product 2 and product 3, respectively. Each collected peaks were rechromatographed by HPLC and the chromatogram were shown in figure 21.

Peak I (salicin at Rt 4.3 minutes) peak II (product 1 at retention time 6.1 minutes), peak III (product 2 at retention time 9.5 minutes) and peak IV (product 3 at retention time 15.7 minutes) were identified, respectively, (table 16) were collected by HPLC fraction collection and confirmed by TLC (figure 22). Other spots in crude products of the reaction observed on TLC were not detectable on HPLC under the condition used. The three glycoside salicin products were further characterized for size and structures and determined for their properties.

(a)



(b)



(c)

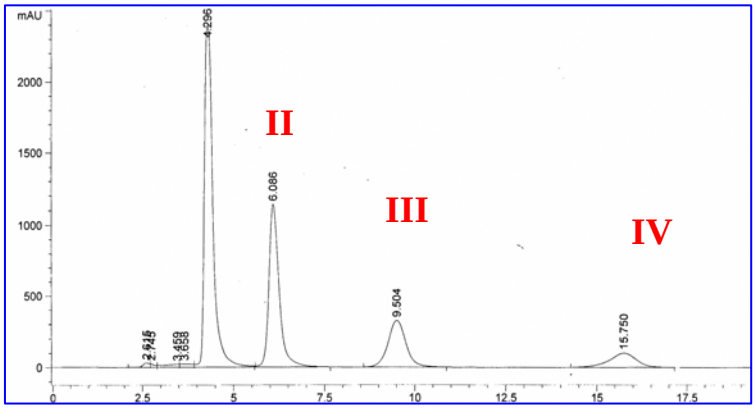


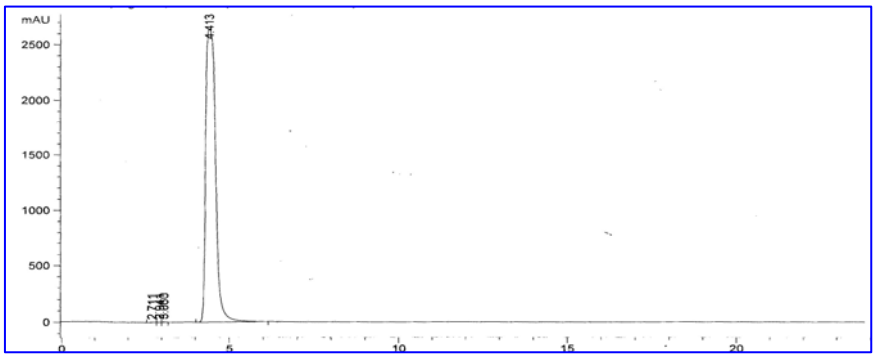
Figure 20 HPLC chromatogram of reaction products of recombinant CGTase with salicin acceptor and beta CD donor.

(a) Reaction at zero time

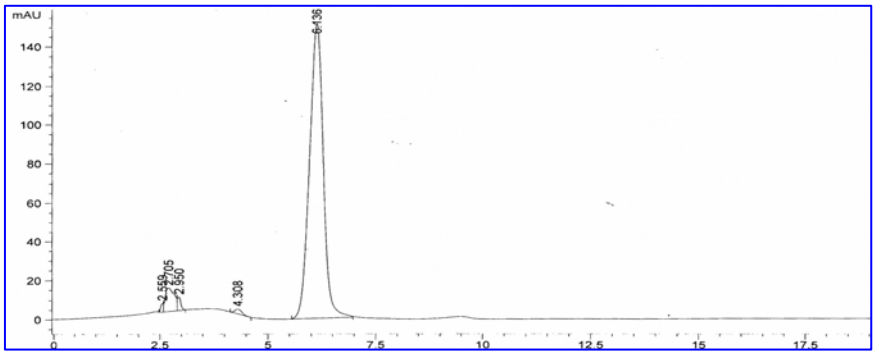
(b) Before optimization

(c) After optimization

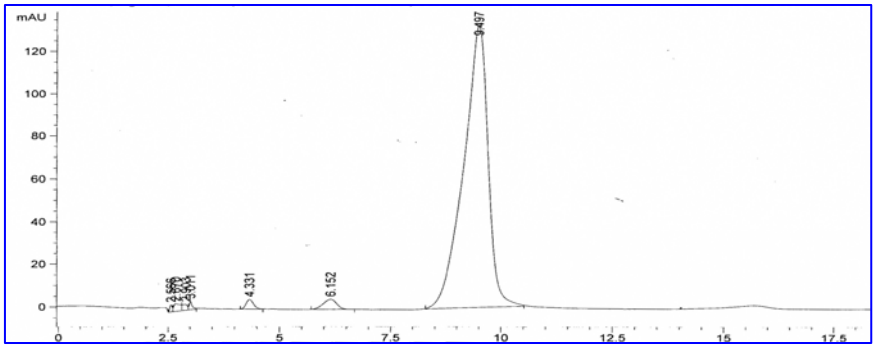
(a) peak I



b) peak II



(c) peak III



(d) peak IV

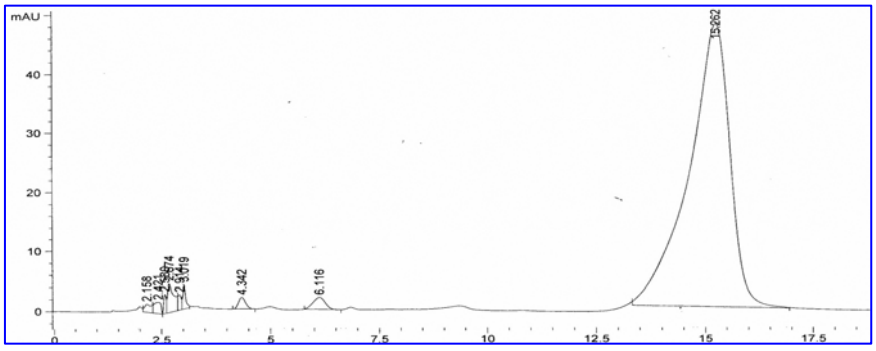


Figure 21 HPLC chromagram of peak I to IV collected from first run of HPLC.

Table 16 Identification of peaks I to IV collected from HPLC.

Peak	RT	Products
I	4.4	salicin
II	6.1	product 1
III	9.5	product 2
IV	15.7	product 3

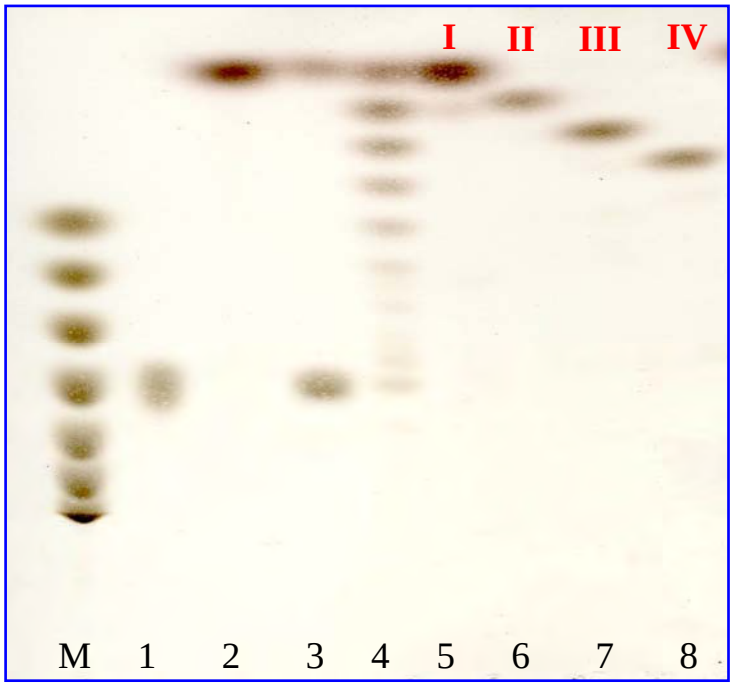


Figure 22 TLC chromatogram of the purified salicin transfer products.

(M) G1(glucose)-G7 (maltoheptaose) standards.
(1) 1%β-CD.
(2) 1%salicin.
(3) reaction mixture at t_0.
(4) reaction mixture incubated 24 hr.
(5) purified salicin, peak I HPLC
(6) purified product 1, peak II HPLC
(7) purified product 2, peak III HPLC
(8) purified product 3, peak IV HPLC

3.5 Characterization of salicin glycosides

3.5.1 Mass Spectrometry

The molecular weights of the recombinant CGTase reaction products were determined by ESI-TOF mass spectrometer as described in section 2.15.1. The molecular weight of product 1 was determined by ESI-TOF mass spectrometry to be $[M+Na]^+$ at m/z of 471.2 (figure 23, table 17) equivalent to the molecular weight of a single glucose unit attached to salicin . Product 2 was determined to be $[M+Na]^+$ at m/z of 633.2 (figure 24, table 17) , indicating two glucose units attached to salicin and product 3 was determined to be $[M+Na]^+$ at m/z of 795.3 (figure 25, table17), indicating three glucose units attached to salicin.

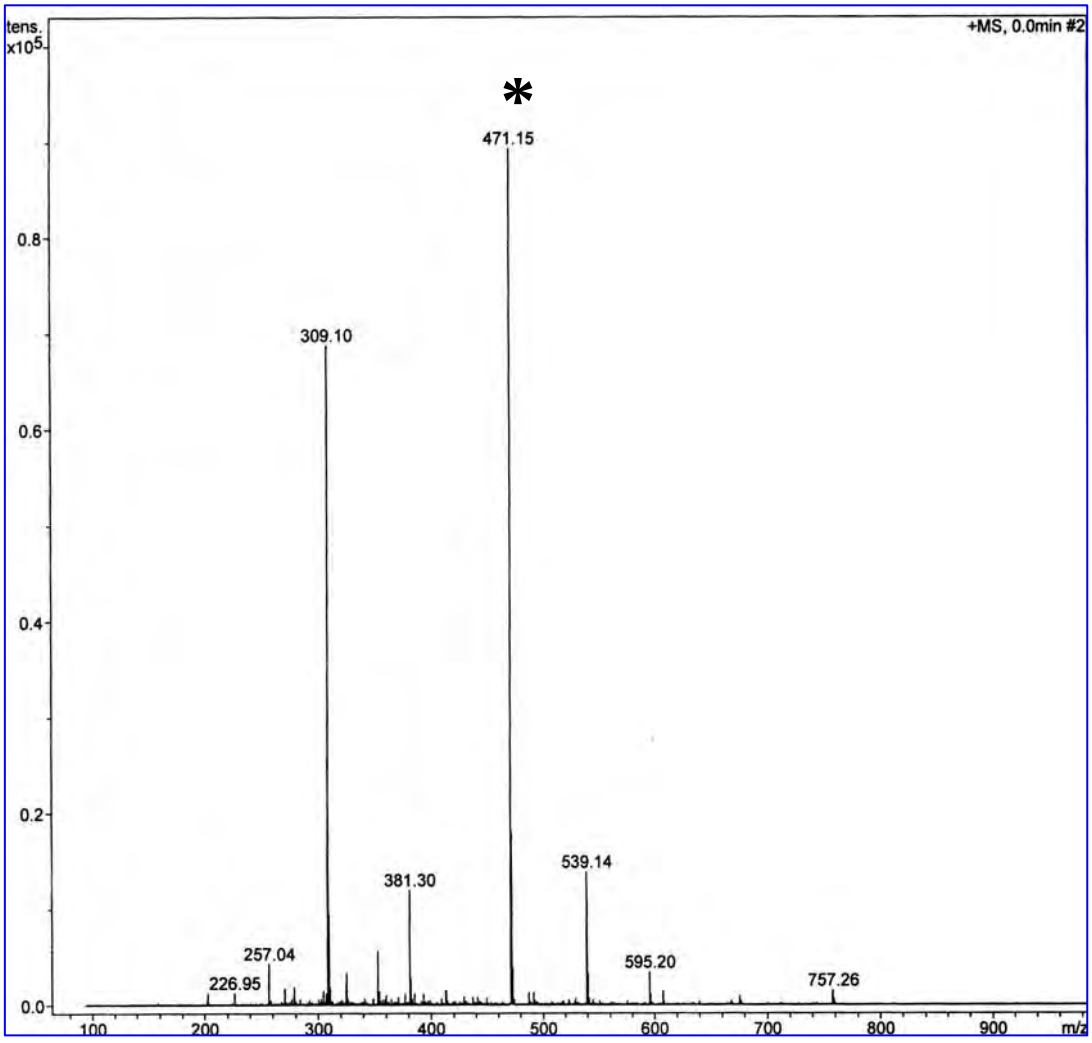


Figure 23 ESI-TOF mass spectrum of product 1 at retention time 6.1 minutes.

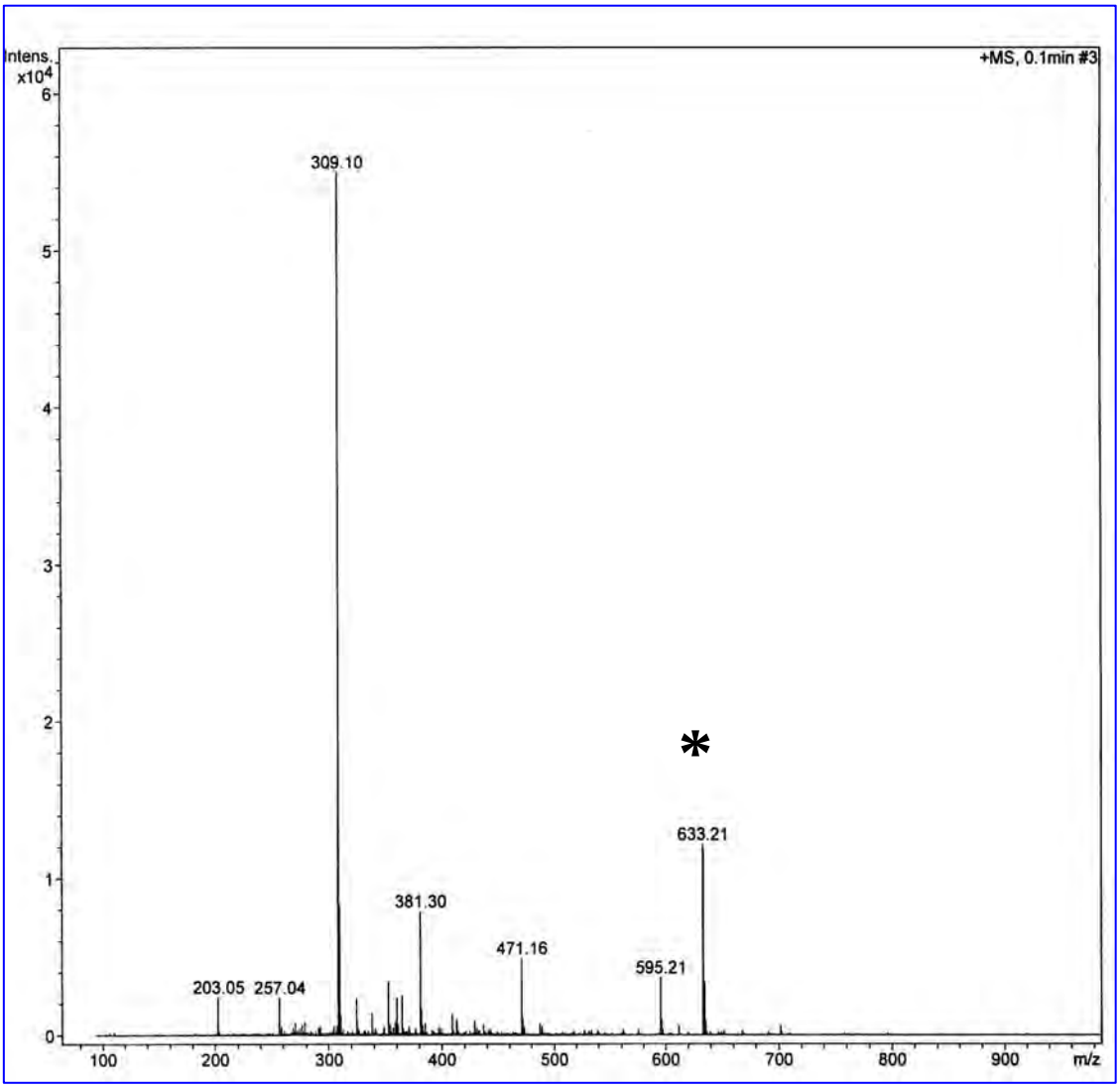


Figure 24 ESI-TOF mass spectrum of product 2 at retention time 9.5 minutes.

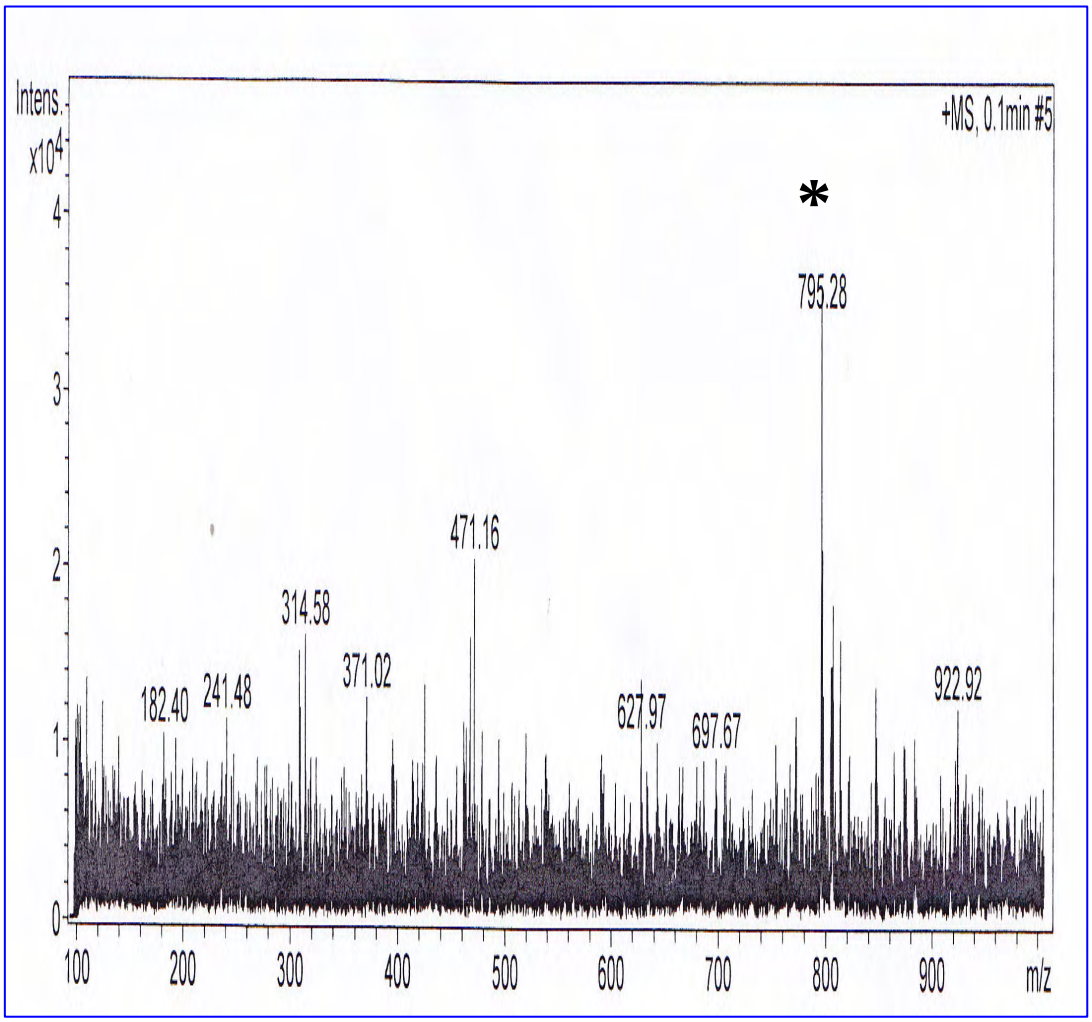


Figure 25 ESI-TOF mass spectrum of product 3 at retention time 15.7 minutes.

Table 17 The theoretical and MS (analyzed by ESI-TOF mass spectrometer) determined molecular weight of salicin and salicin glycoside

Product	**Mass number *(m/z)***	
	Calculated[a] $[M+Na]^+$	**Determined[b] $[M+Na]^+$**
Salicin (Sa)	309.3	309.1
Salicin with one glucose unit (G1-Sa)	471.4	471.2
Salicin with two glucose unit (G1-G1-Sa)	633.6	633.2
Salicin with three glucose unit (G1-G1-G1-Sa)	795.7	795.3

[a] Mass number were calculated from glucose (G1) = 180.15588 , maltose (G1-G1) = 342.300, maltotriose (G1-G1-G1) = 504.4371, Na = 22.9898; mass number of Salicin (Sa) = 286.28, H_2O = 18.0153

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

3.5.2 Nuclear Magnetic Resonance

In ^{13}C nuclear magnetic resonance (NMR) spectrum, carbon signals for glycoside products (the product at retention time 6.1, 9.5 and 15.7 minutes) were assigned to the salicin glycoside. Analysis of ^{13}C-NMR and ^{1}H-NMR data determined the type of glycosidic linkage. The difference was observed with the height, the peak at 62-64 ppm ($-CH_2OH$), 71-82 ppm (aromatic carbon), 102 ppm (α-carbon). Chemical shifts of product in the ^{13}C-NMR spectra were compared with those of salicin (table 18). The position for the terminal glucose linked to the salicin was determined through the chemical shift of the C-4' signal of the glucose moiety in salicin. The ^{13}C-NMR spectra of the product 1 at Rt 6.1 (figure 26) shown the 19 signals, the chemical shift of C-4' in the glucose unit of changed greatly from 71.34 ppm to 80.90 ppm (+9.56 ppm). It indicated that the transferred glucosyl group was at the to C-4' in the glucose unit of salicin (Jung,*et.al*,2009). While, the chemical shift of C-4' in the glucose unit of salicin was changed from 71.34 ppm to 80.92 ppm (+9.58 ppm) in the product 2 and 71.34 ppm to 80.86 ppm (+9.52 ppm) in product 3, too. The ^{13}C-NMR spectra of the product 2 at retention time 9.5 (figure 27) shown the 25 signals, the chemical shift of C-4'' in the transferred glucose unit of product 1 was changed greatly from 71.56 ppm to 81.38 ppm (+9.82 ppm). Similarly, the chemical shift of C-4'' in the glucose unit of product 1 was changed from 71.56 ppm to 81.35 ppm (+9.79 ppm) in the product 3. Due to, the ^{13}C-NMR spectra of the product 3 at retention time 15.7 (figure 28) shown the 31 signals, the chemical shift of C-4''' in the transferred glucose unit of product 2 was changed greatly from 71.53 ppm to 81.45 ppm (+9.92 ppm). In addition, ^{1}H-NMR spectrum (figure 29-31), The difference was observed with the height, the peak at 5.18 ppm (anomeric proton).

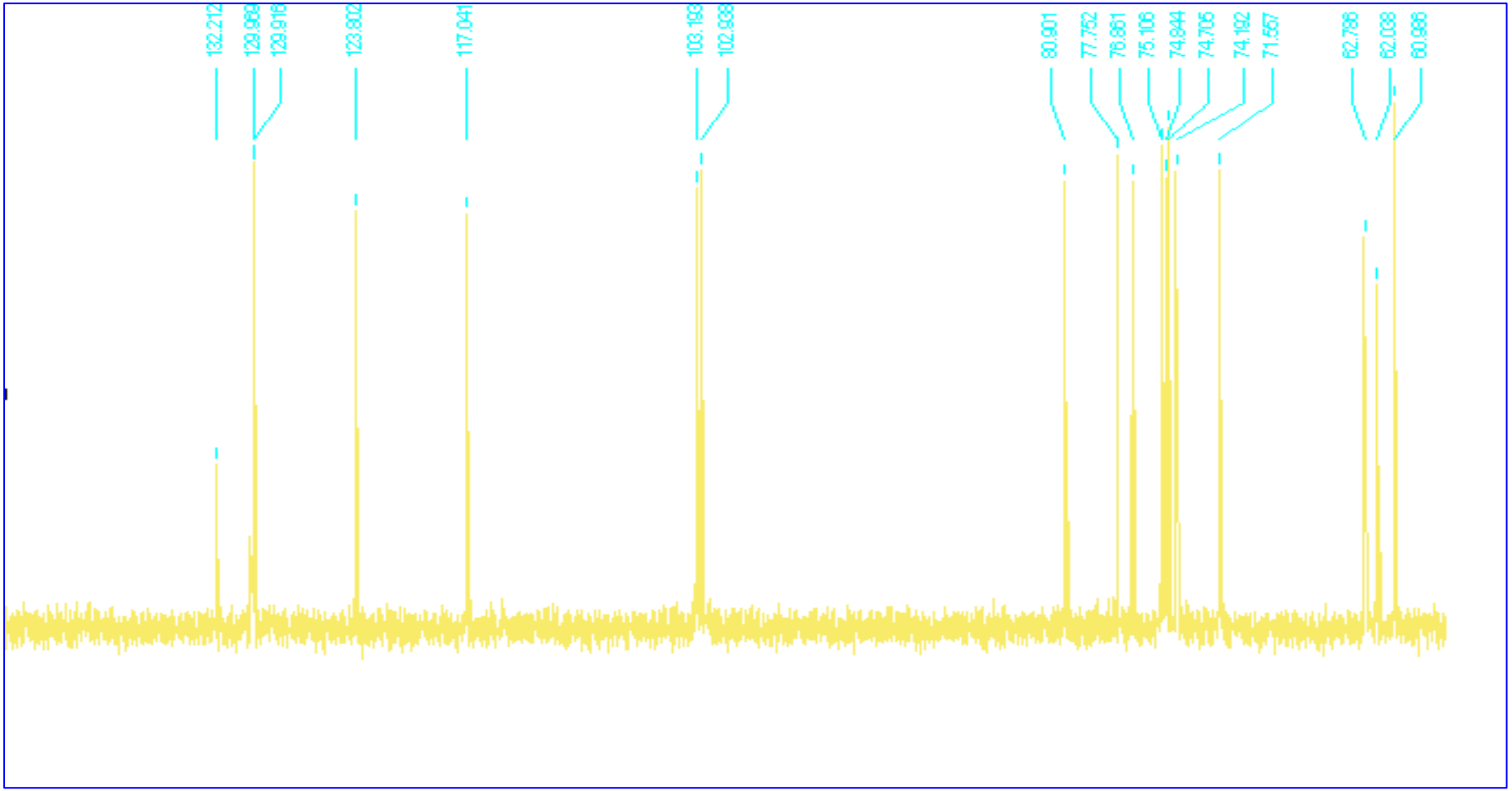


Figure 26 The 400 MHz ^{13}C-NMR spectrum of product 1 at retention time 6.1 minutes.

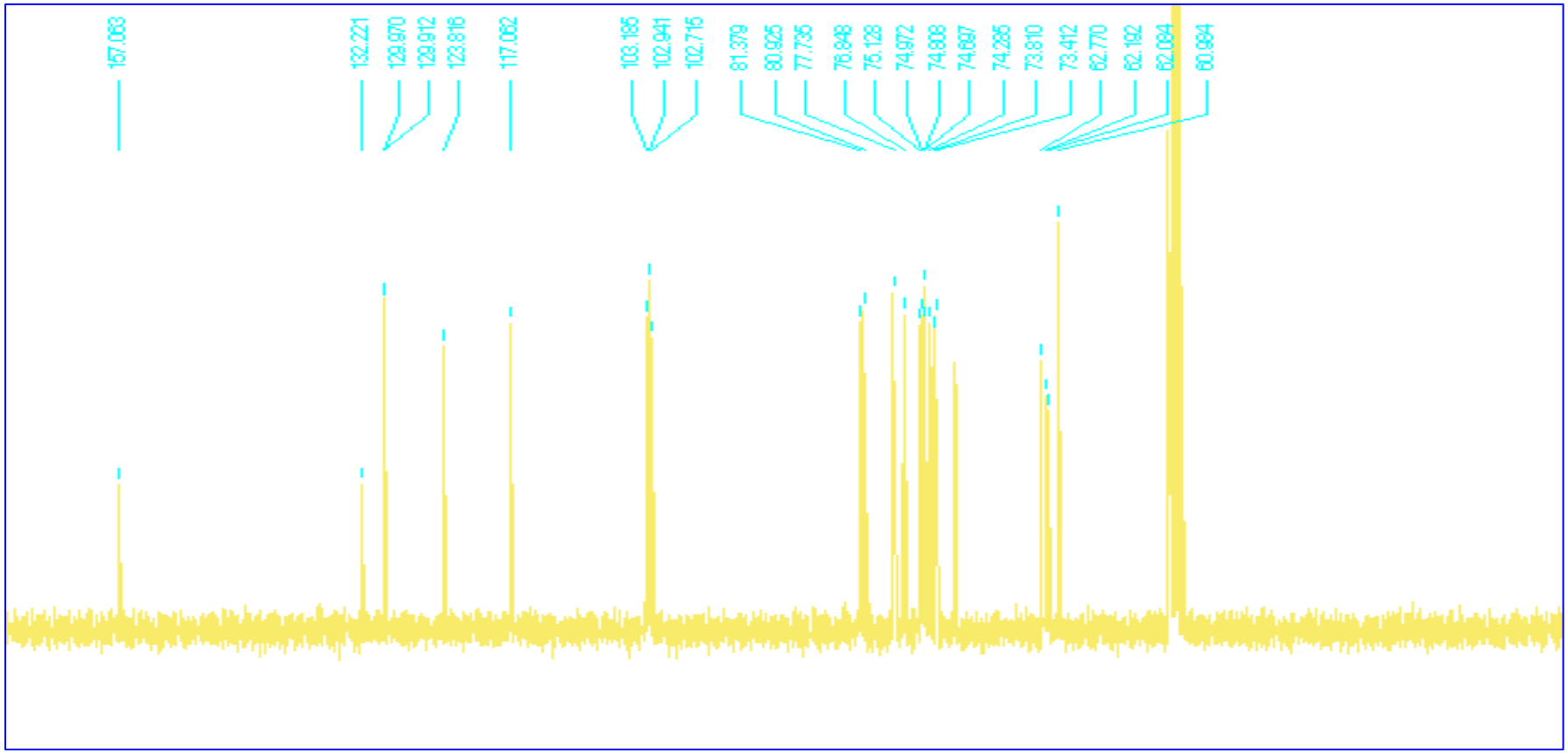


Figure 27 The 400 MHz ^{13}C-NMR spectrum of product 2 at retention time 9.5 minutes.

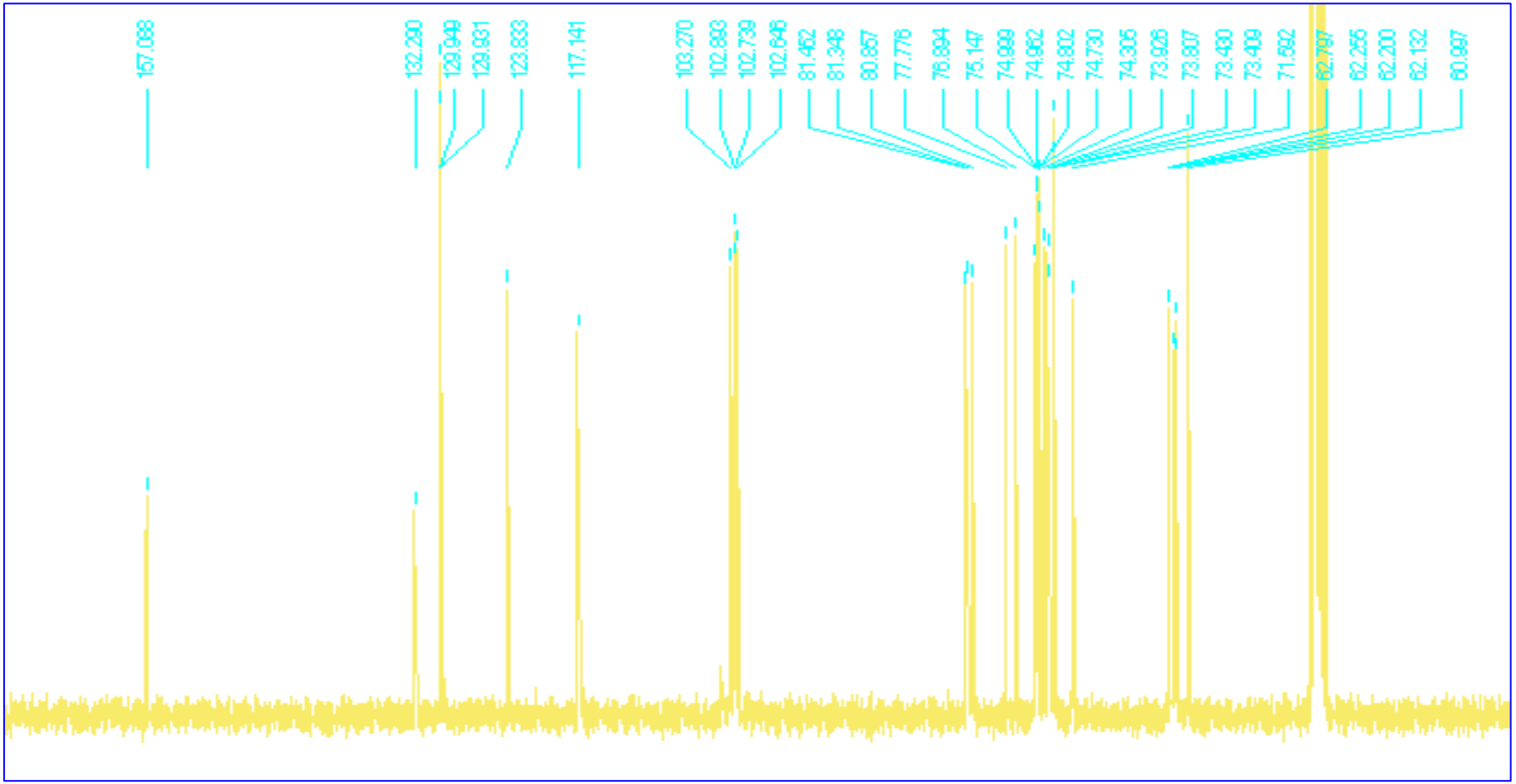


Figure 28 The 400 MHz ^{13}C-NMR spectrum of product 3 at retention time 15.7 minutes.

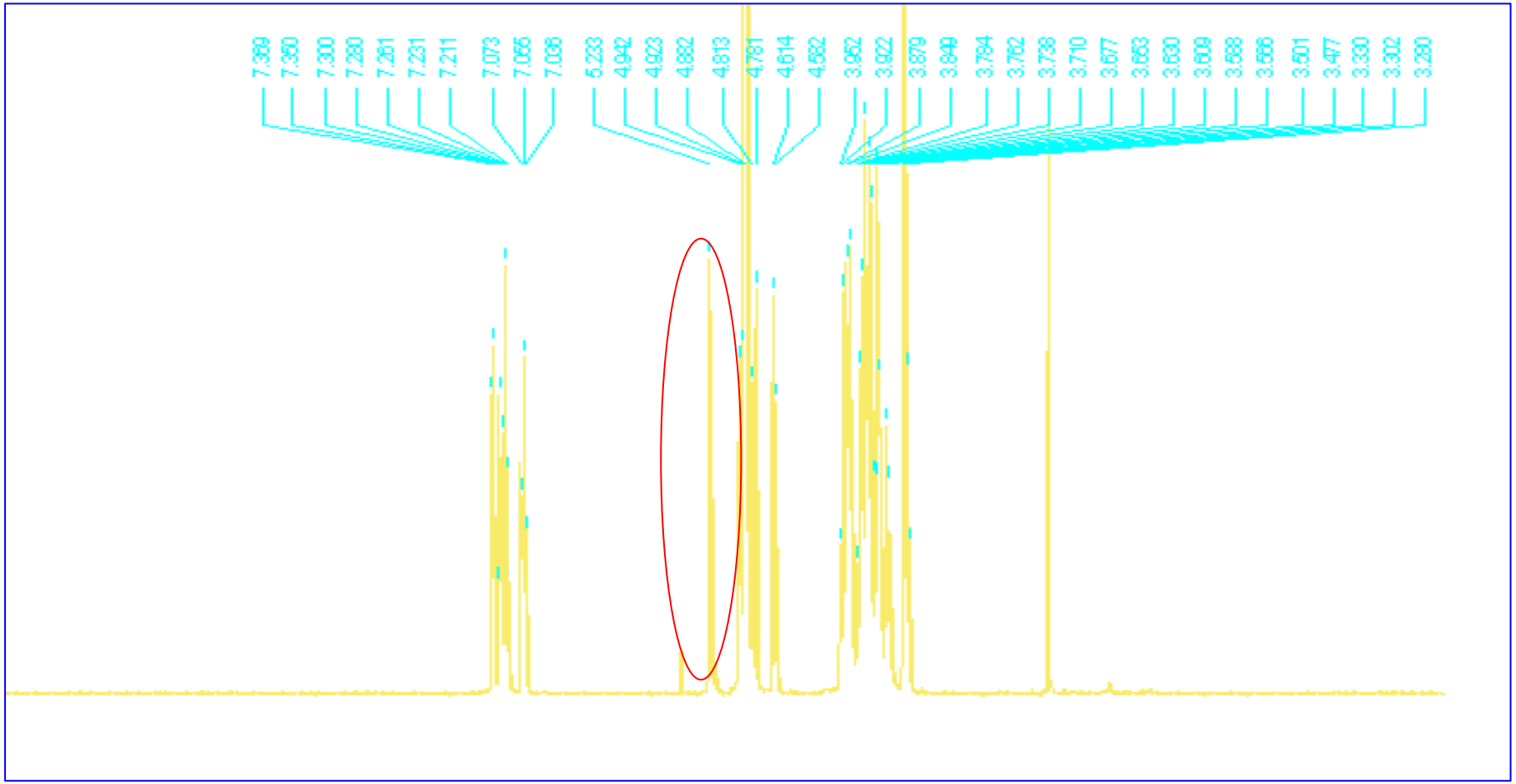


Figure 29 The 400 MHz ^{1}H-NMR spectrum of product 1 at retention time 6.1 minutes.

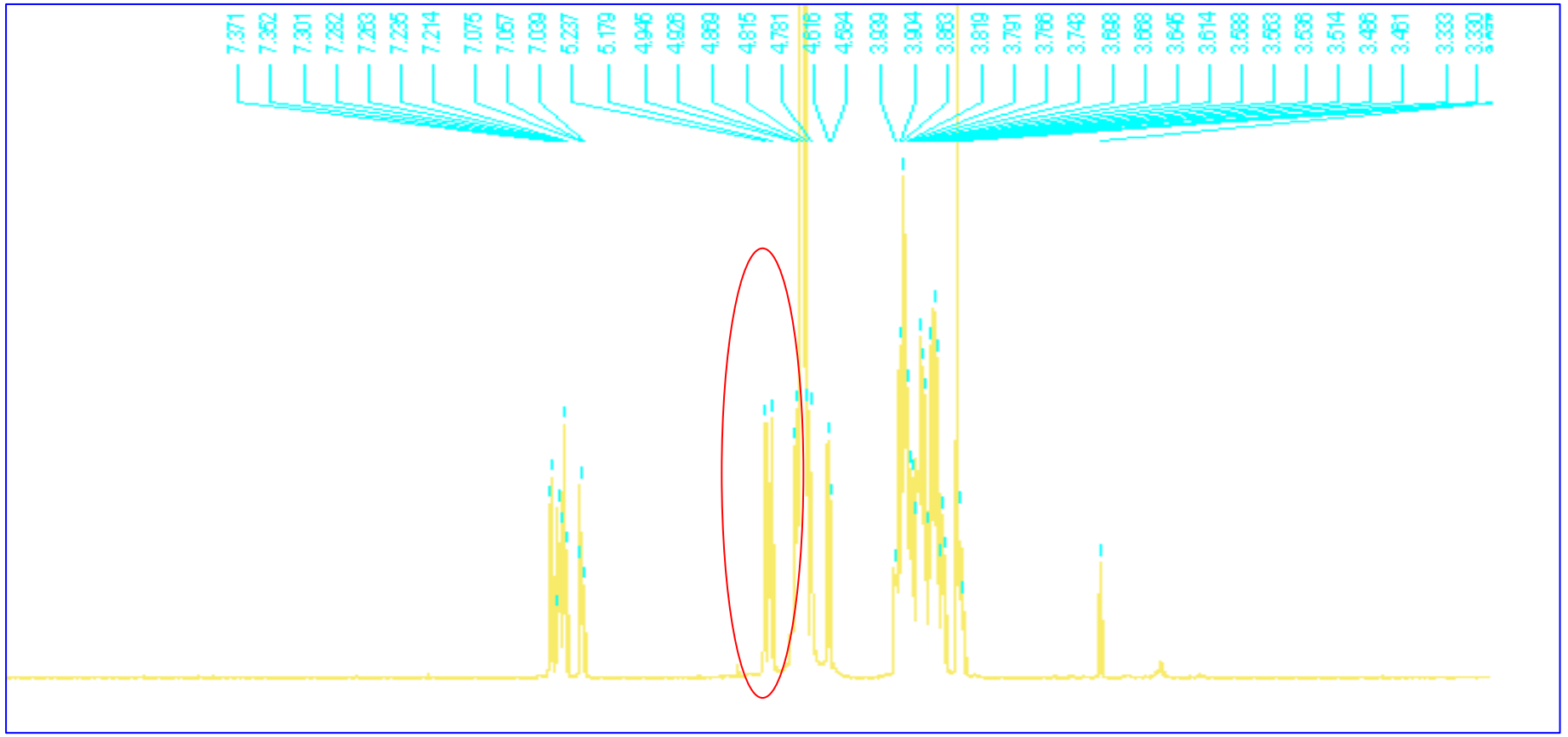


Figure 30 The 400 MHz ^{1}H-NMR spectrum of product 2 at retention time 9.5 minutes.

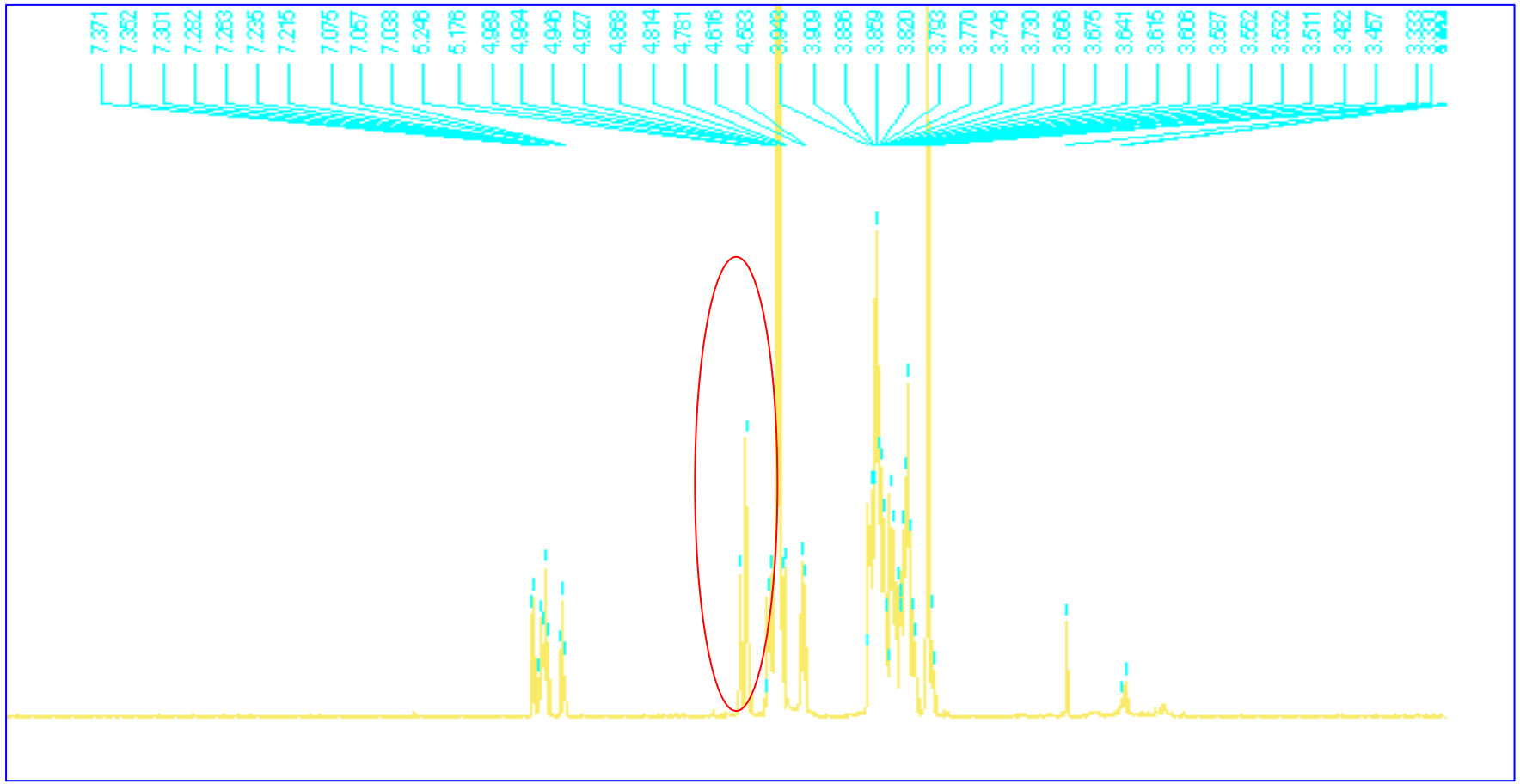


Figure 31 The 400 MHz ^{1}H-NMR spectrum of product 3 at retention time 15.7 minutes.

Table 18 ^{13}C-NMR analyses of salicin transglycosylation products

Carbon atom		**^{13}C NMR**			
		Salicin	**product 1**	**product 2**	**product 3**
Group1 salicyl alcohol		156.99	157.06	157.06	157.09
	2	132.03	132.21	132.22	132.29
	3	132.29	129.75	129.91	129.93
	4	123.62	123.80	123.81	123.83
	5	129.85	129.97	129.97	129.95
	6	116.96	117.04	117.06	117.14
	7	60.98	60.98	60.98	61.00
Group 2 Glucose I	1'	103.29	103.19	103.13	103.27
	2'	75.05	74.70	74.70	74.73
	3'	77.96	77.75	77.73	77.77
	4'	71.34	80.90	80.92	80.86
	5'	78.21	76.86	76.85	76.89
	6'	62.53	62.04	62.08	62.13
Group 3 Glucose II	1''		102.94	102.94	102.89
	2''		74.19	73.81	73.81
	3''		75.11	74.97	74.96
	4''		71.56	81.38	81.35
	5''		74.84	73.41	73.41
	6''		62.73	62.19	62.20
Group 4 Glucose III	1'''			102.71	102.74
	2'''			74.28	74.30
	3'''			75.13	75.15
	4'''			71.53	81.45
	5'''			74.81	74.80
	6'''			62.77	62.80
Group 5 Glucose IV	1''''				102.65
	2''''				73.43
	3''''				75.00
	4''''				71.59
	5''''				73.92
	6''''				62.25

3.6 Determination of properties of salicin glycoside

3.6.1 Solubility in water

The solubility of each salicin glycosides was compared to that of salicin. The excess salicin and salicin glycosides was mixed with water and incubated at 30 °C for 15 minutes. The soluble part of the sample was analyzed by HPLC for determination of the concentrations. From the result, the solubility of salicin was 23.31 mg/ml whereas the solubility of glucosyl salicin, maltosyl salicin and triosyl salicin were 72.23, 134.46 and 207.76 mg/ml, respectively (table 19), corresponding to 3,6 and 9 times higher than that of salicin (table 19).

3.6.2 Anticoagulant activity assayed by measurement of clotting times

To find the anticoagulation effect of modified salicin or salicyl alcohol, blood drawn from rabbit ear was mixed with salicin, glucosyl salicin, maltosyl salicin and tricosyl salicin and the blood clotting time was recorded. Salicin showed blood clots within 5 min under our experimental condition. Glucosyl salicin, maltosyl salicin and tricosyl salicin did not form blood clot, even after 10 min had passed. We also checked the anticoagulation effect of heparin as a positive control. Heparin (0.5 U/ml), a positive control, showed slow blood clotting within 5 min under our experimental condition (table 20).

Table 19 The water solubity from standard curve of salicin, glucosyl, maltosyl and triosyl salicin at 30°C

Compound	Salicin	Glucosyl-salicin	Maltosyl-salicin	Triosyl-salicin	Ratio of water solubility
Solubility (mg/ml)	23.31	72.23	134.46	207.76	1:3:6:9

Table 20 Effect of salicin glycoside for blood clot formation

Time (min)	control	aspirin	salicin	glucosyl-salicin	maltosyl-salicin	triosyl-salicin
10	C	C	C	NC	NC	NC
20	C	C	C	C	C	C

C : clot formation

NC : no clot formation

CHAPTER IV

DISCUSSION

4.1 Purification of recombinant CGTase

Cyclodextrin glycosyltransferase gene from *Paenibaccilus* sp. BT01, isolated from waste of a starch factory in Nakhon Pathom province, was cloned into *Escherichia coli* BL21(DE3) using pET 19b vector. This recombinant cell was named pBT. The optimum condition for growth and for producing recombinant CGTase of these bacteria was previously performed and reported by Tantanarat (2005). To purify CGTase from pBT, starch adsorption and DEAE-cellulose column were used. Since CGTase are extracellular enzyme, starch adsorption (Kato and Horikoshi, 1985, modified by Laloknam, 1977) was selected because it was simple and resulted in higher purification fold and % yield than ammonium sulfate precipitation (Tantanarat,2005). This method based on the interaction between substrate and enzyme or substrate-enzyme affinity binding. Recombinant CGTase was adsorbed by corn starch added into the crude enzyme and the adsorbed enzyme was then elute with maltose in buffer solution, maltose, one of the substrates of CGTase, has higher affinity to CGTase than starch. Thus, maltose was added into the starch adsorbed enzyme for competitive binding of recombinant CGTase. Accordingly, enzyme-substrate that presented in soluble fraction was easily separated from pellet of starch. The enzyme-maltose solution was dialyzed against buffer solution to get rid of maltose from enzyme and the enzyme activity was determined by dextrinizing assay. Therefore recombinant CGTase will degrade starch into oligosaccharides which cannot form complex with iodine, the enzyme activity is defined as amount of enzyme producing the reduction in the intensity of starch-iodine complex. After this step, the enzyme was purified to homogeneity with 4 purification fold and 49% yield in pBT (table 7). Tantanarat (2005) reported 7 purification fold and 89 % yield in purity. However, non-denature polyacrylamide gel electrophoresis (ND-PAGE) still revealed many bands (figure2), so the enzyme were not purified to homogeneity. Second step of purification was perfomed on DEAE-cellulose, an anion exchange chromatography. The advantages of DEAE-cellulose column were high sample

volume can be applied and lower cost than affinity and immunoaffinity columns. The chromatograms (figure 6) showed that significant amount of unwanted proteins were separated from the recombinant CGTase peak. After this step, the enzymes showed one band on ND-PAGE (figure 7). Consequently, DEAE-cellulose column can purify the enzymes to homogeneity, with 5 purification fold and 37% yield in pBT (table 7). Tantanarat (2005) reported 8 purification fold and 71 % yield in purity.

4.2 Productions and detection of transglycosylation products of CGTase from pBT

It is known that CGTase catalyzes the transferring of α-1,4 glucan chains to the C-4 hydroxyl groups of D-glucose. There was report that the configuration of the C-2, C-3 and C-4 hydroxyl group of D-glucose was required for acceptors used in the transglycosylation reaction by CGTase (Nakamura,et al.,1994).When incubation of recombinant CGTase with an acceptor was varied and β-CD a donor, the expected transglycosylated products were detected by TLC (figure 8-11) and monitoring the use of β -CD by β-CD-degrading activity using phenolphthalein method (figure 12, table 8).

Thin-layer chromatography (TLC) was used for primary detection of the reaction products. TLC plate is composed of silica gel as a polar stationary phase. Reaction mixtures were spotted and run in pararel with standards sugars from glucose to heptaose (G1-G7) including the unreacted acceptor and donor (zero incubation time) system, The solvent system was n-buthanol : ethanol : water (5:5:3, v/v/v). After running the solvent system, the plate was air-dried and sprayed with sulfuric acid in methanol, followed by heating at relatively high temperature to degrade organic compounds into black or brown zones of carbon on a white background. Among the tested sugars, salicin was one of the best glucosyl acceptor judging from clear observable and resolvable product spots and the utilization of β–CD. Many fast moving spots more observed on TLC with retention time factors less than glucose standard (G1). Three of these products can be clearly observed at retention time of 6.1, 9.5 and 15.7 minutes on HPLC. Others spots ,which moved in the range of the standard G1-G7 and appeared as very faint bands, were most likely the remaining

oligosaccharides from hydrolysis of β –CD. Some of the sugar acceptors such as 2-deoxy-galactose, acarbose, maltotetraose, lactose, lactuose and raffinose yielded no detectable products; some, such as arabinose, fucose, rhamnose, fructose, 2-deoxy-glucose, sucrose, melibiose, xyllitol, sorbital, manose and maltotriose could react and resulted in products with similar retention times to the G1-G7 standards which were not unexpected. It was reported that amylosucrase from *Deinococcus geothermalis* and *Neisseria polysaccharea* could synthesize α-D-glucopyranosyl-(1,4)-salicin and α-D-glucopyranosyl-(1,4)- α-D-glucopyranosyl- (1,4)-salicin from salicin (Jung, *et al*.,2009). From HPLC analysis of reaction products in figure 15, when reaction mixture at zero time was analyzed, the peak of salicin were detected at retention time 4.3 minute. After 24 hour incubation, three product at retention 6.1, 9.5 and 15.2 minutes were clearly observed.

For β-CD to act as glucosyl donor, it has to be hydrolysed to open the ring. Phenolphthalein is normally pink but can form a stable colorless inclusion complex in the presence of β-CD. Thus, monitoring the intensity of pink color at OD. $_{550}$ can indicate the amount of β-CD present in the slolution. The use of β-CD in the transglycosylation reaction can be monitored from the increase in OD_{550} compared to zero time. The results obtained by phenolphthalein method (table 8 and figure 12) confirmed the results from TLC chromatograms (figures 8-11) that D-xylose, glucose, salicin, cellobiose, palatinose and maltose showed relatively high transglycosylation activities with β-CD (table 8 and figure12) Arabinose, fucose, rhamnose, fructose, 2-deoxy-glucose, sucrose, melibiose, xyllitol, sorbital, manose and maltotriose were poor acceptors, whereas had very low or non detectable activities. The order of acceptor capacity were : xylose > glucose > salicin > cellobiose > palatinose > maltose > arabinose > fucose > rhamnose > fructose > 2-deoxy-glucose > sucrose > melibiose > xyllitol > sorbital > manose > maltotriose > 2-deoxy-galactose, acarbose, maltotetraose, lactose, lactuose and raffinose.

In this experiment, β-CD was used as a glycosyl donor. Different types of glycosyl acceptors including monosaccharides and derivatives, disaccharides and oligosaccharide were tested for this capability. When analyzing the structures these monosaccharides (Appendix I), the importance of pyranose ring with C6 unit and the same configurations of C-2, C-3 and C-4 hydroxyl group as glucopyranose is

evidenced. Comparing to glucose, xylose has the same structure and configuration except that xylose has no $–CH_2OH$ as C6. Arabinose change structure and configuration at C-3, and C-4 and it has no $–CH_2OH$. Mannose has different structure and configuration of C-2, the C-2 hydroxy is changed to hydrogen in 2-deoxy-glucose without the change in configuration and 2-deoxy galactose C-2 hydroxy to C-2 hydrogen. The furanoside structure was not supportive acceptor efficiency as evidenced by the result of fructose has the steric effect of C-3 hydroxy. The result of deoxy sugar, rhamnose C-4 position and fucose C-2 and C-4 position has changed hydroxyl to hydrogen. The sugar alcohol, sorbitol and xylitol showed that they were not good acceptors. The structure around C-6 on acceptors was not essential for acceptor function, but it was recognized by CGTase. Therefore, modification of one of the groups at C-2, C-3 or C-4 position causes impairment in the efficiency as acceptor. For disaccharides and oligosaccharide, it was found that all those tested could be used as acceptors except lactose, lactulose, acarbose and raffinose which has no glucose linked at C-4 at the reducing end and β linkage at this position (cellobiose, salicin) seemed to be more active.

Salicin [2- (hydroxymethyl) phenyl - β - D - glucopyranoside] is a naturally occurring glucoside found in the bark of poplar and willow tree. This compound was used as an analogesic and antipyretic (Yoon, *et.al.*, 2004). It has a D – glucopyranose unit attached by a β-linkage to the phenolic hydroxyl group of salicyl alcohol. When we decided to employ salicin as an acceptor of transglycosyltion reactions of recombinant CGTase, its transglycosylated products can be easily detected on HPLC using a UV detector due to the hydroxyl group on the phenol of compound. In this experiment, several CD derivatives (α-CD, G1-α-CD,G2-α-CD, β-CD, G1-β-CD, G2-β-CD ,OH-β-CD and γ-CD) including G6 and G7 were used in the transglycosyl reaction to compare the total yield (calculated from the decrease in peak area of salicin and the sum of peak area of products 1, 2 and 3 in the HPLC profiles). G6, G7, α-CD, β-CD, and γ-CD were effective glucosyl donors for salicin. The total yield of glycosides were not significantly different at 42.7, 43.1, 44.9, 45.4 and 44.3%, respectively. Derivatives of CD (G1-α-CD, G2-α-CD, G1-β-CD and G2-β-CD and hydroxyl-CD were poor glucosyl donors for salicin since minimal conversion of salicin were observed. β-CD was thus chosen as the suitable glucosyl donor for further study due to easy accessibility and lower cost.

4.3 Optimization of transglycosylation reaction

Before the optimization, the result of transglycosylation reaction, the total transglycosylation yield of salicin glycosides was 61.85% (table 10). Three products were obtained from salicin as an acceptor .The products at retention times of 6.1, 9.5 and 15.9 minutes were obtained on HPLC with yield of 34.94, 17.67 and 9.24% respectively. (using 1% salicin as an acceptor, 1% β-CD as a donor with 70 unit/ml recombinant CGTase, pH 6 at 60°C for 24 hours). In order to produce maximum yield of salicin glycoside products with optimum amount of substrates, the synthesis condition was optimized. Parameter optimized were; concentration of salicin as an acceptor, β-CD as donor , amount of recombinant CGTase (unit/ml), pH , reaction temperature and incubation time. After the optimization of transglycosylation reaction, the optimum condition for salicin glycoside production was 3.0 % salicin as an acceptor, 1.8 % β-CD as a donor with 100 unit/ml recombinant CGTase, pH 6.0 at 50°C for 3 hours. The total transglycosylation yield of salicin glycosides was 77.85 % (table 9). The products at retention times 6.1, 9.5 and 15.9 minutes were obtained on HPLC with yield of 47.23, 20.66 and 9.96 %, respectively.

When the recombinant CGTase concentration was determined, the result showed that the transglycosylation yield was rather constant. The concentration of recombinant CGTase used was 100 unit/ml and the excess amount had no effect on the transglycosylation yield. There were reported that excess CGTase may induce unnecessary side reaction which did not contribute to increase in transglycosylation yield. (Park *et al.*,1997)

Using the condition at 3.0% salicin and 1.8% β-CD with 100 unit/ml recombinant CGTase, pH and temperature were varied. When the effect of temperature on the transglycosylation reaction was varied, the effective pH range was from pH 5 to pH 8, with highest transglycosylation yield at pH 6, the activity was significantly reduced at below pH 5 and above pH 9. Transglycosylation yield was rather constant in the the temperature range 30°C to 60°C, above which transglycosylation yield decreased which should be due to denaturing of recombinant

CGTase at high temperature and loss of recombinant CGTase activity. The cyclization activity of recombinant CGTase from pBT was also reported at this temperature (Tantanarat, 2006). Thus, optimum temperature was set at 50°C. Jung *et al*. (2009) reported the transglycosylation activities of amylosucrase from recombinant DGAS and NPAS were employed to synthesized salicin glycosides using sucrose as a glucosyl donor and salicin as an acceptors. The optimal pHs for rNPAS and rDGAS were pH 6.0 and pH 8.0, respectively. For the optimal hydrolysis temperature was 30°C and 45 °C in rNPAS and rDGAS, respectively.

For the effect of incubation time on the transglycosylation rection reaction was determined, the optimal incubation time was found to be 3 hours. The result showed that though the incubation time was prolonged, the transglycosylation yield was rathere constant.

4.4 Large scale preparation and isolation of salicin glycoside products

To prepare higher amounts of salicin glycoside products for characterization, 20 ml reaction mixtures were performed. When isolate and identify the transfer products, the reaction mixture was separated by HPLC chromatography on Shodex Asahipak NH2-50 4E column. Each of the transfer product fraction was collected, the result showed four peaks (I, II, III and IV) were observed. They were identified as salicin, product 1, product 2 and product 3, respective (figure 20,21).

Three different transfer products were detected by HPLC technique from the recombinant CGTase catalyzed transglycosylation reaction with salicin as an acceptor. The result showed that after 3 hours of incubation time, the transfer product 1 at the retention time 6.1 minutes, product 2 at retention time 9.5 minutes and product 3 at the retention time 15.7 minutes were obtained.

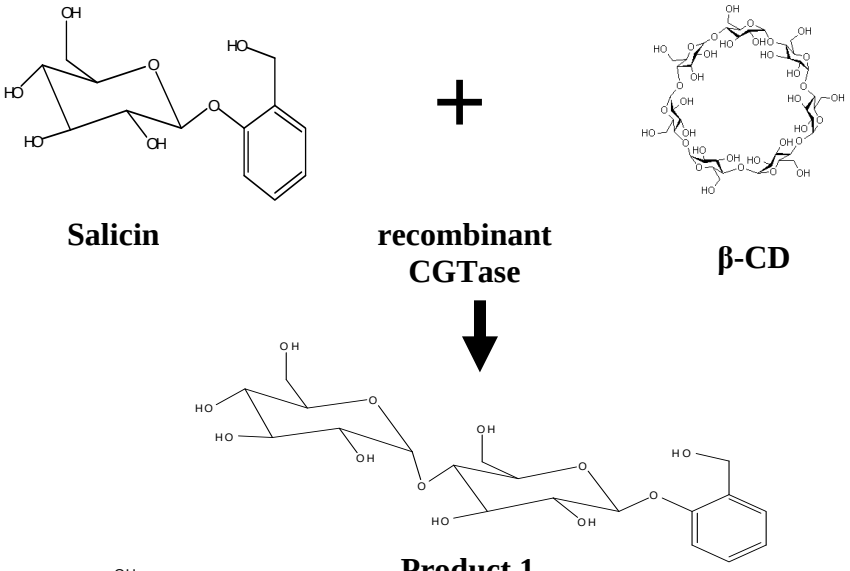
The transfer products should be linear opened-chain oligosaccharides that were produced by cleavage of cyclodextrin ring and transfer glucose unit of maltooligosaccharides to non-reducing end glucose unit of acceptor to form α-1,4-

glucans. This indicated that transfer products should be $(glucosyl)_n$- α-1,4- linkage to salicin.

4.5 Characterization of salicin glycosides

The molecular weights of the recombinant CGTase reaction products were determined by ESI-TOF mass spectrometer. The molecular weight of the product 1 at retention time 6.1 minute was determined to be $[M+Na]^+$ at m/z of 471.2 (table 11 and figure 23). This indicating a single glucose unit attached to salicin . For the product 2 at retention time 9.5 minute determined to be $[M+Na]^+$ at m/z of 633.2 (table 11, figure 24) , indicating two glucose units attached to salicin and the product 3 at retention time 15.7 minute determined to be $[M+Na]^+$ at m/z of 795.3 (table 11, figure 25), indicating three glucose units attached to salicin. These molecular masses corresponded to the calculated molecular masses of glucosyl salicin, maltosyl salicin and triosyl salicin. When various signals of products 1, 2 and 3 in their ^{13}C-NMR and ^{1}H-NMR spectrum. Analysis of ^{13}C-NMR was compared with those of salicin, seven of them (group 1 in table 18) were assigned to the (hydroxymethyl) phenyl group and six (group 2 in table 18) were assigned as glucose units of salicin. The ^{13}C-NMR spectra of product 1 at retention time 6.1 (figure 26) showed 19 signals, including those of salicin molecule Product 2 at retention time 9.5 (figure 27) had 25 signals containing those of salicin and product 1. The product 3 at retention time 15.7 (figure 27) had 31 signals containing those of salicin and product 2. The result, combined with ESI-TOF mass spectrometer analysis, implies that product 1 contained a glucose molecule connected to salicin, whereas two glucose units and three glucose units were attached to the salicin molecule in product 2 and product 3, respectively. The structure of product 1 was identified as α-D-glucopyranosyl-(1→4)-salicin (glucosyl salicin), in which the transferred D-glucose was attached to C-4' of the glucose moiety of salicin by an α-(1→4)-linkage. Consequently, product 2 was determined to be α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-salicin (maltosyl salicin) and product 3 was determined to be α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-(1→4)-α-D-glucopyranosyl–salicin (triosyl salicin) (figure 32). The glucosyl salicin and maltosyl salicin structure from this study, catalyzed by recombinant CGTase from *Paenibacillus* sp.BT01, had the same linkage and configuration as those

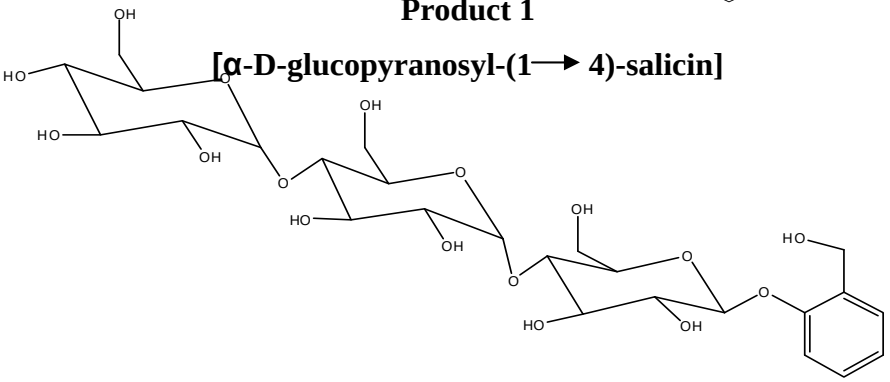
previously reported for salicin glucosides: α-maltosalicin synthesized by transglycosylation of CGTase from *B. macerans* (Yoon *et al*.,2004), the glucosyl salicin and maltosyl salicin by recombinant amylosucrase from *Deinococcus geothemalis* and *Neisseria polysaccharea*. (Jung *et al*., 2009). It remains to be further elucidated whether the glucose units were cleaved from β-CD and added to salicin one at a time or it can also occurred two or more at a time.



Salicin **recombinant CGTase** **β-CD**

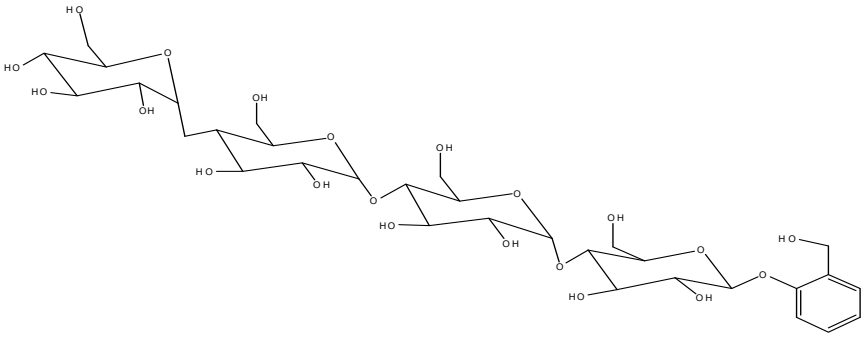
Product 1

[α-D-glucopyranosyl-(1→ 4)-salicin]



Product 2

[α-D-glucopyranosyl-(1→4)- α-D-glucopyranosyl-(1→4)- salicin]



Product 3

[α-D-glucopyranosyl-(1→4)- α-D-glucopyranosyl-(1 →4)-

α-D-glucopyranosyl-(1 → 4)- salicin]

Figure 32 Structures of salicin and transglycosylated salicin derivatives

4.6 Determination of some properties of salicin glycosides.

When the three glycosides produced from the transglycosylation reaction were partially characterized on the similar properties reported for salicin such as water solubility and anti-blood clotting ability, they were found to exhibit superior characteristics, especially products 1, 2 and 3. Its solubility was 3, 6 and 9 times higher than that of salicin.

Among the three salicin glycosides, products 1 and 2 slowed down blood clotting comparing to salicin or heparin under the same conditions. Blood clots can cause severe and life-threatening problems such as after surgery, deep vein thrombosis, pulmonary embolism, and other situations involving excessive blood clotting. Heparin, dextran sulfate (Sakamoto *et al.*, 2002) and chitosan ester (Drozd *et al*., 1992) have been used to prevent formation of blood clots or dissolve blood clots in some circumstances. Thus, glucosyl-salicin, maltosyl-salicin and triosyl-salicin with their high water solubility may offer better choices of anti-blood clotting agents.

From this research work, we reported the production of new glycosides from transglycosylation reaction catalyzed by recombinant CGTase from pBT, using salicin and β-CD as substrates. The new products possess some favorable properties such as high water solubility and high anti blood clotting property which render the possibility of being used in medical application.

CHAPTER V
CONCLUSIONS

1. The recombinant CGTase from *Paenibacillus* sp.BT01 was purified by starch adsorption followed by DEAE-cellulose column chromatography with 5 purification fold and 37 percent yield.

2. The enzyme was able to synthesize oligosaccharides or glycosides from various saccharides. Salicin was one of the best glucosyl acceptor.

3. The recombinant CGTase from *Paenibacillus* sp.BT01 catalyzed the transglycosylation reaction between β-CD and salicin producing at least three transglycosylation products.

4. The optimum conditions for maximum yield were 3.0 % (w/v) salicin as an acceptor, 1.8 % β-CD (w/v) as a donor and 70 unit/ml recombinant CGTase, pH 6 at 50°C for 3 hour.

5. Three products at retention time of 6.1, 9.5 and 15.7 minute were obtained on HPLC,named products 1,2 and 3, with yield of 47.23, 20.66 and 9.96 percent, respectively. Product ratio was only affected by concentration of salicin, with more product1 observed at high salicin concentration.

6. The molecular masses of the salicin glycosides, products 1, 2 and 3, were 471.2 dalton, 633.2 dalton and 795.3 dalton, respectively when analysed by ESI-TOF mass spectrophotometer.

7. The structure of products 1, 2 and 3 were identified as α-D-glucopyranosyl-(1→4)-salicin (glucosyl salicin), α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-salicin (maltosyl salicin) and α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-(1→4)-α-D-glucopyranosyl–salicin (triosyl salicin), respectively.

8. The solubility of glucosyl-salicin, maltosyl-salicin and triosyl-salicin were glucosyl salicin, maltosyl salicin and triosyl salicin were 72.23, 134.46 and 207.76 mg/ml, respectively , corresponding to 3,6 and 9 times higher than that of salicin.

9. Three of the salicin glycosides, glucosyl salicin, maltosyl salicin and triosyl salicin, showed higher water solubility and anti-coagulation activity compared to salicin.

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APPENDICES

Apendix A : Preparation for polyacrylamide gel electrophoresis.

1) Stock reagents.

30% acrylamide, 0.8% bis- acrylamide, 100 ml.

acrylamide	29.2	g.
N,N'-methylene- bis- acrylamide	0.80	g.

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 1M HCl and adjusted volume to 100 ml. with distilled water.

2.0 M Tris-HCl pH 8.8.

Tris(hydroxymethyl)-aminomethane	24.20	g.

adjusted pH to 8.8 with 1M 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 6.8 with 1M HCl and adjusted volume to 100 ml. with distilled water.

1.0 M Tris-HCl pH 6.8.

Tris(hydroxymethyl)-aminomethane	12.10	g.

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

2) Non-denaturing PAGE

7.5 % Separating gel.

30% acrylamide solution	2.5	ml.
1.5 M Tris-HCL pH 8.8	2.5	ml.
distilled water	5.0	ml.
10%$(NH_4)_2S_2O_8$	50	µl.
TEMD	10	µl.

5.0 % Stacking gel.

30% acrylamide solution	0.67	ml.
1.5 M Tris-HCL pH 8.8	1.0	ml.
distilled water	2.3	ml.
10%$(NH_4)_2S_2O_8$	30	µl.
TEMD	5	µl.

Electrophoresis buffer, 1 litre.

Tris(hydroxymethyl)-aminomethane	3.0	g.
Glycine	14.4	g.

adjusted volume to 1 litre with distilled water (pH should be approximately 8.3).

Staining solution, 100 ml.

ethanol	27	ml.
acetic acid	10	ml.
coomassie brilliant blue R-250	0.04	g.
$CuSO_4$	0.50	g.
distilled water	63	ml.

Dissolved the $CuSO_4$ in water before adding the alcohol. Either dissolved the dye in alcohol or added it to the solution at the end. Immersed the gel in stain for approximately 1-2 hours.

Destaining solutions

First destaining solution, 100 ml.

ethanol	12	ml.
glacial acetic acid	7	ml.
$CuSO_4$	0.50	g.
distilled water	81	ml.

Dissolved the $CuSO_4$ in water before adding the alcohol Immersed the gel in two of three 500 ml. changes of this solution until the background was nearly clear. Gentle agitation and slight heating will speed the destaining process.

Second destaining solution, 100 ml.

ethanol	25	ml.
glacial acetic acid	7	ml.
distilled water	68	ml.

Immersed the gel in this solution to removed the least traces of stain and $CuSO_4$.

Note: Prolong soaking of gels with gel support flim backing in acetic solutions may cause the gel to separate from the backing. Staining and destaining steps should be no longer than 3-4 hours.

Appendix B : Preparation for buffer solution.

0.2 M sodium acetate pH 4.0, 5.0, and 6.0

CH3COONa	1.21	g.

Adjusted volume to 100 ml. with distilled water. Adjusted to pH 4, 5 and 6 by 0.2 M acetic acid.

0.2 M phosphate pH 6.0.

KH_2PO_4	3.28	g.
K_2HPO_4	0.16	g.
distilled water	100	ml.

0.2 M phosphate pH 7.0.

KH_2PO_4	1.35	g.
K_2HPO_4	1.67	g.
distilled water	100	ml.

0.2 M phosphate pH 8.0.

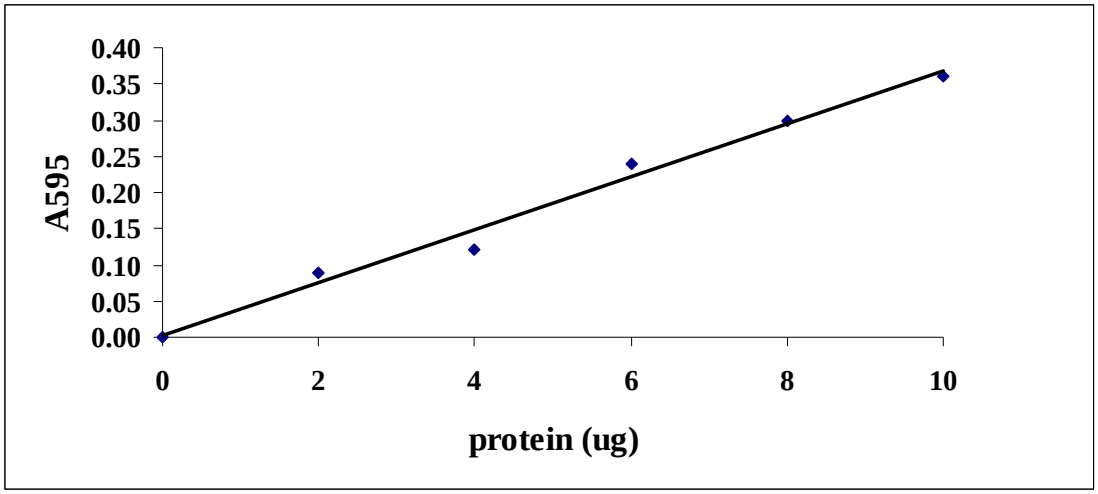
KH_2PO_4	0.48	g.
K_2HPO_4	2.34	g.
distilled water	100	ml.

0.2 M Tris-glycine NaOH pH 8.0, 9.0 and 10.0.

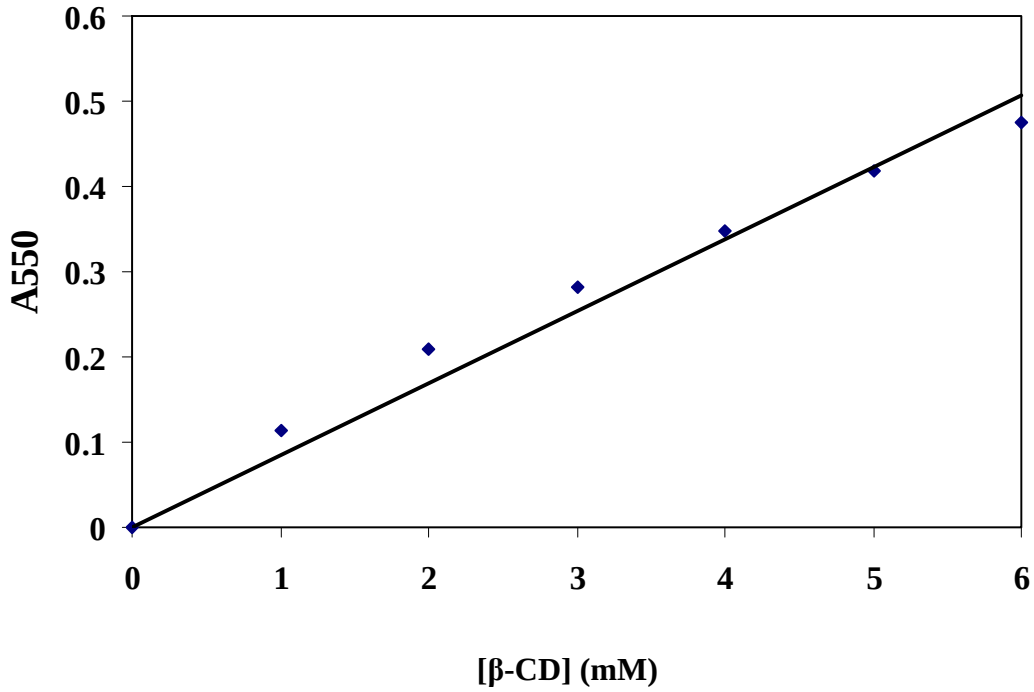
Glycine	1.5	g.

Adjusted to pH 8.0, 9.0 and 10.0 by 1 M NaOH and adjusted volume to 100 ml. with distilled water.

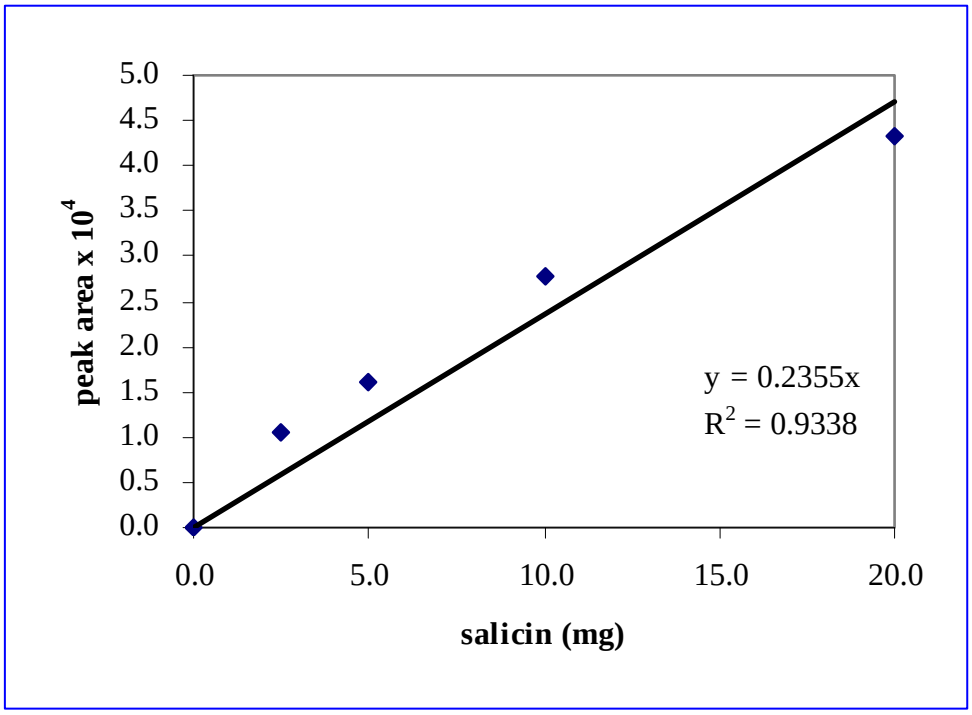
Appendix C : Standard curve for protein determination by Bradford's method.



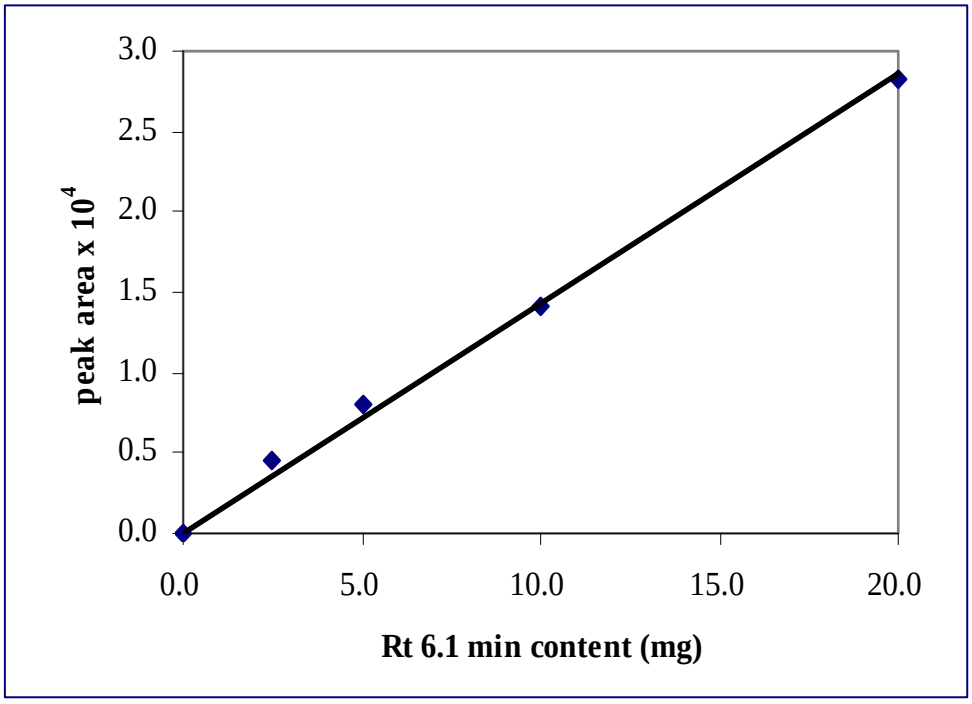
Appendix D : Standard curve of β-cyclodextrin by phenolphthalein method.



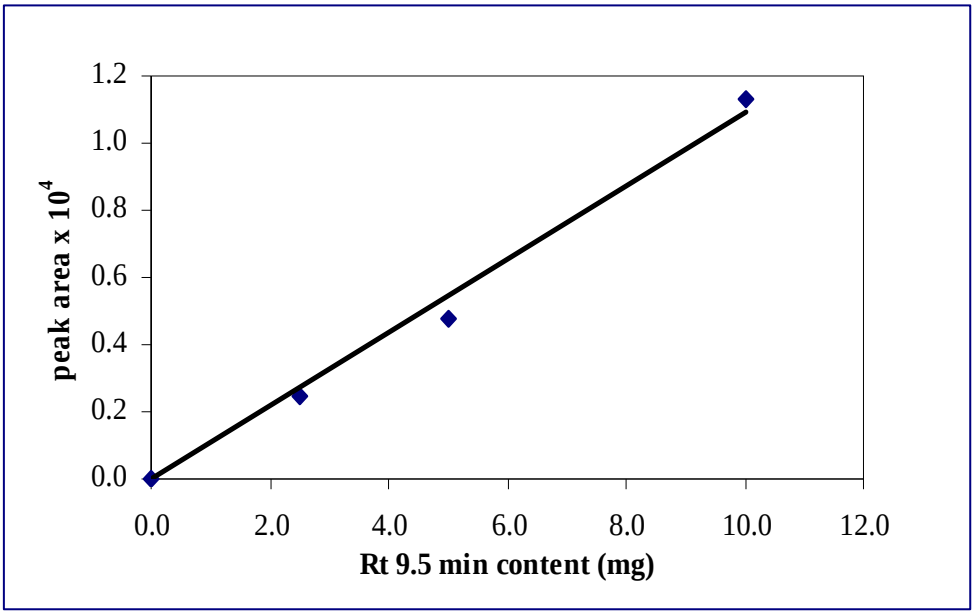
Appendix E : Standard curve of salicin by HPLC.



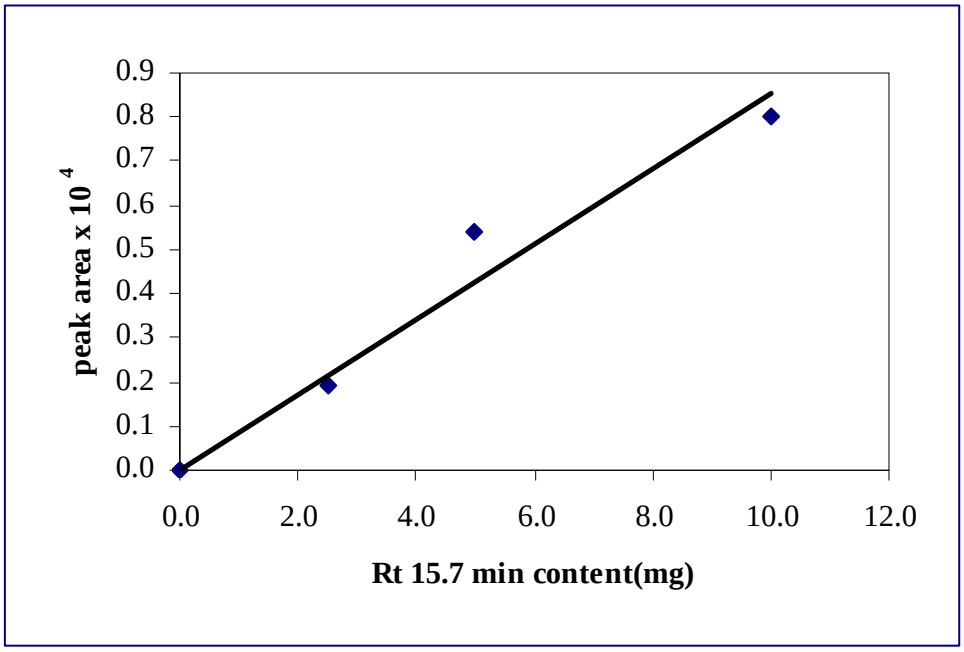
Appendix F : Standard curve of the product at retention time 6.1 minutes by HPLC.



Appendix G : Standard curve of the product at retention time 9.5 minutes by HPLC.

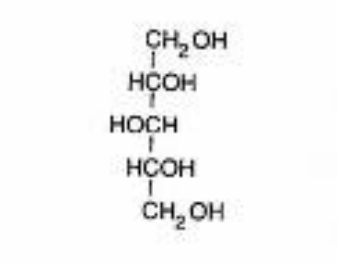


Appendix H : Standard curve of the product at retention time 15.7 minutes by HPLC.

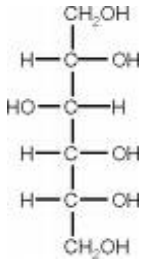


Appendix I : Structure of sugar

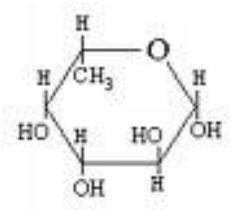
Xylitol



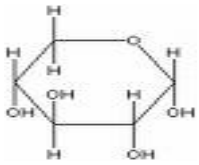
Sorbital



Fucose



Xylose



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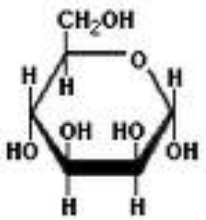

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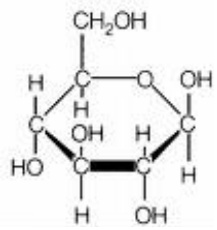

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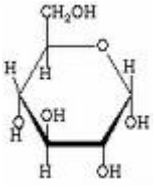

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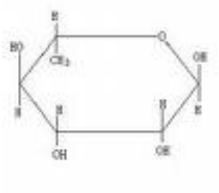
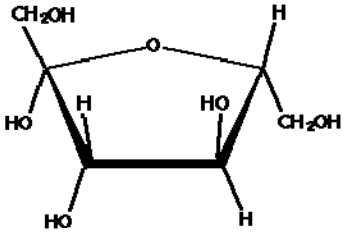


Fructose



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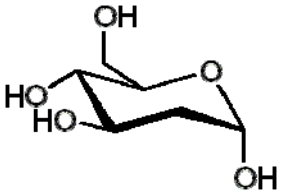

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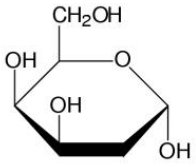

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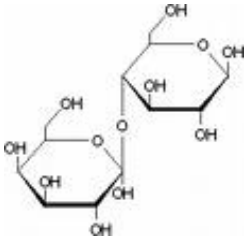

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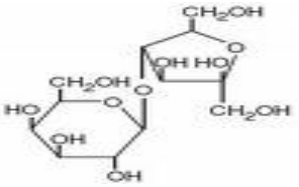

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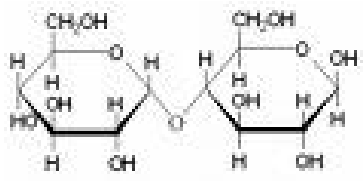
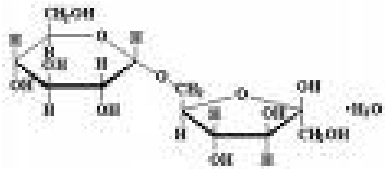
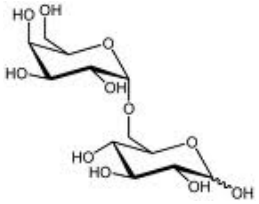


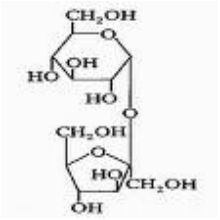
Palatinose



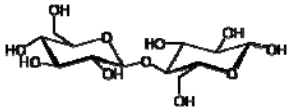
Melibiose



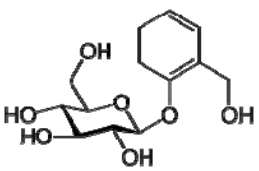
Sucrose



Cellobiose



Salicin



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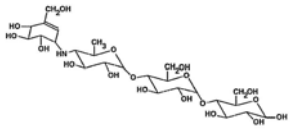
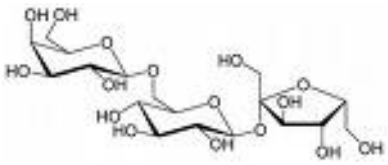
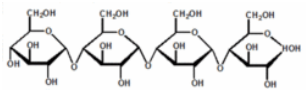


Raffinose



Maltotetraose



Moltotriose

BIOGRAPHY

Miss Prapai Hongsa was born on January 10th, 1977. She graduated with the Bachelor's degree of Science in General Science program from Prince of Songkhla University in 2000, and continued studying for the Master's degree of Science in Biochemistry program, Faculty of Science at Chulalongkorn University in 2008.