APPLICATION OF 4-α-GLUCANOTRANSFERASES FOR THE SYNTHESIS OF FUNCTIONAL GLYCOSIDES AND OLIGOSACCHARIDES

BY

MISS NATTIDA KHAMMANEE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(BIOCHEMISTRY AND MOLECULAR BIOLOGY)
FACULTY OF MEDICINE
THAMMASAT UNIVERSITY
ACADEMIC YEAR 2019

APPLICATION OF 4-α-GLUCANOTRANSFERASES FOR THE SYNTHESIS OF FUNCTIONAL GLYCOSIDES AND OLIGOSACCHARIDES

BY

MISS NATTIDA KHAMMANEE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(BIOCHEMISTRY AND MOLECULAR BIOLOGY)
FACULTY OF MEDICINE
THAMMASAT UNIVERSITY
ACADEMIC YEAR 2019

THAMMASAT UNIVERSITY

FACULTY OF MEDICINE

DISSERTATION

BY

MISS NATTIDA KHAMMANEE

ENTITLED

APPLICATION OF 4-α-GLUCANOTRANSFERASES FOR THE SYNTHESIS OF FUNCTIONAL GLYCOSIDES AND OLIGOSACCHARIDES

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

on August 28, 2020

Chairman Prakorn R.
(Lieutenant Colonel Assistant Professor Prakarn Rudeekulthumrong, Ph.D.)

Member and Advisor
(Associate Professor Jarunee Kaulpiboon, Ph.D.)

Member Pintusorn Hansakul
(Assistant Professor Pintusorn Hansakul, Ph.D.)

Member K. Aree
(Assistant Professor Kalaya Aree, Ph.D.)

Member Natwadee Poomipark
(Natwadee Poomipark, Ph.D.)

Dean Dilok Aw
(Associate Professor Dilok Piyayotai, M.D.)

Dissertation Title	APPLICATION OF 4-α-GLUCANOTRANSFERASES FOR THE SYNTHESIS OF FUNCTIONAL GLYCOSIDES AND OLIGOSACCHARIDES
Author	Miss Nattida khammanee
Degree	Doctor of Philosophy (Biochemistry and Molecular Biology)
Major Field/Faculty/University	Biochemistry and Molecular Biology Medicine Thammasat University
Dissertation Advisor	Associate Professor Jarunee Kaulpiboon, Ph.D.
Academic Year	2019

ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) and amylomaltase (AMase) are the enzymes in 4-α-glucanotransferase family (4-α-GTase). Both enzymes play many important as a catalyst in transglycosylation reaction to produce functional glycosides and oligosaccharides. In this research, the main purpose of this study was to synthesize pinoresinol-α-D-glycosides (PGs) and xylose-α-D-glycosides (XGs) using recombinant CGTase and AMase, respectively. The influence of various parameters on the productions of PGs and XGs was investigated. For PGs, the optimal conditions for synthesizing were to incubate 0.5% (w/v) β-cyclodextrin, 1.5% (w/v) pinoresinol and 80.0 U/mL of CGTase in 20 mM Tris-HCl buffer, pH 9.0 at 50 ^{o}C for 60 hours. Under these conditions, two PG transfer products were detected by TLC and mass spectrophotometer with molecular weights of 543.54 and 682.19 Daltons, corresponding to pinoresinol monoglycoside (PG_1-I and PG_1-II) and pinoresinol diglycoside (PG_2), respectively. The PGs were also prepared in large scale and purified by preparative TLC and HPLC. The structures of PG_1 and PG_2 were confirmed as pinoresinol-α-D-glucopyranoside and pinoresinol-α-D-diglucopyranoside by ^{1}H-NMR and ^{13}C-NMR analysis. The free-radical scavenging and anti-inflammatory activities of

PG_1 and PG_2 were comparatively studied with the original pinoresinol using DPPH radical scavenging reaction and β-glucuronidase inhibition assay. The results found that the free-radical scavenging and anti-inflammatory activities of PG_1 and PG_2 reduced when compared to original pinoresinol. The loss of antioxidant and anti-inflammatory activities might result from the addition of sugar in the position 4-OH of pinoresinol that may have disturbed its electron rotation. However, in fact, the PGs need to be converted to the pinoresinols before they are absorbed and so loss of activity may be minimal. Interestingly, the α-glucosylated compounds which showed the better change of physicochemical properties such as solubility and sweetness could promote a positive effect on the bioavailability of original pinoresinol.

In part of XGs, they were synthesized using tapioca starch (TS) and xylose through the transglycosylation reaction of recombinant AMase from p19bAMY cell. The optimal conditions for XGs synthesis were to incubate 1.5% (w/v) TS, 2% (w/v) xylose and 4 U/mL of AMase in 20 mM Tris-HCl buffer, pH 7.0 at 70 °C for 120 hours. Upon analysis of the products by TLC, at least 2-4 xylose glycosides were observed. These transferred products were formed with molecular weights of 335.10 and 497.20 Daltons as determined by mass spectrometry analysis; these values were in accordance with xylose monoglycoside (XG_1) and xylose maltoside (XG_2), respectively. The XG_1 and XG_2 were also prepared on a large scale and subsequently purified by preparative TLC and HPLC. The structure of XG_1 and XG_2 were confirmed as xylose-α-D-monoglucopyranoside and xylose-α-D-diglucopyranoside by ^{1}H-NMR and ^{13}C-NMR analysis. The long carbohydrate chains of xylose were less effective in sweetness property and acidic resistance than original xylose and sucrose. However, both XG_1 and XG_2 showed more solubility, maillard reaction, and prebiotic properties than original xylose. Thus, the glucosides of xylose have the potential of being incorporated into foods for health benefits.

Keywords: Transglucosylation reaction, Cyclodextrin glycosyltransferase (CGTase), Amylomaltase (AMase), Functional glycosides, Functional oligosaccharides

ACKNOWLEDGEMENTS

My deepest thankfulness goes to my advisor, Assoc. Prof. Dr. Jarunee Kaulpiboon, Department of Pre-clinic Science (Biochemistry), Faculty of Medicine, Thammasat University for guiding the research investigations, designing experiments, assisting the data analysis, writing the manuscript, editing the thesis, and providing financial support for the research.

Sincere thanks to Lt. Col. Assist. Prof. Dr. Prakarn Rudeekulthamrong, Department of Biochemistry, Phramongkutklao College of Medicine, Assoc. Prof. Pintuson Hansakul, Ph.D., Assist. Prof. Kalaya Aree, Ph.D. and Natwadee Poomipark, Ph.D. Department of Pre-clinic Science (Biochemistry) Faculty of Medicine, Thammasat University for their valuable comments and suggestions.

I am grateful to all staff of the Faculty of Medicine, Thammasat University for their kind help and support.

I deeply appreciated Faculty of Medicine, Thammasat University, Thailand for laboratory supporting, and I also thank Drug Discovery and Development Center, Thammasat University, and Center of Scientific Equipment for Advanced Research, Thammasat University for using HPLC, TLC Scanner 4 and TLC Visualizer on special rate service.

Finally, million thanks to my family for mentally support and encouragement.

Miss Nattida Khammanee

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percentage
/	Per
<	Less than
=	Equivalent
>	Greater than
±	Plus-minus
X	Multiplication
Δ	Delta
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µmol	Micromole
A	Absorbance
AMase	Amylomaltase
CDs	Cyclodextrins
CGTase	Cyclodextringlycosyltransferase
DMSO	Dimethylsulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
E.C.	Enzyme commission number
et al.	Et alibi, and colleagues
g	Gram
i.e.	In other word
kDa	Kilodalton
M	Molar
m/z	Mass-to-charge ratio
mA	Milliamp

mg	Milligram
mL	Milliliter
mM	Millimolar
mmol	Millimole
MS	Mass spectrometry
nm	Nanometer
O.D.	Optical density
P	Pinoresinol
PAGE	Polyacrylamide gel electrophoresis
PG_1	Pinoresinol monoglucoside
PG_2	Pinoresinol diglucoside
Rf	Relative mobility
Rt	Retention time
Rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Trolox	6-hydroxy-2,5,7,8-tetramethyl
U	Unit (s)
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
X	Xylose
XG_1	Xylose monoglucoside
XG_2	Xylose maltoside
α-CD	α-Cyclodextrin
β-CD	β-Cyclodextrin
γ-CD	γ-Cyclodextrin

CHAPTER 1
INTRODUCTION

1.1 Background and rationale

4-α-glucanotransferase (4-α-GTase) is an enzyme in alpha-amylase family. It functions as a catalyst in transglycosylation reaction to transfer α-1, 4-glucan from the reducing agent in glucosyl group to the acceptor. The significant enzymes in 4-α-GTase group are cyclodextrin glycosyltransfersase (CGTase, EC 2.4.1.19) which is an extracellular enzyme and amylomaltase (AMase, EC 2.4.1.25) which is an intracellular enzyme. Various CGTases excreted from *Bacillus* spp. For AMase, it was first found in *Escherichia coli* (Hashimoto et al., 1998), and then it was found in other bacteria according to the report like *Thermus aquaticus* (Fujii et al., 2005) and *Corynebacterium glutamicum* (Srisimarat et al., 1998). CGTase and AMase can catalyze the chemical reaction into four different ways (Salva et al., 1997) as the followings: disproportionation, cyclization, coupling and hydrolysis reactions. (a) Disproportionation is the reaction that glucosyl group is displaced in oligosaccharides chain (intermolecular transglucosylation) and produce chain length polymer of oligosaccharides. (b) Cyclization reaction is the reaction of displacing glucosyl group in the same oligosaccharide polymer (intramolecular transglucosylation) and produce cyclodextrin as a product. This reaction is main reaction of both enzymes. (c) Coupling reaction is a reversible reaction of cyclization reaction. Cyclodextrin (glucosyl donor) is hydrolyzed to linear oligosaccharides the reducing agent of glucosyl and the acceptor is a short linear polymer of oligosaccharides and transfers its oligosaccharides to glucosyl acceptor to produce a longer linear polymer. (d) Hydrolysis reaction is a reaction which displaces glucosyl group to H_2O molecule. With hydrolysis reaction, both enzymes show very low activity (Figure 1.1).

There are many reports about transglycosylation abilities of 4-α-GTase such as CGTase, AMase and other enzymes. As reported previously, CGTase and AMase were able to be used as catalyst in the synthesis of functional glycosides and

oligosaccharides using CDs or starches as glucosyl donors and saccharides, alcohols sugar, alcohols or polyphenolic compound as acceptors. Kitahata and others (Kitahata et al., 1976) found that L-sorbose, D-xylose and D-galactose were specific acceptors of CGTase from *Bacillus magaterium* strain no. 5. Cellobiose was an effective acceptor of CGTase from *Bacillus circulans* A11 when used β-CD as a glucosyl donor (Wannapa et al., 2000). This enzyme was specific to 6-carbon-atom monosaccharides and configuration of hydroxyl group at C2- and C4-position like glucose. Besides, Chotipanang et al. study the acceptor specificity in alcohol group; they found that CGTase from *Paenibacillus* sp. RB01 had a specificity on short-chain linear alcohols, especially 30% (v/v) methanol under when using β-CD as a glucosyl donor. Under this reaction, it could produce methyl monoglycoside, methyl diglycoside and methyl triglycoside. These products are called that akyl glycosides and used as surfactant in manufacturing (Chotipanang et al., 2011). With AMase, there were a few studies on their transglycosylation reaction. In 2013, Saehu and coworkers have successfully synthesized anticariogenic oligosaccharides (maltooligosylsucroses: G_2F-$G_{11}F$) through transglycosylation reaction of AMase from *Corynebacterium glutamicum* using starch and sucrose as glucosyl donor and acceptor. They reported that the synthesized products could suppress the activity of glucosyltransferase from *Streptococcus mutans* which is the cause of tooth decay. In the same year, Rudeekulthamrong and others (Rudeekulthamrong et al., 2013) reported that the combination of AMase and transglucosidase could produce the transferred products, prebiotic isomaltooligo-saccharids, from maltotiose. So, the significant benefit of transglycosylation reaction of 4-α-GTase is to make glu/glycosides or to improve the property of oligosaccharides or polyphenolic compound e.g. water solubility, biological action, stability and reducing bitter taste in better way.

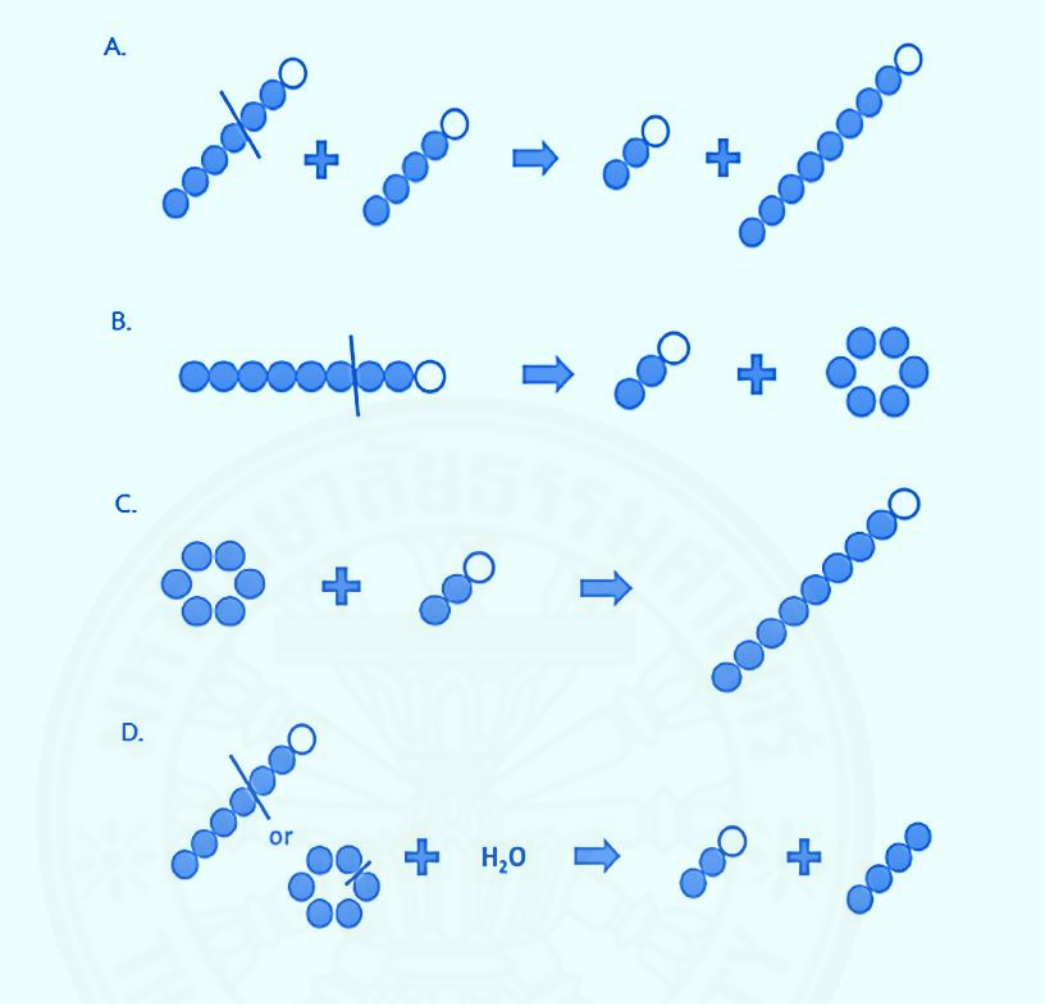


Figure 1.1 The catalytic mechanism of CGTase (A.) disproportionation reaction, (B.) cyclization reaction, (C.) coupling reaction and (D.) hydrolysis reaction. The circles represent glucose residues; the white circles indicate the reducing end sugars.

For example, Kurimoto and coworker reported that glycoside of vitamin E was more stable on oxidative reaction than the original vitamin C (Kurimoto et al., 1997) and hesperidin glycoside which was the glycosylated product from transferring glucosyl group of β-CD to hesperidin showed a 300-fold increase in water solubility (Kometani et al., 1994).

Therefore, according to the transglycosylation reaction of 4-α-CGTase from several reports, this research aims to study the synthesizing of functional glycoside and oligosaccharide using the recombinant CGTase and AMase. This is not only to apply the use of CGTase and/or AMase to synthesize functional glycoside and oligosaccharide products, but also to use these products in other ways like the study of their physiochemical and biological properties, etc. This knowledge will help more understanding about the role of enzyme in synthesizing the new kinds of functional glycosides and oligosaccharides, e.g., phenolic, flavonoid and steroidal (cardiac) glycosides, low-calorie sugar and prebiotic. These glycosides can be used as conventional medicine to treat the illness or as medicine composition to improve their efficacy. Using the extract from herbs, they have several limitations, for example, the quantity of herbs is not sufficient and the extract is varied depending on season. In addition, the knowledge occurred from this research leading improvement bioavailability of products by adding sugar to flavonoid and oligosaccharide through the transglycosylation reaction of the CGTase and AMase. For functional oligosaccharide products, they can be used as sweetener such as sugar alcohols prebiotics and flavoring healthy foods. In addition, the advantage for using enzyme in the synthesis of products is friendly performed with environment and moreover, its process cost in production is lower than the chemical production process that must produce in elevated temperature and high pressure. Besides, the chemical process also uses more procedures which cause pollution. Thus, in this research we here focus on syntheses of functional glycoside and oligosaccharide products by through the transglycosylation reaction which is catalyzed by 4-α-glucanotransferase (CGTase and AMase).

1.2 Objectives

1.2.1 To purify and characterize the cyclodextrin glycosyltransferase and amylomaltase.

1.2.2 To find the optimum conditions for the synthesis of functional glycosides and oligosaccharides with mono- and disaccharides.

1.2.3 To identify the structure of obtained functional glycosides and oligosaccharides.

1.2.4 To investigate the physical and biological properties of functional glycosides and oligosaccharides.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Glycosides

Glycoside is a molecule in which a sugar is bound to another functional chemical group via a glycosidic bond. Glycoside is classified as one of the major organic compounds that can be found from the extraction of high-class plants. Its structure composes of two parts: the sugar part is called glycone and the non-sugar part is called aglycone or genin. Most glycosides have biological effects, so they can be used as medicine. The pharmacological effect of glycosides mostly depends on proportion of aglycone. Glycoside can be divided into many groups (Nakano and Kitahata, 2005) using its aglycone as follows:

2.1.1 Cardiac glycosides

The family "Solanaceae" does not have a corner on poisonous/medicinal plants. There are about 20 species of foxglove (*Digitalis* spp.: figwort family, Scrophulariaceae). Digoxin (Figure 2.1) is a cardiac glycoside which is extracted from foxglove. It often goes under the name of digitalis. Some cardiac patients under treatment for congestive heart failure and atrial arrhythmia carry a supplement of digitalis pills for self-medication if they feel symptomatic and are away from professional help, although its use is declining. Overdosing of digitalis can result in death. People have sometimes confused foxglove with comfrey (*Symphytum* spp.) and brewed a toxic "comfrey tea". However, comfrey contains the pyrrolizidine alkaloid retronecine that is hepatotoxic and linked to liver cancer and probably should not be ingested. Treatments for accidental and purposeful overdoses of these drugs remain a major field of research (Levine et al., 2011).

2.1.2 Cyanogenic glycosides

Cyanide has been a popular poison for homicides and mass murders. It inhibits the mitochondrial enzyme, cytochrome c oxidase, and stops cellular respiration leading to death in minutes. It can be administered as a gas (hydrogen cyanide or prussic acid) or taken orally in compound form (e.g., potassium cyanide, sodium thiocyanate). In 2013, Urooj Khan of Chicago cashed in his lottery ticket for $600,000 only to fall ill the next day and die. It was considered a natural death until a relative pressed the authorities to exhume his body and does toxicology scan. The results indicated that he had been poisoned with cyanide. Although the common sources for cyanide are artificial, more than 1,500 species, mostly angiosperms, produce cyanogenic glycosides as predator deterrents. Although these compounds may produce unpleasant effects in humans, the concentrations are not likely to be lethal. During droughts, the lack of water increases the concentrations of cyanogenic glycosides, and they prove more toxic to insects and other predators that attempt to feed on the leaves, stems, or roots. Amygdalin (Figure 2.2) is present in the almond fruits of *Prunus dulcis*. Its name is derived from the ancient Greek word for “almond”. There are two varieties of almonds, one that tastes sweet (variety dulcis) and one that is bitter (variety amara, also called bitter almond). In the bitter almond, amygdalin is enzymatically converted to the toxic prussic acid and benzaldehyde, the chemical that gives the almond a bitter taste. The edible almonds consumed in the USA are sweet almonds, but bitter almonds can be found in specialty stores. Cyanogenic glycosides may be found throughout many edible fruits, including, apples, peaches, pears, raspberries, cherries, apricots, and plums, but they are especially concentrated in the seeds. If these seeds are swallowed, they generally pass through the digestive system untouched. It is strongly recommended, however, that elderberries not be eaten raw as cooking releases a considerable amount of cyanide from the pulp of the fruits that diffuses harmlessly into the air. Bamboo shoots have high concentrations of cyanogenic glycosides as do cassava roots. The processing of cassava roots (the source of tapioca) requires release of cyanide from the glycoside linamar (Figure 2.2) (also present in lower amounts in lima beans and flax). Otherwise, the cassava roots are very

poisonous. Considerable amounts of cyanide are also released from burning tobacco although the amounts of cyanide inhaled are well below the lethal range.

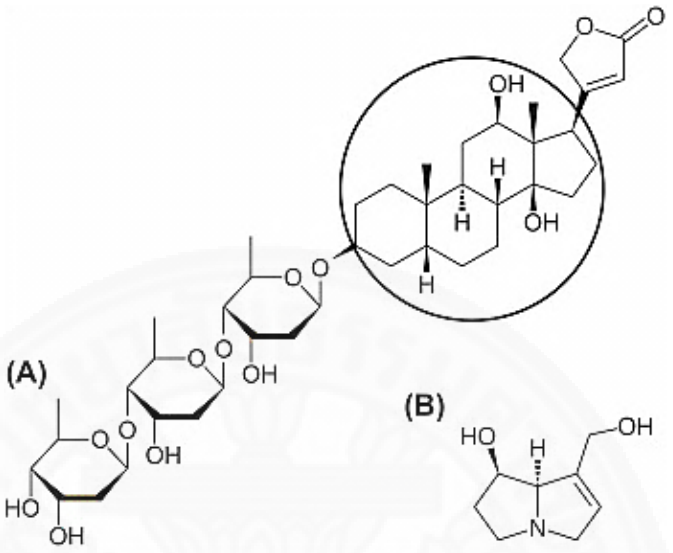


Figure 2.1 (A) Digoxin (digitalis), a cardiac glycoside from *Digitalis* spp. Note the inclusion of a steroid nucleus (circle). **(B)** Retronecine, a pyrrolizidine alkaloid extractable from comfrey.

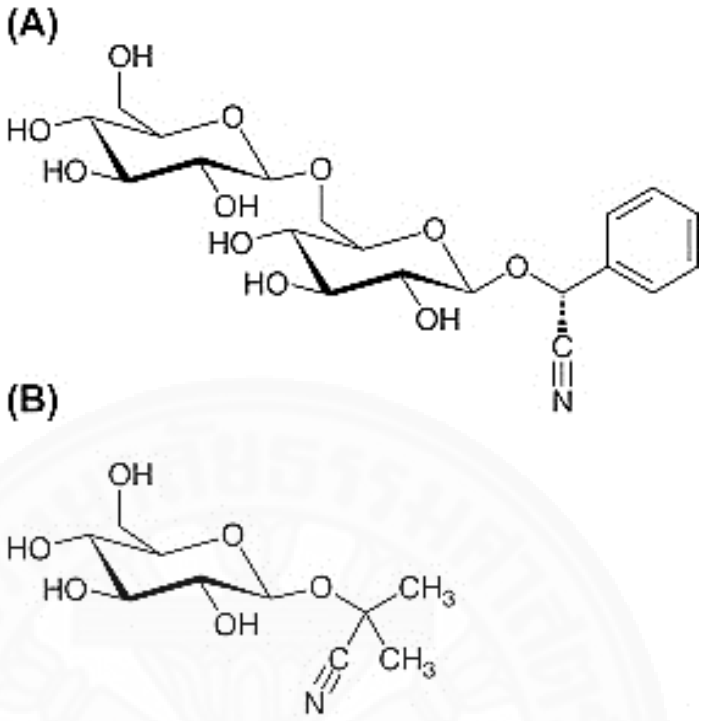


Figure 2.2 Cyanogenic glycosides. **(A)** Amygdalin; **(B)** Linamarin. CN = cyanide grouping (Forensic Plant Science).

2.1.3 Phenolic glycosides

This glycoside group has aglycone as phenol compound. It is used as painkiller, diuretic, artificial flavor and a composition in slain lightening cosmetic like arbutin or hydroquinone-beta-D-glycoside. The phenolic glycosides can be found in several plants like berries and pears etc. They are used as an essential composition in high-end cosmetic. The phenolic glycoside and arbutin can inhibit tyrosinase enzyme which is an important enzyme in melanin production. In addition, the one advantage of arbutin is that it does not make to thin skin. For the structure of arbutin, it composes of glycone as glucose and aglycone as hydroquinone. The hydroquinone also inhibits the production of melanin but it is skin irritant. In contrast, the glycosylated hydroquinone, arbutin, is safer for using as a skin brightening agent (Tomita et al., 1990; Kitao and Sekine, 1994; Nishimura et al., 1994; Sugimoto et al., 2003).

2.1.4 Alkyl glycosides

Alkyl glycosides such as methyl-, propyl- and other are a class of non-ionic surfactants widely used in a variety of cosmetic, household, and industrial applications. This glycoside composes of aglycone as alkyl like methyl and ethyl which is used as surfactant and drug carrier (Larsson et al., 2005; Sarney and Vulfson 1995; Turner et al., 2007). The raw materials are typically starch and fat, and the final products are typically complex mixtures of compounds with different sugars comprising the hydrophilic end and alkyl groups of variable length comprising the hydrophobic end. When derived from glucose, they are known as alkyl polyglycosides. Alkyl glycosides that are uses as surfactant has a significant property that is when adding the surfactant in water it will reduce the surface tension to make the chemical reaction occur easily such as foaming, drenching, cleaning process, etc. Accordingly, this is applied to be a compound in several products like toothpaste, hair conditioner, fabric softener, detergent, dishwashing liquid and soap (Rosen, et al., 1989) or to use myristyl, cetyl and stearyl glycosides as drug carriers due to the fact that these alkyl glycosides can have the configuration like liposomes (Hiroshi et al., 1985).

2.1.5 Alcoholic glycosides

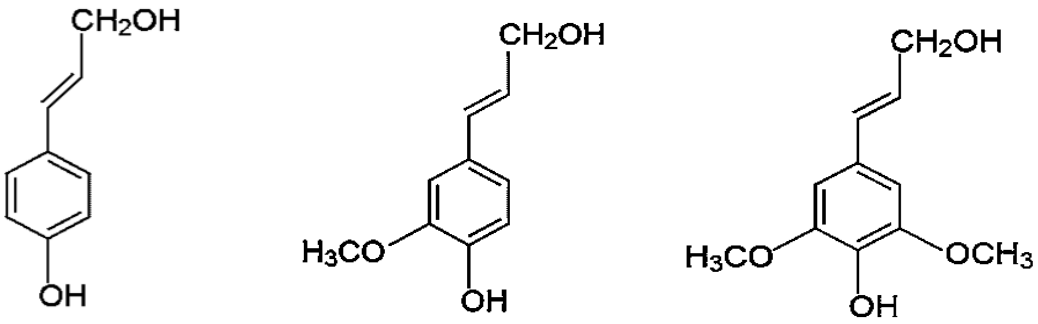
This group consists of aglycone like alcohol. It is used to be analgesic antipyretic drugs, anticoagulants, anti-inflammatory drugs and antihistamine, for example, salicin or 2-(hydroxymethyl) phenyl-β-D-glucopyranoside which is distilled from willow is a drug substance that relieves the pain. But, the limitation of it is when taking the salicin it will irritate the stomach. To add acetylchloride into salicin will form acetyl salicylic acid or aspirin which we use as painkiller nowadays (Jung et al., 2009).

2.1.6 Lignan glycosides

Lignan glycosides have aglycone as lignan. There is C6-C3 (aromatic bonds 3-atom carbon) as a basic formula that para-cinnamic acid will be added to hydroxyl group employing hydroxylase enzyme which are para-coumaroyl alcohol, coniferyl alcohol and cinapyl alcohol as monomers (Figure 2.3). In case the two monomers bonds together (X_2), it will form lignan. Yet, if it bonds as polymer (X_n), it will be called lignin.

Some types of lignan biological synthesis start from coniferyl alcohol (Figure 2.3 C) is oxidized to be coniferyl alcohol radical. When the coniferyl alcohol radical dimerizes, it will form dimer (Figure 2.4 A-D) that is the significant intermediate.

A. B. C.



D.

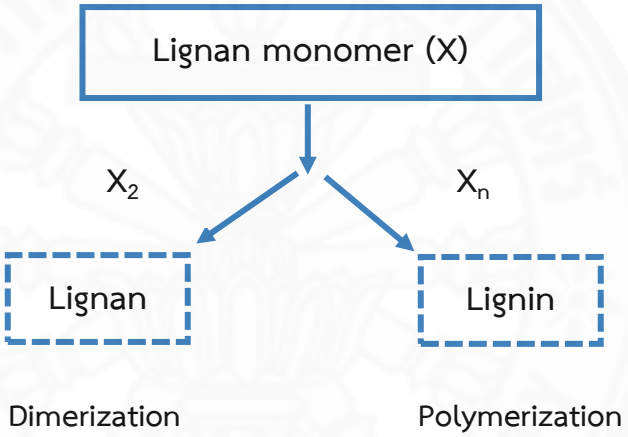
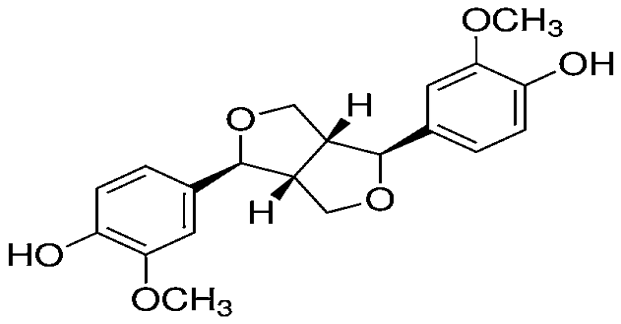
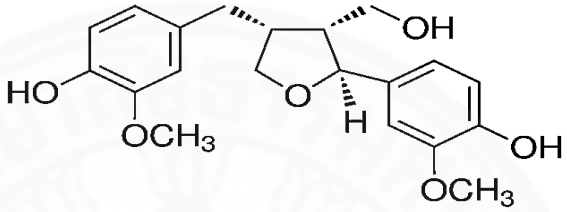


Figure 2.3 Structure of (**A.**) *p*-Coumaroyl alcohol, (**B.**) Coniferyl alcohol (**C.**) Sinapyl alcohol (Monomer of lignans) and (**D.**) Dimerization and polymerization of lignan monomer.

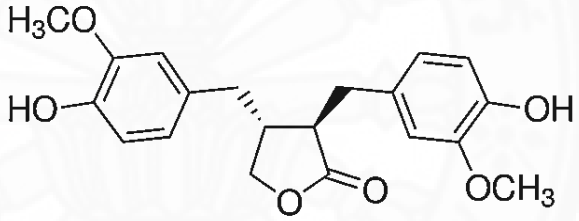
A.



B.



C.



D.

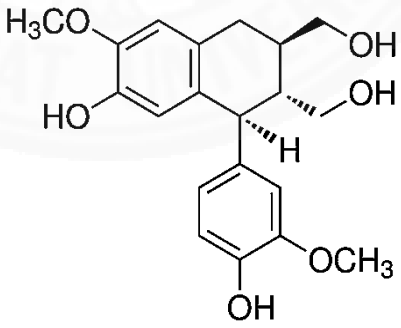


Figure 2.4 Structure of (**A.**) Pinoresinol, (**B.**) Lariciresinol, (**C.**) Matairesinol and (**D.**) Isolariciresinol (Dimer of lignans).

According to the study, it is found that lignan compounds and lignan glycosides active like estradiol. When it gets into the intestine, the estradiol will be active. That is why the estradiol plays an influential role to cure menopause and to

prevent cardiovascular disease and osteoporosis (Kumar and Chopra, 2005; Schroeder et al., 2006; Feng et al., 2007; Peterson et al., 2010; Kim et al., 2015).

In addition, there are also other interesting groups of glycosides i.e., anthraquinone glycosides, saponin glycosides, and flavonoid glycosides.

2.2 Oligosaccharide products

An oligosaccharide is a saccharide polymer containing a small number (typically three to ten) of monosaccharides (simple sugars). Oligosaccharides consist of 2-20 parts of monosaccharides bonded with glycosidic bond. Several types of oligosaccharides can be found in food like vegetables, fruits, milk and honey. It is found that some types of oligosaccharides have high efficiency to boost up the quality of functional food or indigestible oligosaccharides. These is the essential bacteria's food in intestine and increase their population by growing bifidobacteria. It stimulates minerals absorption, prevents heart disease and reduces cholesterol in plasma and glucose in blood. In addition, there is the use of oligosaccharides to apply with chemical properties and physical properties of food as well (Nakakuki et al., 2002) or to manufacture anticariogenic oligosaccharides. To extract oligosaccharides by means of natural or chemical method is quite difficult. However, to extract by using the enzyme is more efficient and highly specified, so this is suitable for manufacturing in industries. To use the enzyme synthesizing oligosaccharides can be made by displace some parts of the agent's and the acceptor's oligosaccharides line. The relation of substrate's structure and specificity of the enzyme will determine the structure of glycosidic bond. There is a research that used the enzyme displacing glucosyl group such as glucansucrase, cyclodextrin glycosyltransferase and alpha-amylase to add the glucosyl group to other acceptors.

2.3 Cyclodextrin glycosyltransferase (CGTase)

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is an extracellular enzyme that produces cyclodextrins (CDs) from starch substrate. CDs are non-reducing

cyclic oligosaccharides with the special structure of a doughnut. The interior of CDs is hydrophobic and its external surface is hydrophilic. Due to this feature, CDs are able to form inclusion complexes with either organic or inorganic guest molecules (Abdel et al., 2011). This can lead to the change of physiological and chemical properties of guest molecules. CGTase is an important industrial enzyme in which is used to produce cyclodextrins. It is inducible enzyme that is produced only by microbial cells. CGTase genes from more than 30 bacteria have been isolated and several of the enzymes have been identified and biochemically characterized. For a better understanding of the reaction mechanism and function of CGTase, the enzyme has been analyzed at gene level and protein level with regard to its structure and the similarity of different CGTase subgroups. The biological role of enzyme is also proposed, based on its application in biotechnology for the production of CDs, functional oligosaccharides and glycosides with novel properties (Qi and Zimmermann, 2005).

For the catalytic mechanism of CGTase in detail, CGTase can catalyze four related reactions; cyclization, coupling, disproportionation and hydrolysis (Figure 2.1). Cyclization reaction is a reaction in which cyclodextrins are 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 is transferred to an acceptor maltooligosaccharide substrate. Another reaction of CGTase, a linear maltooligosaccharide is cleaved and the new reducing end is transferred to an acceptor maltooligosaccharide substrate in disproportionation reaction. 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 its hydrolysis of amylase or the linearization of cyclodextrin (Kitahata et al., 1976). As described, the major product of cyclization reaction is α-, β- and γ-cyclodextrins. CGTase is a member of the α-amylase family of glycosyl hydrolase. 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).

2.3.1 Structure of CGTase

Structures 3D of five CGTase from *Bacillus circulans* strain 8 (Klein et al., 1991), *Bacillus* sp. Strain 1011 (Harata et al., 1996), *B. circulans* strain 251 (Penninga et al., 1994), *Thermoanaerobacterium thermosulfurigenes* strain EM1 (Knegtel et al., 1996), and a strain of *Bacillus stearothermophilus* (PDB: 1CYG) have been resolved. All of which contain of five domains, A, B, C, D, and E, with common domain structure and organization. Domain A includes the central $(\beta/\alpha)_8$ barrel with the bulging domain B, which together forms the portion of substrate binding and catalytic subsites (Lawson et al., 1994). Domain E is a starch-binding domain. The functions of domains C and D, nevertheless, are not known. The catalytic domain of the CGTase, sometimes referred to as domains A/B, is the key domain for the CD-producing activity as well as the CD product specificity (Figure 2.5).

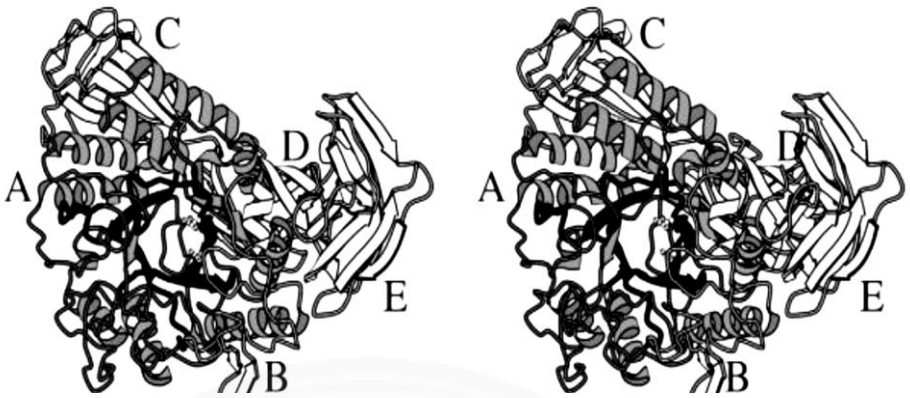


Figure 2.5 Stereo-view of the structure of a representative member of the α- amylase family, *Bacillus circulans* strain 251 cyclodextrin glycosyltransferase (BC 251 CGTase) (Uitdehaag et al., 2002).

Domain A: The catalytic $(\beta/\alpha)_8$ barrel (gray), that CGTase shares with the other members of the α-amylase family. This domain contains the sequence regions that are conserved in the α-amylase family (black).

Domain B: A loop protrudes that forms part of the active site), and which is also present in many other α-amylase family enzymes (white).

Domain C, D and E:**T**hree additional domains are present (white) contain binding sites for raw starch.

2.3.2 Application of CGTase

The application of CGTase is mainly used through intra-and inter-transglycosylation reactions.

With the cyclization reaction (intratransglycosylation reaction), CGTase enzyme is able to produce CDs from starch in which CDs are used in various industries. CDs consist of 6-8 glycosyl units, as well known as α-, β- and γ-CDs. CD molecules have the ability to form inclusion complexes with a variety of compounds. The formation of inclusion complexs result in change in chemical or physical property of the compounds (Challa et al., 2005). For example, in CDs application, CDs is used as an artificial chaperone for protein folding (Machida et al., 2000) and this is available in the form of a useful commercial kit. In addition, CDs can form molecular inclusion complexes (host-guest complexes) with a wide range of solid, liquid, and gaseous compounds and hence have found various applications in the field of food. CDs are widely used as food additives for stabilization of flavors, elimination of undesired tastes or other undesired compounds and to protect browning reactions. Moreover, CDs are used in the preparation of controlled release powdered flavors, candy and chewing gum to retain flavor and smell for longer duration (Mabuchi; and Ngoa, 2001). β-CD is used to remove the cholesterol from egg, butter and milk in the production of low cholesterol dairy products (Szejtli, 2004). The important advantage for the use of CDs in cosmetics manufacturing is suppressing the volatilization of perfumes by stabilizing and controlling the release of the fragrance from the inclusion complexes. The interesting application of CDs in biotechnological field is the use of CDs artificial chaperone to refold the inactive inclusion bodies into its biologically active form (Kitahata et al., 1974). For inter-tranglycosylation reaction, the acceptor specificity of CGTase is rather broad. A number of hydroxyl containing compounds such as glycoside, sugar alcohols, vitamins, flavonoids, etc. may act as CGTase acceptor, in many case with high efficiency (Aga et al., 199; 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 for the synthesis of glycoside series (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 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.

In recent year, the demand for glycoside and oligosaccharides products in food, drink, and cosmetic industries increases, as glycosides and oligosaccharides usually have better characteristics, e.g. solubility, stability and bioactivity than their parent compounds. For example, 3-*o*-α-maltosyl-L-ascorbate with high stability under oxidative conditions was synthesized from α-maltosyl fluoride and L-ascorbic acid catalyzed by mutated CGTase (Hee-Jeong et al., 2015). For an example of a functional oligosaccharide, (Wongsangwattana 2010) reported that the intermolecular transglycosylation reaction of *Paenibacillus* spp. An CGTase can synthesize the cellobiose oligosaccharides and was used as probiotic food. Thus, it is highly possible that the intermolecular transglycosylation activity of the CGTase can be used for the synthesis of new oligosaccharides and glycosides depending on enzyme specificity and donor/acceptor substrate. Both of oligosaccharide and glycoside can lead to the application as probiotic, anticonstipation, antioxidant, anticariogenic and anti-inflammatory agents etc. (Wongsangwattana et al., 2010; Aramsangtienchai et al., 2011; Kim et al., 2000).

2.4 Amylomaltase (AMase)

AMase (EC 2.4.1.25) are classified as bacterial intracellular hydrolases of the 4α-glucanotransferase (4α-GTase) family, was first found in *Escherichia coli* in 1948 as a maltose-inducible enzyme (Doudoroff et al., 2002). The enzyme catalyzes both intramolecular and intermolecular α-1,4-glucan transfer through four activities: cyclization, coupling, disproportionation and hydrolysis. Cyclization, the only

intramolecular transglycosylation reaction of this enzyme, produces cycloamylose (large-ring cyclodextrin, LR-CD) from linear glucan. This reaction is reversible, by a reaction called coupling. In disproportionation reaction, glucosyl group is transferred from a donor to an acceptor oligosaccharide, resulting in oligosaccharides with variable length. While in hydrolysis reaction, water is a glucosyl acceptor. Cyclization and disproportionation are the two main reactions of amylomaltase. Interest in LR-CDs, a cyclic oligosaccharide that is composed of 9 or more glucose units with high aqueous solubility led to more studies of amylomaltase (Terada et al., 1999). In addition, this enzyme was later reported in most thermophilic bacteria and archaea such as *Clostridium butyricum* (Goda et al., 1997), *Aquifex aeolicus* (Bhuiyan et al., 2003), *Thermotoga maritime* (Liebl et al., 2011), *Thermus aquaticus* (Terada et al., 1999), *Thermus thermophiles* (Van der Maarel et al., 1994), *Thermus brockianus* (Bang et al., 2006), *Thermus scotoductus* (Seo et al., 2007), *Corynebacterium glutamicum* (Srisimarat et al., 2011) and *Thermococcus litoralis* (Jeon et al., 1997). The enzyme was reported in plants, e.g. potato and cassava (Tantanarat et al., 2014). Amylomaltase catalyzes an intermolecular transglucosylation reaction, transferring glucosyl residues from donor to acceptor at free OH group of C_4 to produce longer linear oligosaccharides. The enzyme has four different activities: disproportionation, cyclization, coupling and hydrolysis, with the first two as main (Takaha et al., 1999). For AMase has many applications. Firstly, it is uses for the synthesis of a prebiotic isomaltooligosaccharide (Lee et al., 2002), an anticariogenic maltooligosylsucrose (Saehu et al., 2013) and for the production of a thermoreversible starch gel for gelatin substitute in food products, have been reported, in addition, this enzyme possesses is used in the production of LR-CDs via cyclization reaction, all CDs have inner cavities capable of trapping guest molecules of appropriate size and hydrophobicity inside. Improvement of solubility or stability of the guest molecules, such as the drugs prednisolone and digitoxin, is obtained upon forming inclusion complexes with LR-CDs (Tomono et al., 2002). Secondly, amylomaltase is used to modify starch and produce thermo-reversible starch gel with gelatin-like party and can be used as fat and cream alternatives in dairy products to improve creaminess in low-fat yogurt (Bang et al., 2006). Lastly, the enzyme can be used in the production of linear

oligosaccharidesand glycoside products through intermolecular transglucosylation reaction. Short-chain isomaltooligosaccharides (IMOs) as DP_2-DP_6 with prebiotic properties synthesized by combination of amylomaltase and transglucosidase have been reported (Rudeekuthamrong et al., 2013).

2.4.1 Structure of AMase

AMase comprises a catalytic core $(\beta/\alpha)_8$-barrel domain (A), three inserted subdomains (B1, B2, and B3), and a loop covering the active center, as shown by the crystal structure of the 55-kDa *Thermus aquaticus* AMase (Taq AMase) (Figure. 2.6). The catalytic mechanism of AMase was revealed by crystallographic snapshots of *Thermus thermophilus* AMase with a covalently bound intermediate and an acceptor analog in subsites −1 and +1, respectively. AMase do not contain canonical carbohydrate-binding domains or motifs, but a cocrystal structure with the inhibitor acarbose has identified a secondary carbohydrate-binding site between the unique B2 domain and the core $(\beta/\alpha)_8$-barrel in addition to the primary binding region around the active site comprising D293, E340, and D395. On the basis of all available structures and biochemical data, a continuous binding site for up to seven consecutive carbohydrate moieties, −4 to +3 with respect to the enzymatic cleavage site, has been postulated for AMase. Further interactions with native polymeric substrates have only been depicted in conceptual models, whereas the characteristic interaction of these enzymes with CAs or polymeric substrates remains unknown. They reported the crystal structure of Taq AMase in complex with a 34-meric CA (CA34), in which, in combination with a series of catalytic assays, revealed the mechanisms of substrate recognition and CA ring-size determination by AMase as well as a contribution of a nonstandard aspartate-derived amino acid, succinimide, to carbohydrate interaction. The observed conformation of CA34 in the substrate crevice provides a model of the interaction of AMs with the helical amylose of starch.

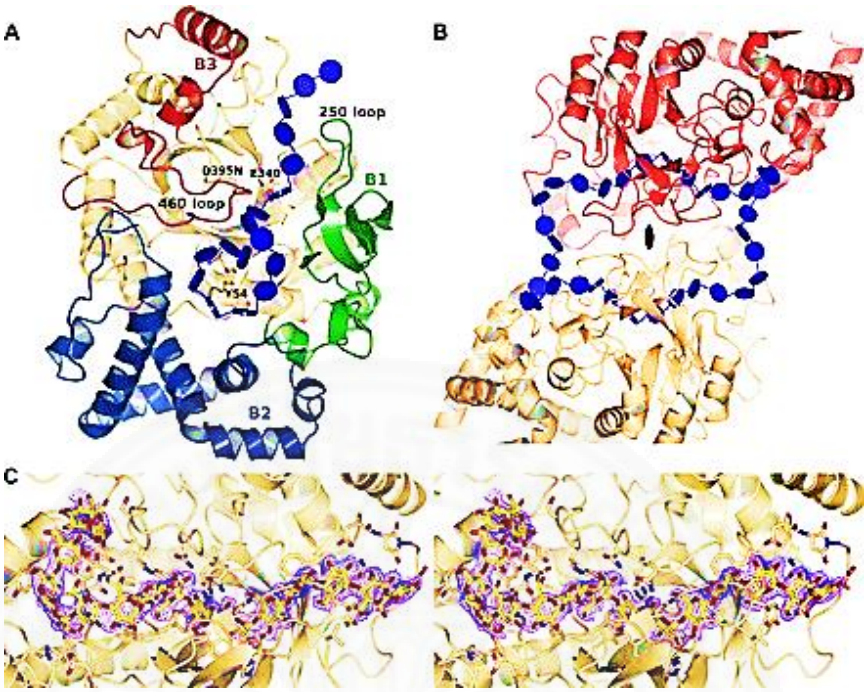


Figure 2.6 Cocrystal structure of CA34 bound to Taq AMase. (A) Overview of the Taq AMase fold. In addition to the (β/α) 8-barrel, three subdomains (B1, B2, and B3) are present. (B) Representation of the crystallographic dimer bridged by the bound CA34 (shown as blue glycoblock representation). The symmetry-related monomers are shown in red and beige. (C) Binding of CA residues 1 to 16 to one monomer of Taq AMase shown in stereo. The corresponding 2mFo–DFc electron density around the CA after refinement is contoured at 1σ.

2.5 Enzymatic synthesis of glycoside

- A low-water organic solvent two-phase system suitable for glycosylation of hydrophobic substrates is described. Almond β-glycosidase adsorbed on polymeric supports has been shown to catalyze alkyl-β-glycoside synthesis via a transferase reaction or through direct condensation of the glucosidic bond. High concentrations of glucosyl donors were present in the aqueous phase, while water-immiscible primary alcohols, which form the organic phase, served as acceptors of glucose. Reaction yield appeared to be thermodynamically controlled. The influence of various support materials, glucosyl donors, and glucosyl acceptors on reaction rate and product yield was investigated (Vulfson et al., 1990).

- A basic possibility of enzymic synthesis of alkyl glycosides in a system of the Aerosol-OT (AOT) reverse micelles was studied. Octyl beta-D-galactopyranoside and octyl beta-D-glucopyranoside were synthesized from the corresponding sugars (lactose or glucose) and octyl alcohol under catalysis with glycolytic enzymes, beta-galactosidase and beta-glucosidase, respectively. The transglycosylation/hydrolysis ratio was shifted toward transglycosylation by using octyl alcohol, one of the substrates, as an organic solvent. The alkyl glycosides were thus obtained in one step from a hydrophilic mono- or disaccharide and a hydrophobic aliphatic alcohol. The direction of the reaction was shown to depend on the pH of aqueous solution immobilized in nerves micelles. The maximum yields were 45% and 40% for octyl galactoside and octyl glycoside, respectively; they markedly exceeded the yields of enzymic syntheses in a two-phase system reported previously (Kouptsova et al., 2001).

- Cyclodextrin glucanotransferase [1, 4-α-D-glucan 4-α-D-(1, 4-glucano)-transferase, cyclizing ; CGTase, EC 2.4.1.19] from an alkalophilic Bacillus species produced hesperidin monoglycoside and a series of its oligoglycosides by the transglycosylation reaction with hesperidin as an acceptor and soluble starch as a donor. The formation of the glycosides was more effective at alkaline pHs than at neutral or acidic pHs, because of higher solubility of the acceptor. The structure of the purified monoglycoside was identified as 4G-α-D-glucopyranosyl hesperidin by FAB-MS, α-, β-glucosidase and glucoamylase treatments, and methylation analysis. The

solubility of both hesperidin mono and diglycoside in water was about 300 times higher than that of hesperidin, and they were found to have a stabilizing effect on the yellow pigment crocin, from fruits of Gardenia jasminoides, against ultraviolet radiation (Kometani et al., 1994).

- The microorganism *Talaromyces duponti* ATCC 20805 can produce an extracellular glucosyltransferase (EC 2.4.1.24) with a high transglucosylating activity. A 100-h culture using maltodextrins as the carbon source gave an enzyme production of 11 U ml^{-1}. The enzyme was further purified to homogeneity by fractionation steps involving ammonium sulfate precipitation, hydrophobic interaction chromatography, ion-exchange chromatography, and chromatofocusing. The enzyme gave a single protein band on gel electrophoresis but two protein bands on isoelectrofocusing (pI values 4.19 and 4.26). It is composed of two isoforms both showing transglucosylase activity. The two isoforms could be separated and their carbohydrate content evaluated. These were equal to 2.30 and 2.84% (w/w). The optimum pH and temperature for the enzymatic reaction are 4.5 and 70 °C, respectively. The transglucosidase retained 50% activity after 73.2 hours at 60°C. The high thermostability of the enzyme makes it particularly suitable for α-butylglucoside synthesis in biphasic medium by transfer of glucosyl moieties from maltooligosaccharide donors onto butanol (Bousquet et al., 1998).

- Alkyl glycosides with long carbohydrate groups are surfactants with attractive properties but they are very difficult to synthesize. Here, a method for extension of the carbohydrate group of commercially available dodecyl-beta-d-maltoside (DDM) is presented. DDM was converted to dodecyl-beta-d-maltooctaoside (DDMO) in a single step by using a CGTase as catalyst and alpha-cyclodextrin (alpha-CD) as glycosyl donor. The coupling reaction is under kinetic control and the maximum yield depends on the selectivity of the enzyme. The *Bacillus macerans* CGTase favored the coupling reaction while the Thermoanaerobacter enzyme also catalyzed disproportionation reactions leading to a broader product range. A high ratio alpha-CD/DDM favored a high yield of DDMO and yields up to 80% were obtained using the *B. macerans* enzyme as catalyst (Svenson et al., 2009).

- The glycosylation of alpha- and delta-tocopherols using *Klebsiella pneumoniae* and cyclodextrin glucanotransferase (CGTase) was investigated. *K. pneumoniae* converted alpha- and delta-tocopherols into the corresponding beta-glucosides in 10 and 8% yield, respectively. CGTase glycosylated alpha-tocopheryl beta-glucoside to alpha-tocopheryl beta-maltoside (51%) and alpha-tocopheryl beta-maltotrioside (35%). On the other hand, delta-tocopheryl beta-glucoside was converted into the corresponding beta-maltoside (45%) and beta-maltotrioside (29%) by CGTase. The beta-glucoside of alpha-tocopherol, and beta-glucoside and beta-maltoside of delta-tocopherol showed inhibitory effects on IgE antibody production and on histamine release from rat peritoneal mast cells (Shimoda et al., 2009).

- The production of β-maltooligosaccharides of capsaicin was investigated using *Lactobacillus delbrueckii* and cyclodextrin glucanotransferase (CGTase) as biocatalysts. The cells of *L. delbrueckii* glucosylated capsaicin to give its β-glucoside. The β-glucoside of capsaicin was converted into the corresponding β-maltoside and β-maltotrioside by CGTase. On the other hand, β-melibioside and β-isomaltoside of capsaicin, which were two new compounds, were synthesized by chemical glycosylation. The β-glucoside, β-maltoside, β-melibioside, and β-isomaltoside of capsaicin showed inhibitory effects on IgE antibody production (Shimoda et al., 2010).

- Enzymatic synthesis of epicatechin (EC) glucosides was performed through the transglucosylation reaction catalyzed by the cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* spp. RB01. The enzyme showed the same product specificity for the three donor substrates, starch, β-cyclodextrin and maltoheptaose (G7). Using β-cyclodextrin as the glucosyl donor, several EC glucoside products were obtained at an overall minimal yield of 18.1%. The structures of the four main products were elucidated by MS and NMR techniques as EC-3'-*O*-α-d-glucopyranoside (EC3A), EC-3'-*O*-α-d-diglucopyranoside (EC3B), EC-3'-*O*-α-d-triglucopyranoside (EC3C) and EC-4'-*O*-α-d-glucopyranoside (EC4A). Of these, EC3A was the major product while EC4A, unique for this CGTase, was formed in the lowest amount. The water solubility and stability against UV irradiation of EC3A were significantly higher than that of EC. Although the antioxidant activity was 1.5-fold lower,

the advantage of the enhanced solubility and stability makes the EC3A glucoside more beneficial as food ingredient than its parent EC (Pornpun et al., 2011).

2.6 Enzymatic synthesis of oligosaccharide

- Synthesis of maltooligosylsucroses by the recombinant amylomaltase from *Corynebacterium glutamicum* as a N terminal (His) 6 chimera is reported. The suitable glucosyl donor was found to be raw tapioca starch. The optimal condition was 2.0% (w/v) sucrose, 2.5% (w/v) raw tapioca starch and 9 U/ml of amylomaltase at 30 ^{o}C for 48 hours, giving an overall 82% yield of maltooligosylsucrose products. After purified by Bio-Gel P-2 size exclusion column chromatography, the main products were determined by MS and NMR analysis to be maltosyl-, maltotriosyl-, maltotretraosyl- and maltopentaosyl-fructosides (G2F, G3F, G4F and G5F, respectively, where G = glucosyl unit and F = fructose) with an α-1,4 linkage between the added glucosyl unit and the sucrose. The low cariogenic property of these maltooligosylsucrose products was confirmed by analyzing the effect on the synthesis of water insoluble glucan, acid fermentation, plaque formation and cell aggregation of Streptococcus mutans when compared to those exerted by sucrose. Moreover, by adding sucrose to maltooligosylsucrose products at ratios of 1:1, 1:2 and 1:4, the inhibitory effects on glucosyltransferase activity of S. mutans by 7, 33 and 50%, respectively, were observed. These results suggest that the obtained maltooligosylsucrose products have an anticariogenic property and could be used to substitute for sucrose in food or related products (Saehu et al., 2013).

- Acceptor specificity for intermolecular transglucosylation reaction of amylomaltase from *Corynebacterium glutamicum* was investigated using starch as glucosyl donor and various saccharide acceptors. Maltooligosaccharides (G_1–G_4), mannose, palatinose and sucrose were efficient acceptors; the best one was glucose. This amylomaltase preferred hexose sugar containing the same configuration of C2-, C4- and C6-hydroxyl groups as glucopyranose. Palatinose was chosen as suitable acceptor for the synthesis of palatinose glucosides (PGs). The optimal condition was to incubate 5 U/ml amylomaltase with 7.5 mM palatinose and 1.0% (w/v) soluble

potato starch at 30 °C for 24 hours. In addition to PGs, maltooligosaccharides were also produced as by product. The product yield was 67.9%, in which the ratio of PGs to maltooligosaccharides was 1:1. Then PGs were separated by Bio-Gel-P2 column chromatography and analyzed by HPAEC. PG_1–PG_{13} was identified with PG_1 and PG_2 as major products. NMR analysis showed that the PGs produced are novel products, PG_1 and PG_2 were a tri- and tetra-saccharide with the structure [*O*-α-d-glucopyranosyl-(1⟶4)]$_n$-*O*-α-d-glucopyranosyl-(1⟶6)-d-fructofuranose, where *n* = 1–2. PG was less sweet than palatinose and sucrose, more hygroscopic with similar prebiotic activity as palatinose. PGs thus have potential to replace sucrose or palatinose in food products for health benefits (Naumthong et al., 2015).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Equipments

Autoclave: HV-110, HIRAYAMA, Japan

Autopipette: Pipetman, Gilson, France

Amino HPLC column: Sphere Clone 5u ODS 250x4.6 mm, Phenomenex, U.S.A.

Biosafety carbinet: Model Power wave XS, BioTek, U.S.A.

Brix refractometer: PAL-1, Atago, Japan

C18 HPLC column: Sphere Clone 5u ODS 250x4.6 mm, Phenomenex, U.S.A.

Centrifugal evaporator: Model Concentrator 5301, Eppendorf, Germany

Centrifuge filter units: Amicon®.Ultra, Merck Millipore, Ireland

Densitometer: GS-800 Calibrated Densitometer, Bio-Red, U.S.A

Dialysis tubing: Model D-9527, Sigma, U.S.A.

HisTrap FF™ column chromatography: GE Healthcare, UK

HPLC: Agilent 1260 Infinity Quaternary LC, Agilent Technologies, Germany

Incubator: Model UNB 500, Memmert, Germany

Magnetic stirrer: Model 500P-2, PMC, U.S.A.

Mass spectrophotometer: MicrOTOF, Bruker, Germany

Microcentrifuge: Model Legend Micro 17, Thermo scientific, UK

Microplate spectrophotometer: Model Power WaveTM Xs, BioTek, U.S.A.

NH_2 HPLC column: Phenomenex, U.S.A.

Orbital incubator shaker: Model 240, Thermo scientific, U.S.A.

pH Meter: Model PHS-3BW, BANTE instrument, China

Power supply: Model Power PACTM Basic, Bio-Rad, U.S.A.

Quick spin: Model C1301B-230, Labnet, Korea

Refractometer: MASTER-URC/NM 2793-E01, ATAGO®, Japan

Refrigerated centrifuge: Allegra® X-15R, Beckman coulter, U.S.A.

Scanning densitometer: Model GS-800 Calibrated Densitometer, Bio-Rad, U.S.A.

Sonicator: Vibra Cell™ VCX130, sonics, U.S.A.

Spectrophotometer: Model UV-1601, Shinmadzu, Japan

TLC plate heater: CAMAG, Swiss

Vortex mixer: Genie 2 G-560E, Scientific IndustriesTM, U.S.A.

Water bath: Model WNB Basic, Memmert, Germany

3.2 Chemicals

Acetic acid: BDH Prolabo chemicals, UK

Acetonitrile: Sigma, U.S.A.

Agar: Merck, Germany

Ammonium persulfate: Sigma, U.S.A.

Ampicillin: Sigma, U.S.A.

Aquacide II: Merck, Germany

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

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

Bromophenol blue: Merck, Germany

Calcium chloride dehydrate: BDB Prolabo chemicals, UK

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

Corn starch; Knorr, Thailand

β-Cyclodextrins: Bio Research Corporation of Yokohama, Japan

Dimethyl sulfoxide: Sigma, U.S.A.

Ethyl acetate: BDH Prolabo chemicals, UK

Glucose luquicolor (Glucose oxidase kit): Human, Germany

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

Hydrochloric acid: Carlo Erba Reagents, France

Iodine: Baker chemical, U.S.A.

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

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

Magnesium sulfate: BDH Prolabo chemicals, UK

Mannose: Wako, Japan

Methanol deuterium: Merck, Germany

β-Mercaptoethanol: Fluga, Switzerland

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

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

Galactose: Sigma, U.S.A.

Glucose: BDH Prolabo chemicals, UK

Fructose: Sigma, U.S.A.

Lactose: Sigma, U.S.A.

Maltoheptaose: Tokyo Chemical Industry, Japan

Maltohexaose: Sigma, U.S.A.

Maltopentaose: Sigma, U.S.A.

Maltose: Sigma, U.S.A.

Maltotetraose: Sigma, U.S.A.

Maltotriose: BioChemika, Japan

Palatinose: Sigma, U.S.A.

Sucrose: Sigma, U.S.A.

Hesperitin: Sigma, U.S.A.

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

Pinoresinol: Sigma, U.S.A.

Potassium dihydrogen phosphate: Merck, Germany

di-Potassium hydrogen phosphate: Merck, Germany

Potassium iodine: Mallinckrodt, U.S.A.

Quercetin-3-glucoside: Sigma, U.S.A.

Quercitrin hydrate: Sigma, U.S.A.

Rutin hydrate: Sigma, U.S.A.

Sodium carbonate anhydrous: BDH Prolabo chemicals, UK

Sodium chloride: Merck, Germany

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

Sodium hydroxide: Merck, Germany

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

Sulfuric acid: Carlo ERBA Reagents, France

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

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

Tapioca starch (Flomax®): a gift from Siam Modified Starch Co., Ltd, Thailand

Tris base: Vivantis, U.S.A.

Tris hydrochloride: Vivantis, U.S.A.

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

Water (HPLC grade): Merck, Germany

Xylose: Sigma, U.S.A.

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

PART 1: Synthesis and characterization of functional glycosides

3.3 Enzymatic resource

The pBC recombinant cell was constructed by inserting the CGTase gene with signal peptide sequence from *Bacillus circulans* A11 on pET19b-based plasmid and transformed into *E. coli* strain BL21 (DE3) host cell.

3.4 Bacterial cultivation and enzyme production

3.4.1 Starter inoculum

The pBC transformed *E. coli* strain BL21 (DE3) (GenBank accession no. AF302787) (Kaulpiboon et al., 2010) was transferred from 50% glycerol stock into Luria Bertani (LB) medium containing 100 µg/ml ampicillin and grown overnight at 37 °C with rotary shaking 200 rpm.

3.4.2 Enzyme production

1% (v/v) of the starter inoculum was transferred into 300 ml LB medium in 1,000 ml Erlenmeyer flask and cultured at 37 °C with rotary shaking at 250 rpm. When the tubidity of the culture at wavelength 660 nm reached OD. 0.4-0.6, IPTG was added to final concentration of 0.2 mM to induce CGTase gene expression and cultivation was continued at 37 °C for 24 hours. After cultivation, bacterial cells were removed by refrigerated centrifugation at 4,800 rpm for 50 minutes at 4 °C. Culture broth with crude CGTase was collected and kept at 4 °C for dextrinizing activity and protein determination.

3.5 Purification of CGTase

Crude CGTase from the pBC recombinant cell culture was purified by starch adsorption (Kuttiarcheewa, 1994).

3.5.1 Starch adsorption

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

3.6 Enzyme analysis

Characterization of molecular weight of the recombinant CGTase was also performed by Native-PAGE and SDS-PAGE (Bollag and Edlestein 1996).

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

The starch degrading activity of CGTase was determined from the migration of protein (enzyme) on non-denaturing gel. Separating gel was 7.5% (w/v) acrylamide while the stacking gel used here was 5% (w/v) acrylamide. The purified protein and crude extract were mixed with sample buffer before load into the wells. Tris-glycine buffer pH 8 was used as electrode buffer. The system was preceded by setting the constant current of 15 mA per slap using a Mini-Gel electrophoresis unit from cathode towards anode. The electrophoresis was allowed to perform at room

temperature. When the dye front reached the bottom of the gel, the gel was cut in to 2 parts for the determination by protein staining and activity staining.

3.6.2 Coomassie blue staining (Protein staining)

A gel from Native-PAGE was subjected to protein staining by the addition of 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid and incubated for 30-60 minutes. Subsequently, the gel was destained with several changes of destaining solution 10% (v/v) methanol and 10% (v/v) acetic acid.

3.6.3 Dextrinizing activity staining (Fuwa et al., 1954)

For dextrinizing activity staining, the native gel was soaked with 2% (w/v) soluble starch in 0.2 M phosphate buffer pH 6.0 at 40 ^{o}C for 10 minutes. The gel was rinsed several times with distilled water to remove the remaining starch on gel. After that 10 ml of I_2 staining reagent (0.2% (w/v) I_2 in 2.0% (w/v) KI) was added and incubated at 40 ^{o}C for color development. The starch degrading activity was observed by the clear zone on the blue background on the gel.

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

The denaturing gel electrophoresis was carried out on 7.5% (w/v) separating gel and 5% (w/v) of stacking gel. Electrophoresis buffer was Tris-glycine pH 8.0, containing 0.1% SDS. Protein samples were treated with 5x sample buffer (1% w/v) bromophenol blue and 50% (v/v) glycerol in 1 M Tris-HCl pH 6.8) and boiled for 5 minutes before loading onto the gel. The system was operated at 15 mA of constant current per slap using a Mini-Gel electrophoresis unit from cathode towards anode. After electrophoresis, the gel was stained with Coomassie brilliant blue for visualization of the separated protein. The approximate protein bands fractionated on the gel were determined by comparing the travel distance with known molecular weight protein markers. The molecular weight marker proteins were myosin (250 kDa), phosphorylase (150 kDa), β-galactosidase (100 kDa), bovine serum albumin (75 kDa) and ovalbumin (50 kDa).

3.7 Assay of CGTase activity

The CGTase activity was determined as described below:

3.7.1 Dextrinizing activity

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

3.7.2 Coupling activity by glucose oxidase assay

Glucose oxidase kit was used to measure the enzymatic activity in changing β-CD to glucose (Miwa et al., 1972). The reaction mixture (150 µl) contained 60 µl of 1% (w/v) β-CD, 60 µl of 1% (w/v) various acceptors and 30 µl of the enzyme in 0.2 M phosphate buffer, pH 6.0. The reaction mixture was performed at 50 °C for 5 minutes and stopped by boiling for 5 minutes. Then, a 10 µl aliquot from reaction mixture was added into 1 ml of glucose oxidase reagent. The glucose oxidase reaction was incubated at 37 °C for 5 minutes and then the absorbance at 500 nm was measured. The glucose concentration was calculated from the equation (3.1):

$$\text{Glucose concentration (mol/ml)} = 5.55 \times \frac{A_{sample}}{A_{std}} \quad (3.1)$$

The absorbance of the standard solution (A_{std}) was measured by using 10 µl a standard glucose (5.55 µmol/ml) with 1 ml of glucose oxidase reagent.

3.8 Determination of transglycosylation reaction efficiency

Transglycosylation reaction efficiency was judged by transglycosylated product yield on TLC plate with mobile phase. The yield was quantitated using a scanning densitometer and a Quantity One® 1-D analysis program. Glucose spot which was run on the same TLC plate was used as standard. In addition, the yield of transglycosylated product was also analyzed by High performance liquid chromatography (HPLC) which was calculated from the ratio of peak area of product to the initial peak area of acceptor in HPLC profile, using the equation (3.2):

$$\text{product yield (\%)} = \frac{\text{Peak area of product}}{\text{Peak area of acceptor at } t_0} \times 100 \quad (3.2)$$

3.9 Detection of products

3.9.1 Thin Layer Chromatography (TLC) analysis

Reaction products were analyzed by applying samples on TLC plate, and developed in a System composing of ethyl acetate/ acetic acid/ water (3:1:1, v/v) as a mobile phase (Funayama et al., 1993) for detection of glucosyl products. After running twice, the plate was dried thoroughly and dipped in the same procedure as a mobile phase. The intensity of glucosyl product spots was evaluated from glucose standard value.

3.9.2 High Performance Liquid Chromatography (HPLC) analysis

The products were also analyzed by HPLC (Agilent Technologies 1260, Germany) using a 5 µm reverse phase C18 column (4.6 x 250 mm) (Phenomenex, USA) and detected with UV detector at 210 nm. The reaction mixture of glycosides product was filtered through nylon membrane of 0.45 µm disc filter before injection and eluted with methanol: water (40:75; v/v) using a flow rate of 1.0 ml/min at 25 °C. Product yield were calculated as described in Section 3.8 (Equation 3.2).

3.10 Optimization of functional glycosides production

3.10.1 Optimization of DMSO concentration

To investigate the effect of DMSO concentration on pinoresinol solubility, the reaction mixtures containing 1% *(w/v)* β-CD donor and 2% *(w/v)* pinoresinol acceptor in different DMSO concentrations ranging from 0-100 % *(v/v)* were incubated with 10 U/ml CGTase in 20 mM phosphate buffer *(pH 6.0)* at 45 °C for 24 hours. The reactions were stopped by boiling for 5 minutes and then runned on TLC plate as described in Section 3.9.1 to find the optimal DMSO concentration for pinoresinol glycosides production. The amount of pinoresinol glycosides was measured by scanning densitometer. The appropriate DMSO concentration was judged from the highest yield of the pinoresinol glycosides products.

3.10.2 Optimization of substrate ratios (β-CD*:* Pinoresinol)

β-CD was selected as an appropriate donor for the synthesis of pinoresinol glycosides by transglycosylation reaction of CGTase. The retio of β-CD*:* pinoresinol which was varied as 1:1, 1:2, 1:3, 1:4, 2:1, 2:2, 3:3 and 4:1 in phosphate buffer pH 6.0 was mixed pinoresinol and 10 U/ml CGTase in 20 mM phosphate buffer pH 6.0. The reaction mixture was incubated at 45 °C for 24 hours and the reaction was stopped by boiling for 5 minutes. The reaction mixture was runned on TLC plate as described in Section 3.9.1 to find the optimal substrate ratios for pinoresinol glycosides production. The amount of pinoresinol glycosides was measured by scanning densitometer. The appropriate substrate ratio was judged from the highest yield of the pinoresinol glycosides products.

3.10.3 Optimization of enzyme concentration

To find the optimum concentration of CGTase, β-CD substrate ratios (Section 3.10.2) which were selected from previous investigation were incubated with varying amount of CGTase (10, 20, 40 and 80 U/ml) in 20 mM phosphate buffer pH 6.0 at 45 °C for 0-72 hours. The optimum of enzyme unit and incubation time was decided from the amount of pinoresinol glycoside products on TLC analysis as described in Section 3.9.1.

3.10.4 Optimization of pH

The reaction mixture was performed by incubation at optimal substrate ratios CGTase unit and incubation time from the previous Section in buffer with pH ranging from 3.0-11.0. Different inorganic substances were used as buffer e.g. acetate buffer (pH 3.0-5.0), phosphate (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and Glycine-NaOH (pH 9.0-11.0). The optimal pH for the production of pinoresinol glycosides was judged from TLC analysis as described in Section 3.9.1.

3.10.5 Optimization of temperature

The optimal temperature for the synthesis of pinoresinol glycosides was determined in the reaction mixture which consisted of an optimum concentration of substrate ratio, CGTase unit and incubation time as reported in Section 3.10.2 and 3.10.3, respectively. The reaction mixture was performed at optimal pH (Section 3.10.4) and various temperatures (35, 40, 45, 55, 50, 60, 65 and 70 °C). The optimal temperature for the production of pinoresinol glycosides was judged from TLC analysis as described in Section 3.9.1.

3.11 Large scale production of pinoresinol glycosides

Large scale production of pinoresinol glycosides was performed under the optimum conditions obtained from previous section (Section 3.10). In the initial experiments, transglycosylation reaction was performed in the reaction mixture of 150 µl. To produce large amount of products, the total volume was raised to 50 ml. After the incubation was complete, the reaction mixture was concentrated by evaporation at 45 °C before purification pinoresinol glycosides by HPLC as described in Section 3.9.2.

3.12 Structural analysis of pinoresinol glycosides

3.12.1 Mass spectrometry (MS) analysis

A mass spectrometry (Bruker, Germany) was used to determine the molecular mass of chemical compounds by separating ionic molecules according to

their mass-to-charge ration (*m/z*). The molecular mass was calculated by the equation (3.3):

(molecular mass + number of protons)/charge = mass-to-charge ration (*m/z*) (3.3)

The pinoresinol glycosides products were dissolve in 50% (v/v) methanol and subjected into mass spectrometry device. Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was recorded on microTOF at the Service Unit of the National Center for Genetic Engineering and Biotechnology (BIOTECH, Thailand). The compounds were ionized by electrospray ionization in the sodium positive-ion mode using capillary voltage of 5,000 volts. The 4 liters per minute flow of nitrogen gas at 150 ^{o}C was performed to nebulize the analytic solution to droplets using nebulizer pressure at 1 bar.

3.12.2 Nuclear Magnetic Resonance (NMR) analysis

Structural identification of pinoresinol and the synthesized pinoresinol glycosides was performed by ^{1}H and ^{13}C NMR spectrum with Bruker AVANCE III HD 400 NMR (Bruker Daltonik GmbH, Bremen, Germany) using stand Bruker NMR software at the Biological Service Unit of the National Center for Genetic Engineering and Biotechnology (BIOTECH, Thailand. The operation was 400 MHz ambient temperature. The spectra were obtained with 2 mg of each sample dissolved in methanol-D_4(CD_3OD).

3.13 Evaluation of the physiochemical and biological properties of pinoresinol glycoside products

3.13.1 Solubility test

Excess amounts of pinoresinol glycosides (PG_1-I, PG_1-II and PG_2) were mixed with 200 µl of water, vortexed for 5 minutes at room temperature to disperse into solution, and then each sample was incubated at 30 ^{o}C for 15 minutes to allow the saturation. pinoresinol glycosides were then filtered through a 0.45 µm membrane to remove insoluble particles and subjected to quantitative reverse phase C18 HPLC analysis in order to determine the concentration of soluble pinoresinol glycosides (Aramsangtienchai et al., 2011).

3.13.2 β-Glucuronidase inhibition assay

For this assay, 0.1 ml of 2.5 mM p-nitrophenyl-β-D-glucopyranosiduronic acid in 0.1 M acetate buffer (pH 7.4) was incubated with 1 mg of pinoresinol or its glycoside derivatives for 5 minutes, followed by the addition of 0.1 ml of β-glucuronidase. The reaction mixture was then being incubated for 30 minutes, followed by the addition of 2 ml of 0.5 N NaOH to halt the reaction. The amount of reaction product formed was measured at 410 nm and calculated using the Equation (3.4):

$$\text{Anti-inflammatory activity (\%)} = \left(1 - \frac{A_{410} \text{ of test sample}}{A_{410} \text{ of control}}\right) \times 100 \quad (3.4)$$

A 1 mM salicylic acid solution was used as a reference drug for comparison (Nia et al., 2003).

3.13.3 DPPH radical scavenging assay

The antioxidant activity of pinoresinol and pinoresinol glycosides (PG_1-I, PG_1-II and PG_2) was evaluated using the DPPH radical scavenging reaction (Abe et al., 2000). Each of pinoresinol and its glycosides was dissolved or diluted in the required volume of ethanol to yield the final pinoresinol glycosides concentration in range of 1-600 μM. DPPH was then added to a final of 507 μM. After 10 minutes in the dark, the absorbance of the mixture was measured at 517 nm. The DPPH radical scavenging activity was evaluated by analyzing the percentage decrease in the absorbance ofthe sample, compared to the blank (ethanol). The IC50 value designates the inhibitory concentration at which the absorbance was reduced by 50%.

3.13.4 Sweetness test

The relative sweetness of 1% (w/v) of the tested pinoresinol glycosides was determined by measuring the Brix values using a refractometer (RX-1000, Atago Co., Ltd., Tokyo, Japan) (Kikuchi et al., 2004). Sucrose was used as the reference compound to plot the standard curve of the concentrations (%, w/v) versus the degrees Brix (Bx). One-degree Brix is defined as 1 g of sucrose in 100 ml of solution.

PART 2: Synthesis and characterization of functional oligosaccharides

3.14 Enzymatic resource

The p19bAMY recombinant cell was a pET-19b based plasmid containing the AMase gene from *Thermus aquaticus* and transformed into *E. coli* strain BL21 (DE3) host cell.

3.15 Bacterial cultivation and enzyme production

3.15.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant cell (p19bAMY) was transferred from 50% glycerol stock into Luria Bertani (LB) medium containing 100 µg/ml ampicillin and grown overnight at 37 ^{o}C with rotary shaking 250 rpm.

3.15.2 Enzyme production

1% (v/v) of the starter inoculum was transferred into 300 ml LB medium in 1,000 ml Erlenmeyer flask and cultured at 37 ^{o}C with rotary shaking at 250 rpm. When the turbidity of the culture at wavelength 660 nm reached OD. 0.4-0.6, IPTG was added to final concentration of 0.8 mM to induce AMase gene expression and cultivation was then continued at 37 ^{o}C for 4 hours with 250 rpm rotary shaking. After 4-hours cultivation, bacterial cells were harvested by centrifugation at 4,800 rpm for 30 minutes at 4 ^{o}C and then washed twice by 20 mM sodium phosphate buffer (pH 7.4). The *E. coli* transformant cell were resuspended in the same buffer and then disrupted by sonication for at 40% amplitude for 3 cycles of 5 minutes' pulse and 5 minutes' pause (Vibra Cell^{TM} VCX130, Sonics, U.S.A.). Cell debris was removed by centrifugation at 10,200 rpm for 2 hours. The supernatant was collected for determination of enzyme activity using starch transglycosylation assay as described in section 3.17 and protein concentration was determined by the method of Bradford (1976) as mentioned in section 3.16.

3.16 Purification of AMase

The crude AMase from the p19bAMY recombinant cell culture was purified by HisTrap FF^{TM} column chromatography. The HisTrap FF^{TM} column (0.7 x 2.5 cm) was equilibrated with 5 columns volume of 20 mM phosphate buffer, pH 7.4 containing 0.5 M NaCl and 20 mM imidazole at flow rate 1 ml/min. The crude enzyme from section 3.15.2 was applied to HisTrap FF^{TM} column (1 ml, two columns consecutively) and washed with the same buffer until the A_{280} of eluent decreased to a steady baseline. The bound proteins were eluted by 500 mM imidazole in the same buffer at the flow rate of 1 ml/min. Fraction of 2 ml were collected. The active fraction was pooled for determination of enzyme activity by starch transglycosylation assay as described in section 3.17 and protein concentration was determined by the method of Bradford (1976) as mentioned in section 3.16.

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

3.17 Assay of AMase activity

The AMase activity was determined as described below:

3.17.1 Starch transglycosylation activity

The starch transglycosylation activity of AMase was assayed by measuring the iodine method (modified from Park, et al., 2007). The reaction mixture contained 250 µl of 0.2% (w/v) potato soluble starch, 50 µl of 1% (w/v) maltose, 100 µl of the enzyme and 600 µl of 20 mM phosphate buffer (pH 7.4). The reaction was performed at 70 ^{o}C for 10 minutes and stopped by boiling for 10 minutes. Then, 100 µl aliquot was withdrawn and mixed with 1 ml of iodine solution (0.02% (w/v) I_2 in 0.2% (w/v) KI) and the absorbance at 600 nm was monitored. One unit of starch

transglycosylation activity was defined as the amount of enzyme that produces a 1% reduction in the absorbance per minute under the described conditions.

3.17.2 Disproportionation activity

The disproportionation activity was measured by the glucose oxidase method (Miwa et al., 1972). The reaction mixture contained 30 µl of 5% (w/v) maltotriose and 100 µl of the enzyme (0.24 mg/ml) in 20 mM phosphate buffer (pH 7.4). The reaction was performed at 70 °C for 10 minutes and stopped by the addition of 30 µl of 1 N HCl. Then, 10 µl aliquots of reaction mixture was added into 1 ml of glucose oxidase reagent. After that the mixture was incubated at 37 °C for 5 minutes and then the absorbance at 500 nm was measured. One unit of disproportionation activity was defined as the amount of enzyme required for the production of 1 µmol of per minute under described conditions.

3.18 Enzyme analysis

Characterization of molecular weight of the recombinant AMase was also performed by Native-PAGE and SDS-PAGE.

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

The starch degrading activity of AMase was determined from the migration of protein (enzyme) on non-denaturing gel. Separating gel was 7.5% (w/v) acrylamide while the stacking gel used here was 5% (w/v) acrylamide. The purified protein and crude extract were mixed with sample buffer before load into the wells. Tris-glycine buffer pH 8 was used as electrode buffer. The system was preceded by setting the constant current of 15 mA per slap using a Mini-Gel electrophoresis unit from cathode towards anode. The electrophoresis was allowed to perform at roomtemperature. When the dye front reached the bottom of the gel, the gel was cut in to 2 parts for the determination by protein staining and activity staining.

3.18.2 Coomassie blue staining (Protein staining)

A gel from Native-PAGE was subjected to protein staining by the addition of 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid and incubated for 30-60 minutes. Subseguently, the gel was destained with several changes of destaining solution 10% (v/v) methanol and 10% (v/v) acetic acid.

3.18.3 Starch transglycosylation activity staining

For starch transglycosylation activity staining, the native gel was soaked with 2% (w/v) soluble starch in 0.2 M phosphate buffer pH 6.0 at 40 ^{o}C for 10 minutes. The gel was rinsed several times with distilled water to remove the remaining starch on gel. After that 10 ml of I_2 staining reagent (0.2% (w/v) I_2 in 2.0 % (w/v) KI) was added and incubated at 40 ^{o}C for color development. The starch degrading activity was observed by the clear zone on the blue background on the gel.

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

The denaturing gel electrophoresis was carried out on 7.5% (w/v) separating gel and 5% (w/v) of stacking gel. Electrophoresis buffer was Tris-glycine pH 8.0, containing 0.1% SDS. Protein samples were treated with 5x sample buffer (1% w/v) bromophenol blue and 50% (v/v) glycerol in 1 M Tris-HCl pH 6.8) and boiled for 5 minutes before loading onto the gel. The system was operated at 15 mA of constant current per slab using a Mini-Gel electrophoresis unit from cathode towards anode. After electrophoresis, the gel was stained with Coomassie brilliant blue for visualization of the separated protein. The approximate protein bands fractionated on the gel were determined by comparing the travel distance with known molecular weight protein markers. The molecular weight marker proteins were myosin (250 kDa), phosphorylase (150 kDa), β-galactosidase (100 kDa), bovine serum albumin (75 kDa) and ovalbumin (50 kDa).

3.19 Detection of products

3.19.1 Thin layer chromatography (TLC) analysis

Reaction products were analyzed on TLC silica gel 60 F254 (20 cm in height) (Merck, Germany), and twice developed in a System I composing of isopropyl alcohol/ethyl acetate/water (3:1:1, v/v) as a mobile phase for finding the best donor in the synthesis of oligosaccharide products (Saehu et al., 2013). The TLC chromatograms were visualized by dipping with concentrated sulfuric acid-methanol (1:9, v/v) followed by heating at 120 °C for 15 minutes. The intensity of synthesized product spots was quantitated by a scanning densitometer. Glucose spot (50 µg) was set as standard value.

3.19.2 High performance liquid chromatography (HPLC) analysis

The products were also analyzed by HPLC (Agilent Technologies 1260, Germany) using a 5 µm amino column (4.6 x 250 mm, Phenomenex, USA) and detected with RI detector at 280 nm. The reaction mixture of oligosaccharide product was filtered through nylon membrane of 0.45 µm disc filter before injection and eluted with acetonitrile: water (80: 20, v/v) using a flow rate of 0.5 ml/min at 40 °C. Product yield was calculated as described in Section 3.19.3 (Equation 3.2).

3.19.3 Determination of transglycosylation reaction efficiency

Transglycosylation reaction efficiency was judged by transglycosylated product yield on TLC plate with mobile phase. The yield was quantitated using a scanning densitometer and a Quantity One® 1-D analysis program. Glucose spot which was run on the same TLC plate was used as standard. In addition, the yield of transglycosylated product was also analyzed by High performance liquid chromatography (HPLC) which was calculated from the ratio of peak area of product to the initial peak area of acceptor in HPLC profile, using the equation (3.2):

$$\text{Product yield (\%)} = \frac{\text{Peak area of product}}{\text{Peak area of acceptor at } t_0} \times 100 \quad (3.2)$$

3.20 Determination of donor and acceptor specificities

3.20.1 Donor specificity

The glucose-rich donors, α-, β-, γ-CD, tapioca starch, pea starch and potato starch were investigated for transglycosylation activity by AMase. The reaction mixture (150 µl) containing final concentration of 1% (w/v) xylose acceptor, 1% (w/v) glycosyl donors, and 2 U/ml AMase in 20 mM phosphate buffer, pH 6.0. The reaction was incubated at 65 °C for 24 hours. Then, a 15-µl aliquot concentration was determined by the glucose oxidase method. Samples were also analyzed using thin layer chromatography (TLC) as described in Section 3.19.1. The intensity of functional oligosaccharide product spots was quantitated relative to glucose spot in the same TLC plate. The highest intensity of functional glycoside or oligosaccharide spots were set to 100%.

3.20.2 Acceptor specificity

Purified recombinant AMase in phosphate buffer, pH 6.0, was incubated with 1.5% (w/v) tapioca starch as a glycosyl donor and two types of acceptor: 2% (w/v) of monosaccharides and disaccharides. The group of monosaccharide were such as glucose, fructose, ribose, xylose, mannose and galactose. For the group of disaccharides, maltose, sucrose, lactose, trehalose, cellobiose and palatinose were used. Each reaction mixture was incubated at 65 °C for 24 hours. The products from various acceptors were analyzed by TLC plate using separately appropriate systems (Section 3.19.1.).

3.21 Optimization of functional oligosaccharide production

3.21.1 Optimization of tapioca starch concentration

Tapioca starch (TS) was selected as an appropriate donor for the synthesis of xylose glycosides by transglycosylation reaction of AMase. The amount of tapioca starch which was varied on a final concentration of 0.5-2.0% (w/v) in phosphate buffer pH 6.0 was mixed xylose and 2 U/ml AMase in 20 mM phosphate buffer pH 6.0. The reaction mixture was incubated at 65 °C for 24 hours and the reaction was stopped

by boiling for 5 minutes. The reaction mixture was runned on TLC plate as described in Section 3.19.1 to find the optimal TS concentration for xylose glycoside production. The amount of xylose glycosides was measured by scanning densitometer. The appropriate tapioca starch concentration was judged from the highest yield of the xylose glycoside products.

3.21.2 Optimization of xylose concentration

Optimization of xylose concentration for the synthesis of novel glycoside product was performed by varying xylose acceptor ranging from 0.5-2.0 % (w/v) with the fixed concentration of TS donor. The reaction mixture was incubated with AMase (2 U/ml) at 65 ^{o}C in 20 mM phosphate buffer, pH 6.0 for 24 hours. After incubation completed, the reaction was stopped by boiling for 5 minutes. The reaction mixture was then runned on TLC plate as described in Section 3.19.1 to find the optimal concentration of xylose in the synthesis of xylose glycosides. The amount of xylose glycosides was measured by scanning densitometer. The optimal xylose concentration was evaluated from the highest yield of the xylose glycoside products.

3.21.3 Optimization of enzyme concentration

To find the optimum concentration of AMase, TS substrate concentration (Section 3.21.1) and optimal xylose acceptor (Section 3.21.2) which were selected from previous investigation, were incubated with varying amounts of AMase (2-32 U/ml) in 20 mM phosphate buffer pH 6.0 at 65 ^{o}C for 24 hours. The optimal enzyme concentration was decided from the amount of xylose glycoside products on TLC analysis as described in Section 3.19.1.

3.21.4 Optimization of pH

The reaction mixture was performed by incubation of an optimal concentration of TS donor, xylose acceptor and AMase from the previous Section in buffer with pH in ranging from 3.0-11.0. Different inorganic substances were used as buffer e.g. acetate buffer (pH 3.0-5.0), phosphate (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and Glycine-NaOH (pH 9.0-11.0). The optimal pH for the production of xylose glycosides was judged from TLC analysis as described in Section 3.19.1.

3.21.5 Optimization of incubation time

The reaction mixture which consisted of an optimal concentration of tapioca starch (Section 3.21.1) was incubated with appropriate xylose concentration (Section 3.21.2) and optimal AMase unit (Section 3.21.3) under optimal pH (Section 3.21.4) for transglycosylation reaction. The reaction mixture was incubated for various times (0, 6, 12, 24, 48, 72, 96, 120 and 144 hours). The suitable incubation time for the production of xylose glycosides was chosen from TLC analysis as described in Section 3.19.1.

3.21.6 Optimization of temperature

The optimum temperature for the synthesis of xylose glycosides was determined in the reaction mixture which consisted of an optimum concentration of tapioca starch, xylose and AMase as reported in Section 3.21.1, 3.21.2 and 3.21.3 respectively. The reaction mixture was performed at optimal pH (Section 3.21.4), incubation time (Section 3.21.5) and various temperature (30, 35, 40, 45, 50, 55, 60, 65 and 70 ^{o}C). The optimal temperature for the production of xylose glycosides was judged from TLC analysis as described in Section 3.19.1.

3.22 Large scale production of xylose glycosides

Large scale production of xylose glycosides was performed under the optimum conditions obtained from previous section (Section 3.21). In the initial experiments, transglycosylation reaction was performed in the reaction mixture of 150 µl. To produce large amount of products, the total volume was raised to 50 ml. After the incubation was complete, the reaction mixture was concentrated by evaporation at 45 ^{o}C before purification of xylose glycosides by HPLC as described in Section 3.19.2.

3.23 Structural analysis of xylose glycosides

3.23.1 Mass spectrometry (MS) analysis

A mass spectrometry (Bruker, Germany) was used to determine the molecular mass of chemical compounds by separating ionic molecules according to

their mass-to-charge ration (*m/z*). The molecular mass was calculated by the equation (3.3):

(molecular mass + number of protons)/charge = mass-to-charge ration (*m/z*) (3.3)

The xylose glycosides products were dissolved in 50% (v/v) methanol and subjected into mass spectrometry device. Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was recorded on microTOF at the Service Unit of the National Center for Genetic Engineering and Biotechnology (BIOTECH, Thailand). The compounds were ionized by electrospray ionization in the sodium positive-ion mode using capillary voltage of 5,000 volts. The 4 liters perminute flow of nitrogen gas at 150 ^{o}C was performed to nebulize the analytic solution to droplets using nebulizer pressure at 1 bar.

3.23.2 Nuclear Magnetic Resonance (NMR) analysis

Structural identification of P xylose and the synthesized xylose glycosides was performed by ^{1}H and ^{13}C NMR spectrum with Bruker AVANCE III HD 400 NMR (Bruker Daltonik GmbH, Bremen, Germany) using stand Bruker NMR software at the Biological Service Unit of the National Center for Genetic Engineering and Biotechnology (BIOTECH, Thailand). The operation was 400 MHz ambient temperature. The spectra were obtained with 2 mg of each sample dissolved in methanol-D_4 (CD_3OD).

3.24 Evaluation of the physiochemical and biological properties of xylose glycosides products

3.24.1 Solubility test

Excess amounts of xylose glycosides product were mixed with 200 µl of water, vortexed for 5 minutes at room temperature to disperse into solution, and then each sample was incubated at 30 ^{o}C for 15 minutes to allow the saturation. Xylose glycosides were then filtered through a 0.45 µm membrane to remove insoluble particles and subjected to amino column HPLC analysis in order to determine the concentration of soluble xylose glycosides (Aramsangtienchai et al., 2011).

3.24.2 Sweetness test

The relative sweetness of 1% (w/v) of the tested xylose product was determined by measuring the Brix values using a refractometer (RX-1000, Atago Co., Ltd., Tokyo, Japan) (Kikuchi et al., 2004). Sucrose was used as the reference compound to plot the standard curve of the concentrations (%, w/v) versus the degrees Brix (Bx). One-degree Brix is defined as 1 g of sucrose in 100 ml of solution.

3.24.3 Maillard reaction test

200 mM Phosphate buffer (pH 5-7) containing 0.5 % (w/v) glutamic acid and 10 % (w/v) xylose sugar transfer product or sucrose were put in Eppendrop tubes that were sealed and incubated for 1 hour at 100 ^{o}C. The coloring caused by the color caused by the Maillard activity was determined at A_{420} (Kaulpiboon et al., 2015).

3.24.4 Acidic resistance test

Acidic resistance of xylose glycosides product was investigated in 100 mM acetate buffer, pH 3.0 containing 1.0% (w/v) xylose and xylose glycosides product. The reaction mixture was incubated at 37 ^{o}C for 24 hours. Undegrade oligosaccharides by acidic condition were determined by HPLC as described in section 3.19.2.

3.24.5 Prebiotic activity test

Starter inoculum of probiotic *Lactobacillus brevis* BCC26712 and non-probiotic *Escherichia coli* ATCC® 25922, were cultured in 50 ml of MRS broth at 37 ^{o}C with rotary shaking at 100 rpm for 24 hours.Then, they were continually cultured in MRS broth containing 1% (w/v) of xylose and xylose glycosides product at 37 ^{o}C with rotary shaking at 100 rpm for 48 hours. An in vitro xylose and XG_1 utilization test were performed by following the optical density at 600 nm and the decrease in the pH of the medium for 48 hours. In addition, co-culture experiment of probiotic *L. brevis* and pathogenic *E. coli* was also performed in MRS broth concentration of XG_1 at 37 ^{o}C for 24 hours. After that, a 200 µl co-culture (at 1: 10,000 dilutions) was spread on MRS agar plate. The ability of probiotic bacteria that inhibits growth of pathogenic bacteria was evaluated by counting colonies of each different strain.

CHAPTER 4
RESULTS AND DISCUSSION

PART 1: Synthesis and characterization of functional glycosides

4.1 Bacterial cultivation and enzyme preparation

1% (v/v) of starter inoculum of the recombinant pBC cells (Kaulpiboon et al., 2010) was transferred into 300 ml LB medium in 1,000 mL Erlenmeyer flask and cultured at 37 °C with 250-rpm agitation for 24 hours. The method of cultivation and the enzyme production were described in Section 3.4. After cultivation, the cells were removed by centrifugation at 4,800 rpm for 45 minutes at 4 °C. As an extracellular enzyme, the obtained supernatant was used as crude CGTase. The dextrinizing activity (Section 3.7.1) and protein (Section 3.8) of crude CGTase were 199 U/ml and 0.25 mg/ml, respectively (Table 4.1). According to the production of CGTase, the quality and the quantity of obtained enzymes are depending on their sources. Various sources of microorganisms were used in many studies. *Bacillus firmus* was used to produce CGTase and obtained 1,463 U/ml of total activity (U/ml) and 2,080 mg/ml) of total protein (Yim et al., 1997).

4.2 Purification of CGTase by starch adsorption

The crude enzyme was continuously purified by starch adsorption as described in Section 3.5.1. The total activity of enzyme was 2,096.19 U/ml by dextrinizing activity assay and the recovered proteins were 0.20 mg. The specific activity of the enzyme was 10,480.95 U/mg protein by calculating from the equation (4.1):

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

The purification table is shown in Table 4.1. Enzyme purity was 7-fold with 97% yield. For other examples of CGTase purification, Jia et al (2017) reported that after the four purification steps, the CGTase enzyme from *Bacillus circulans* A11 was purified to 350.5-fold with a yield of 9.3%. According to the starch adsorption purification as a step of purification, various types of starches could be used for enzyme purification. Wheat starch gave the lowest enzyme in the absorbent (separation material) activity of 208.6 U/ml, while tapioca and corn starches gave enzyme activity 1214 unit/ml (Trisiriroj et al., 1996). When compared tapioca and corn starches, CGTase gained higher specific activity of enzyme when corn starch was used. The interaction between starch and enzyme was not only adsorption but also substrate-enzyme affinity type. So, it possible that CGTase may be lost its activity during purification step. The absorbed enzyme was eluted by maltose solutions that have higher affinity than starch in binding to CGTase. The enzyme could be easily separated from maltose solution by dialyzing with TB1 buffer before determined its dextrinizing activity.

4.3 Enzyme analysis by polyacrylamide gel electrophoresis (PAGE)

The CGTase from each step of purification was monitored by 2 types of electrophoresis.

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

Purified CGTase was determined by native polyacrylamide gel electrophoresis (Native-PAGE) and stained for both protein and dextrinizing activity as described in Section 3.6.1. By Native-PAGE analysis, protein staining revealed that the enzyme was highly purified through the first starch adsorption step. A single protein band was observed on the protein staining gel which was consistent with activity band of the enzyme as shown in Figure 4.1A-B, Lane 2. Moreover, it was also confirmed on SDS-PAGE with the one band appearance (Figure 4.2, Lane 2). This result illustrated that it was desirable even working on only 1 step of purification. So, the starch adsorption is an appropriate method for the CGTase purification.

Table4.1 Purification table of CGTase from the pBC transformant cell

Purification step	Activity* (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude CGTase	199.00	0.25	796.00	1.00	100.00
5% (w/v) Starch absorption	2,096.19	0.20	10,480.95	13.17	97.00

* Dextrinizing activity

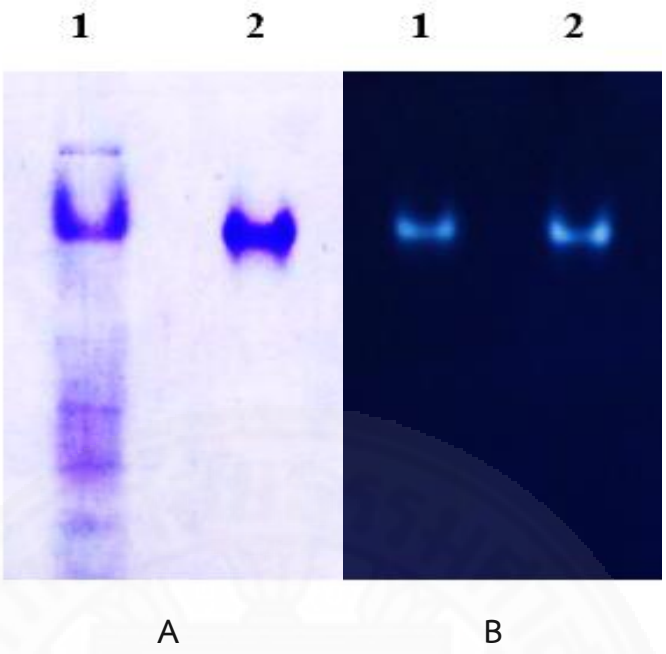


Figure 4.1 10% non-denaturing PAGE of CGTase from each purification step.

(A): Coomassie brilliant blue staining

Lane 1: Crude enzyme 30 µl (4 µg)

Lane 2: 5% Starch adsorption 30 µl (4 µg)

(B): Dextrinizing activity staining

Lane 1: Crude enzyme 10 µl (0.2 U)

Lane 2: 5% Starch adsorption 4 µl (0.2 U)

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

The CGTase from each step of purification was also examined for its purity and protein pattern by SDS-PAGE analysis as described in Section 3.6.4. The crude enzyme showed many bands of protein (Figure 4.2). On the other hand, the purified enzyme of starch adsorption step showed only 1 band of protein as same as the one band from Native-PAGE. This result implied that the enzyme was true purified by one step of starch adsorption. By SDS-PAGE, the molecular mass of CGTase was also determined and found that it was approximately 72 kDa compared to the standard protein's marker (Figure 4.3). The CGTase molecular weight is vary depending on the CGTase source. The size of CGTase is in the range of 70-75 kDa. In comparison with other studies, Yim et al. (1997) reported that the purified CGTase enzyme from *Bacillus firmus* had an estimated molecular weight of 75 kDa. Similarly, the purified CGTase consisted of a single band on SDS-PAGE with a molecular weight of approximately 70 kDa, which was consistent with data reported for the CGTases purified from other *Bacillaceae* species, such as *Bacillus pseudalcaliphilus* 20RF (Atanasova et al., 2011).

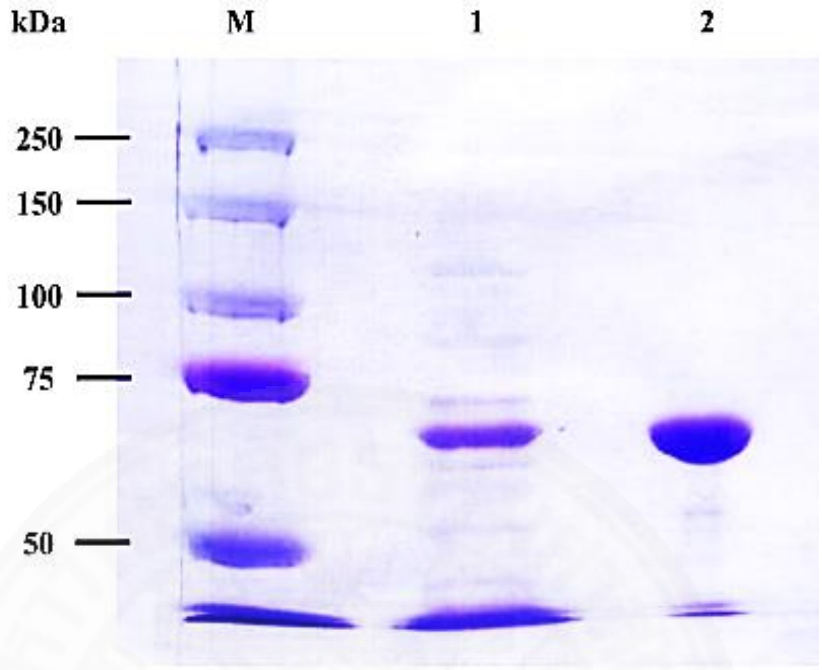


Figure 4.2 10% SDS-PAGE of CGTase from each purification step.

Lane M: Marker	10 µl
Lane 1: Crude enzyme	30 µl (4 µg)
Lane 2: 5% Starch adsorption	30 µl (4 µg)

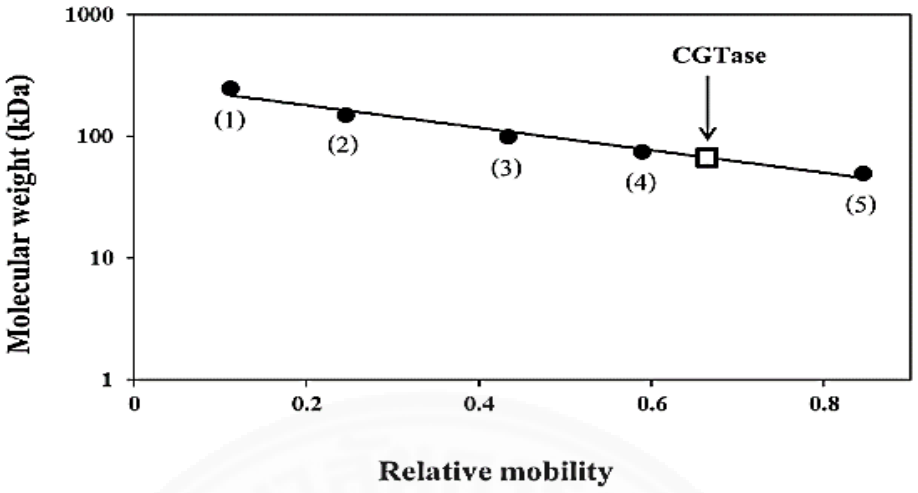


Figure 4.3 Calibration curve for molecular weight of CGTase by 10% SDS-PAGE.

(1) = 250,000 Da (Myosin)

(2) = 150,000 Da (Phosphorylase)

(3) = 100,000 Da (β-galactosidase)

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

(5) = 50,000 Da (Ovalbumin)

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

4.4 Biosynthesis of lignan pinoresinol glycosides by CGTase and their detection

4.4.1 Donor specificity

When the recombinant CGTase was used for the synthesis of lignan pinoresinol glycosides, it was found that this enzyme could catalyze the transglucosylation to transfer glucose molecule from donors to acceptor, and the occurred glucose from the reaction was measured by glucose oxidase assay. As the result, the cyclic oligosaccharide donor (α-, β-, γ-CD) and starch donor group could act on pinoresinol, as the acceptor molecule. β-CD gave the highest transglucosylation activity so it was set as 100% relative avtivity, followed by α-CD,γ-CD, tapioca starch (TS), pea starch (PS) and potato starch (PoS) (Figure. 4.4). In addition, all of the reaction mixtures from different glucosyl donors were also detected using TLC analysis which gave least two main products (Figure. 4.5). When the intensity of each product spot was compared, it was observed that β-CD donor showed highest yield of pinoresinol glycosides. Therefore, based on the relative transglucosylation activity and TLC analysis, β-CD was chosen as a suitable glucosyl donor.

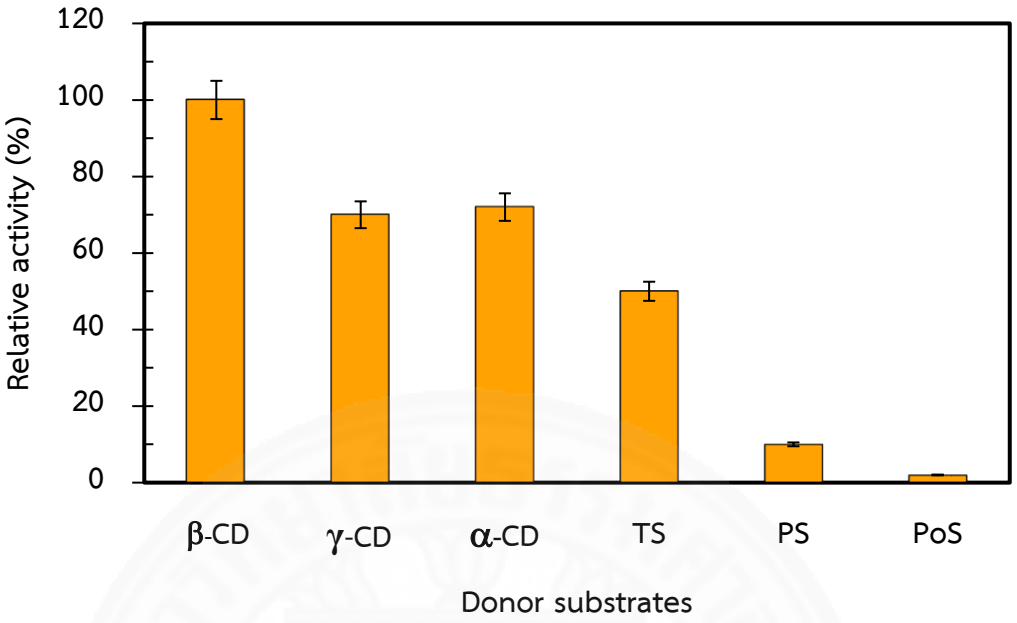


Figure 4.4 Relative intensity of pinoresinol-α-glycosides from transglycosylation reaction of cyclodextrin glycosyltransferase from various 1% (w/v) donors to 1% (w/v) pinoresinol acceptor.

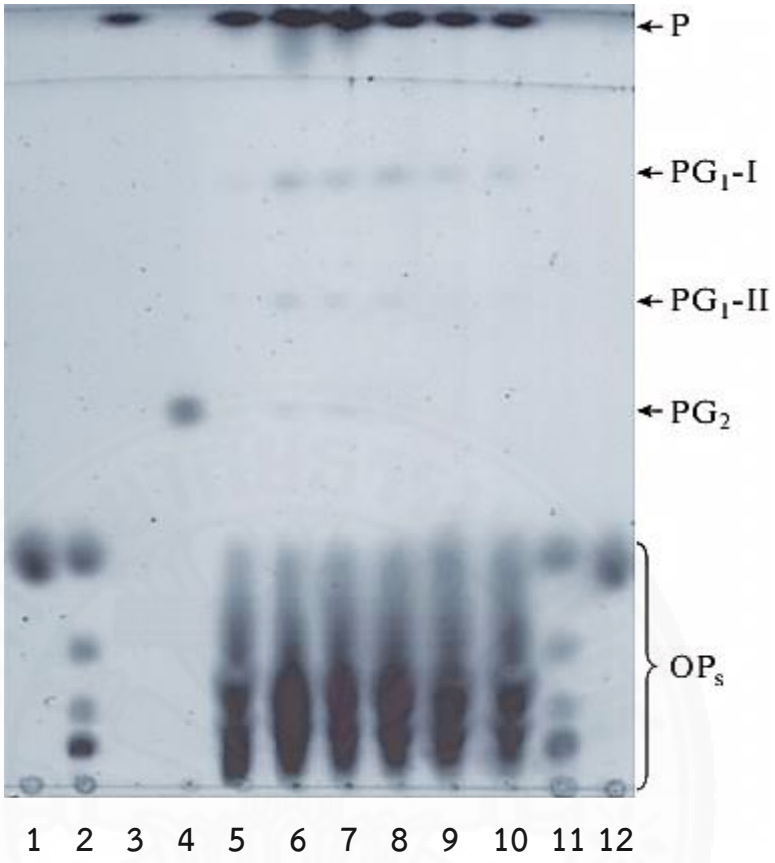


Figure 4.5 TLC analysis of products obtained after transglycosylation of pinoresinol by different donors and CGTase from *B. circulans* A11. Standards: lanes 1, 12-G_1 (10 ug) lanes 2, 11: G_1-G_4 (10 µg), lane 3-P (5 µg), lane 4-PG_2 (5 µg). Products from enzyme reaction between pinoresinol and various donors: lane 5-α-CD, lane 6-β -CD lanes 7-γ-CD, lane 8-pea starch, lane 9-potato starch, and lane 10-tapioca starch. OPs-oligosaccharide products.

4.4.2 Optimization of pinoresinol glycoside production

To optimize the production process of pinoresinol glycosides, five parameters were studied. The amount of of pinoresinol glycosides was analyzed by TLC technique. The intensities of the transfer products on the TLC plate were measured using a densitometer with GS-800 program (Bio-Rad Laboratories, CA, USA) and compared with the glucose standard curve.

4.4.2.1 Effect of substrate ratio (β-CD: pinoresinol)

Using the conditions described in previous section with variable ratios of β-CD: pinoresinol, it was found that product PG_1-I and PG_1-II increased slightly when β-CD: pinoresinol ratios were 1:1, 1:2 and 1:3 respectively. However, at 1:4, the product yields decreased. For the β-CD: pinoresinol ratios of 2:1, 2:2, 3:1 and 4:1, although the transglucosylation transfer efficiency showed a trend of higher percent, the increased product yields were greater for oligosaccharide products rather than glycoside product. The relative intensities (%) of the products formed are presented as means in Table 4.2. The maximum glycoside product was obtained when the reaction was incubated with 0.5% (w/v) β-CD and 1.5% (w/v) pinoresinol (1:3 ratio) compared to other ratios. Thus, the β-CD: pinoresinol ratios of 1:3 was chosen as the optimal ratio for the production of pinoresinol glycosides.

4.4.2.2 Effect of enzyme unit and incubation time

The optimization of the pinoresinol glycoside production for a higher yield was performed by varying enzyme units and incubation times together. The relative amount of the formed-glycoside product is shown in Figure. 4.4. Various enzyme units of recombinant pBC CGTase (10-80 units, determined with dextrinizing activity) were tested depending on incubation times (0-72 hours). The maximum amount of glycoside products was revealed when enzyme units and incubation time were increased to 80 units (from 10 units) and an incubation time of 60 hours, respectively (Figure. 4.6). Thus, the chosen enzyme unit and incubation time for incubating reaction in the further step were 80 units and 60 hours.

Table 4.2 Influence of different substrate ratios on the transglycosylation transfer efficiency (TE) of substrates and their obtained products. The relative amount of the product formed is presented as the mean intensity percentage of replicate sample. All experiment was conducted for 24 hours at pH 6.0 and 45 ^{o}C using 40 U CGTase.

Substrate reaction mixture (β-CD: Pinoresinol)								
Products	**1:1**	**1:2**	**1:3**	**1:4**	**2:1**	**2:2**	**3:1**	**4:1**
PG_1-I	0.65	1.19	1.28	0.60	1.20	0.87	0.69	0.34
PG_1-II	0.40	0.50	0.55	0.37	0.39	0.50	0.25	0.02
PG_2	0.04	0.06	0.06	0.04	0.05	0.04	0.02	0.00
Oligosaccharide	89.71	91.58	91.49	89.99	57.25	88.63	95.29	96.74
Total TE	90.80	93.33	93.38	91.00	88.99	89.94	96.25	97.09
Acceptor substrate remaining	9.20	6.67	6.62	9.00	11.01	10.06	3.75	2.90

4.4.2.3 Effect of temperature

The effect of temperature on pinoresinol glycoside production was determined in the range of 35-70 °C. The reaction was performed by incubating 0.5% (w/v) β-CD and 1.5% (w/v) pinoresinol with 80-U CGTase in 20 mM phosphate buffer pH 6.0 at various temperatures of 35-70 °C for 60 hours. From Figure 4.7, it was found that CGTase could catalyze the transglucosylation to pinoresinol from β-CD at the temperature ranging from 35-70 °C with the highest amount of pinoresinol glycosides (PGs) formed at 50 °C. The relative maximal amount of the product formed was determined to be 3.87% of the total products. At temperatures >50 °C, the product formed decreased dramatically. So, the optimum temperature of 50 °C was chosen for the production of lignan pinoresinol glycosides.

4.4.2.4 Effect of pH

The effect of pH on CGTase-catalyzed transglucosylation reaction from β-CD to pinoresinol for pinoresinol glycoside production was performed. Twelve conditions using different pHs in four kinds of buffer were investigated. The four buffers consisted of acetate buffer pH 3-5, phosphate buffer pH 5-7, Tris-HCl buffer pH 7-9 and glycine-NaOH buffer pH 9-11 using the same concentrations as the main environment of the reaction mixture. The method used is as described previously with optimum conditions at 50 °C for 60 hours. As shown in Figure 4.8, the highest amount of pinoresinol glycoside was at a reaction pH of 9.0.

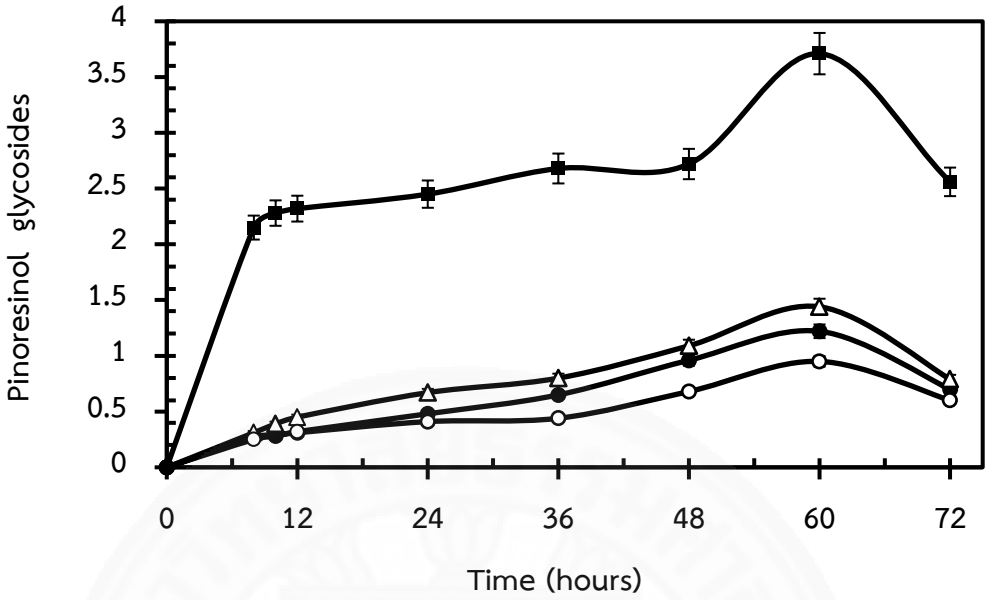


Figure 4.6 Influence of enzyme activity and time on pinoresinol glycoside production. The reaction conditions containing 0.5% (w/v) β-CD and 1.5% (w/v) pinoresinol were incubated with CGTase at **o**, 10; ●, 20; Δ, 40 and ■, 80 units in 20 mM phosphate buffer (pH 6.0) at 45 °C for 0-72 hours.

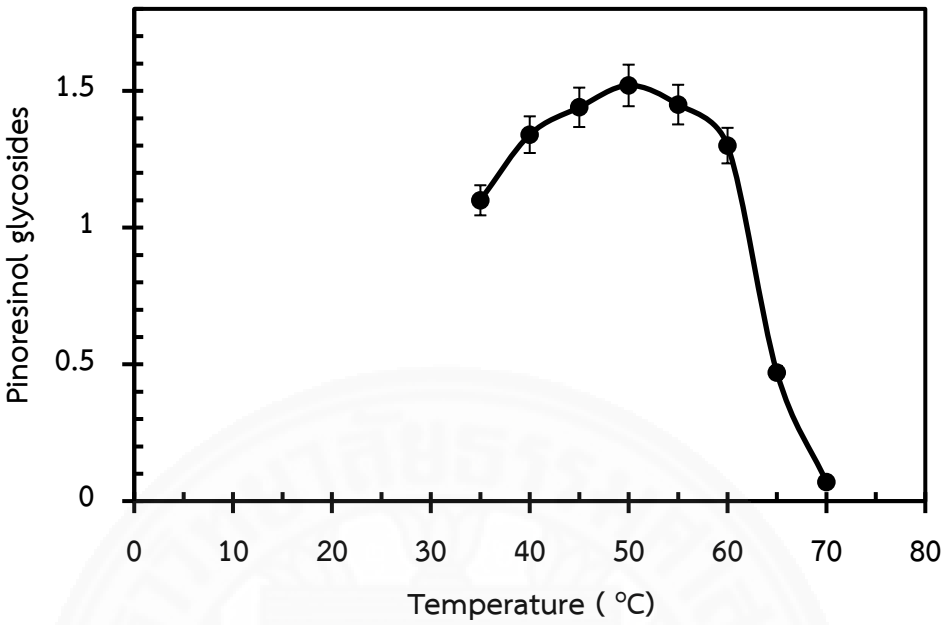


Figure 4.7 Effect of temperature on pinoresinol glycoside production. The reaction conditions containing 0.5% (w/v) β-CD and 1.5% (w/v) pinoresinol were incubated with 80-unit CGTase in 20 mM Phosphate buffer (pH 6.0) at 72 hours.

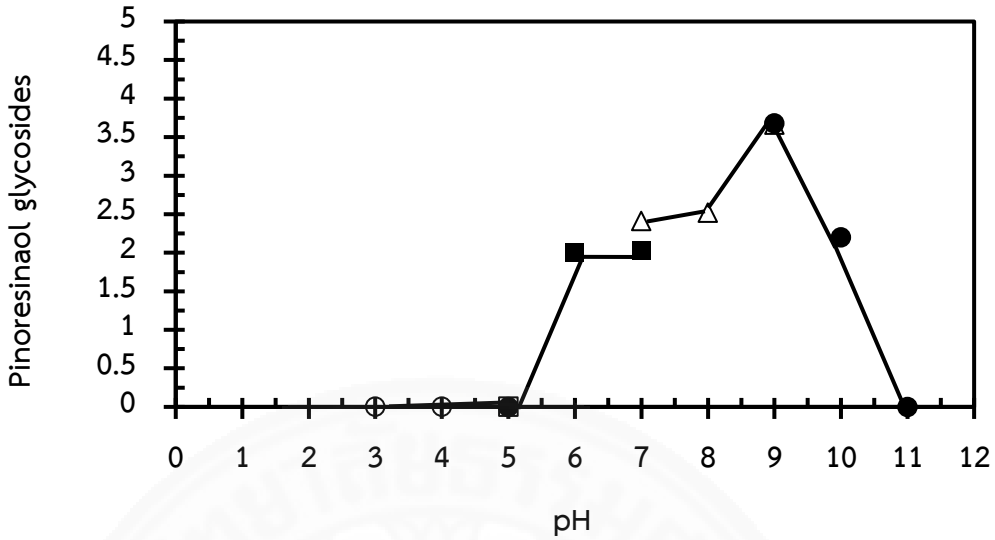


Figure 4.8 Influence of pH on pinoresinol glycoside product. The reaction conditions containing 0.5% (w/v) β-CD and 1.5% (w/v) pinoresinol were incubated with 80-unit CGTase in 20 mM o; acetate buffer, ■; phosphate buffer, Δ; Tris -HCl buffer, ●; glycine-NaOH buffer at 50 °C for 60 hours.

4.5 Large-scale production and purification of pinoresinol glycosides

Before optimization, the 20-unit CGTase was incubated with a reaction mixture containing 1% (w/v) β-CD and 1% (w/v) pinoresinol in 20 mM phosphate buffer pH 6 at 50 ^{o}C for 24 hours. The relative intensities of pinoresinol glycoside products were determined to be 0.56% of total products (Figure. 4.9, Lane 6); after optimization the yield increased to 7.6%. Thus, the relative intensity of pinoresinol glycosides after optimization increased almost 14-fold compared to before optimization (Figure. 4.9, Lane 8). At the optimal condition, HPLC was also used for identifying the glycoside products. HPLC profiles reveal 2 products at R_t = 6.23 min of pinoresinol diglycoside (PG2) and R_t = 17.01 min of pinoresinol monoglycoside (PG_1) (Figure. 4.9 and 4.10). In addition, the reaction mixture for the synthesis of pinoresinol glycosides was upscaled to a volume of 50 ml, and the reaction proceeded under the same optimal conditions. Pinoresinol glycoside products from the large-scale production were then purified using PLC. The three pinoresinol glycoside products (Product PG_1-I, PG_1-II and PG_2) with different R values were scraped separately from the PLC plate and dissolved with methanol. The purified product was applied again onto the TLC plate to determine its purity. The TLC plate results showed that each pinoresinol glycoside product was isolated cleanly, based on their polarities. Besides PLC separation, the formed products from β-CD as a glucosyl donor and pinoresinol as an acceptor using recombinant CGTase from *Bacillus circulans* A11 were also purified by HPLC using an C18-reverse phase column (4.6 x 250 mm, Phenomenex Inc.). The 100-µl products was eluted with a 40:75 mixture of methanol - water at a flow rate of 1.0 ml/min at 25 ^{o}C and was detected using a UV detector at 210 nm in which pinoresinol monoglycoside was the main product and pinoresinol diglycoside as minor product. With HPLC analysis, pinoresinol monoglycoside (product PG_1-I and PG_1-II), which was linked with glucose at different positions, was observed at the same retention time.

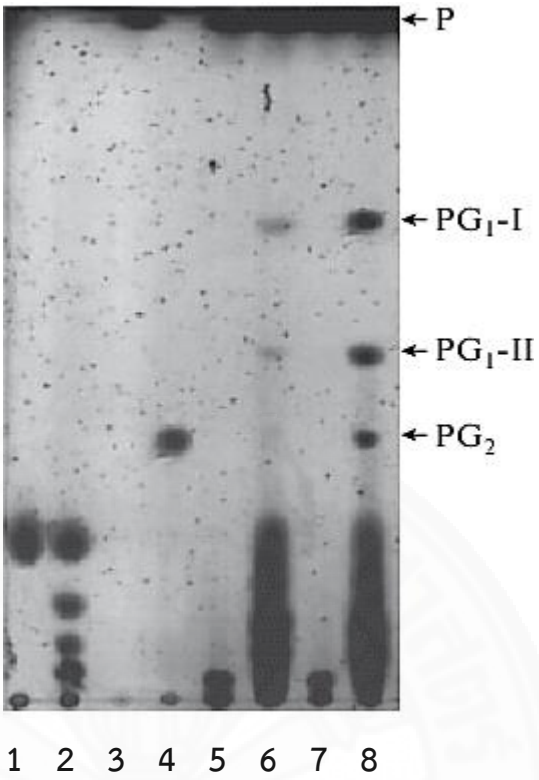


Figure 4.9 TLC chromatogram of products from transglycosylation reaction of CGTase from *B. circulans* A11 before and after optimization. Standards: lane 1-G_1, lane 2-G_1-G_4 (10 µg), lane 3-P, lane 4-PG_2, lane 5-reaction mixture at 0 hour without optimization, lane 6-reaction mixture at 24 hours without optimization, lane 7-reaction mixture at 0 hour before optimization, lane 8-reaction mixture at 24 hours after optimization.

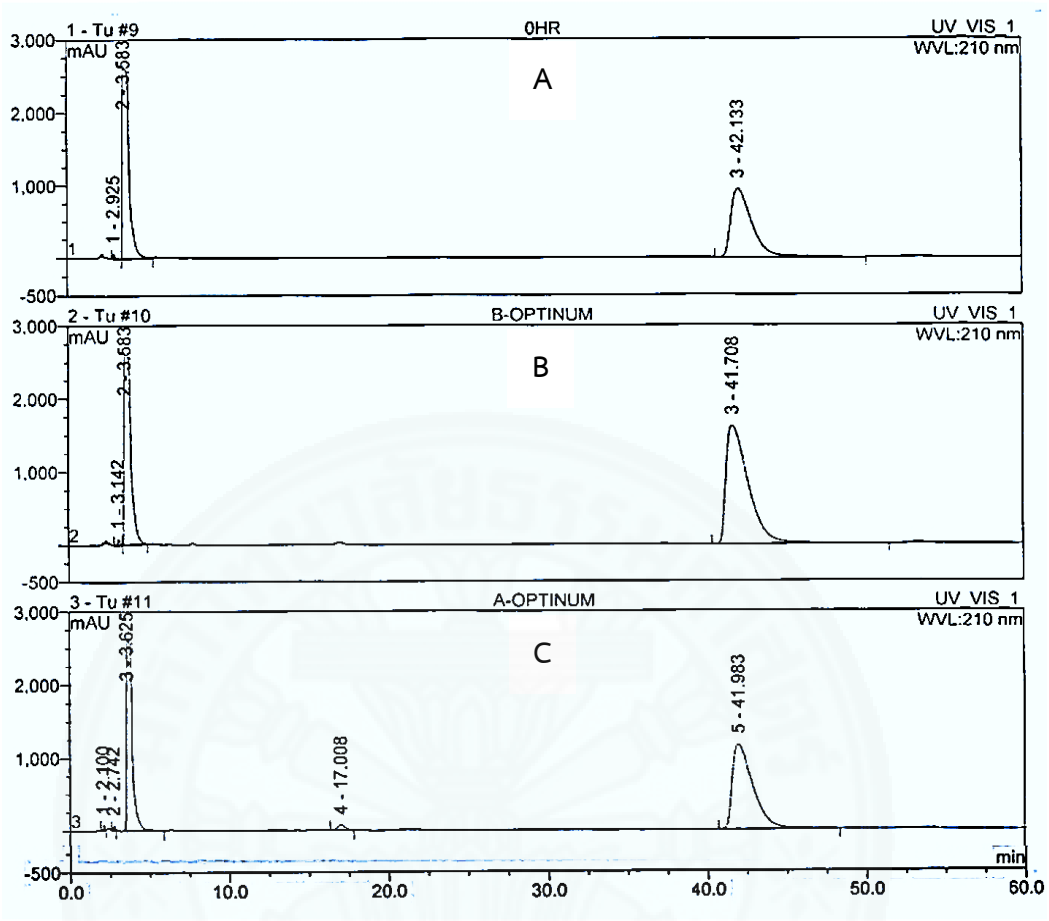


Figure 4.10 HPLC chromatogram of reaction products of CGTase with pinoresinol and β-CD (A. at 0 hour of incubation, B. before optimization and C. after optimization).

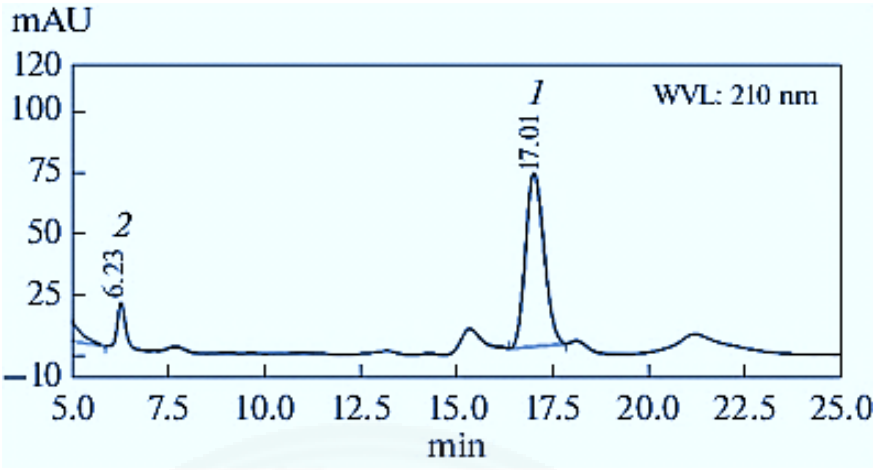


Figure 4.11 HPLC chromatogram (zoom of Figure 4.10C) of reaction products of CGTase with pinoresinol and β-CD at optimal condition.

4.6 Structural analysis of pinoresinol glycosides

The structure of pinoresinol glycosides were determined by enzyme treatment, mass spectrometry (MS) and nuclear magnetic resonance (NMR) analysis as described in Section 3.12.

4.6.1 Enzyme treatment

The structures of the synthesized pinoresinol glycosides were preliminary investigated by enzyme treatment of the PG_2 product with α-glucosidase (EC 3.2.1.20), glucoamylase (EC 3.2.1.3), and several digestive enzymes (Rat intestinal acetone powder as a crude enzyme extract was purchased from Sigma Aldrich (I1630-10G); the final deglucosylated products were analyzed by TLC. The digestive enzyme is a mixture of maltase, isomaltase, lactase, sucrase, α-glucosidase, enterokinase, amylase, lipase and peptidase (Yang et al., 2005). After one hour of digestion with α-glucosidase and the mixed digestive enzymes, the PG_2 disappeared while the intensities of pinoresinol monoglycoside (PG_1) and other maltooligosaccharide spots increased (Figure 4.12). However, when digestion with α-glucosidase and digestive enzyme was performed over 24-hours, only pinoresinol and glucose products were observed. This result implies that PG_1 (observed at 1-hour digestion) and PG_2 are pinoresinol glycosides with an α-configuration; the treatment with α-glucosidase showed only pinoresinol and maltooligosaccharide spots on TLC while PG_1, and PG_2 were absent. With glucoamylase digestion which cleaves all glycosidic bonds except those between pinoresinol and glucose (Chotipanang et al., 2011)., both PG_1, and PG_2 were not hydrolyzed, suggesting that the glucosyl residues are on different sides of the pinoresinol molecule. Thus, PG_2 should be renamed pinoresinol- di- alpha- D-glucopyranoside (Glu- Pinoresinol- Glu) rather than pinoresinol maltopyranoside (Pinoresinol-Glu-Glu) (Figure. 4.12).

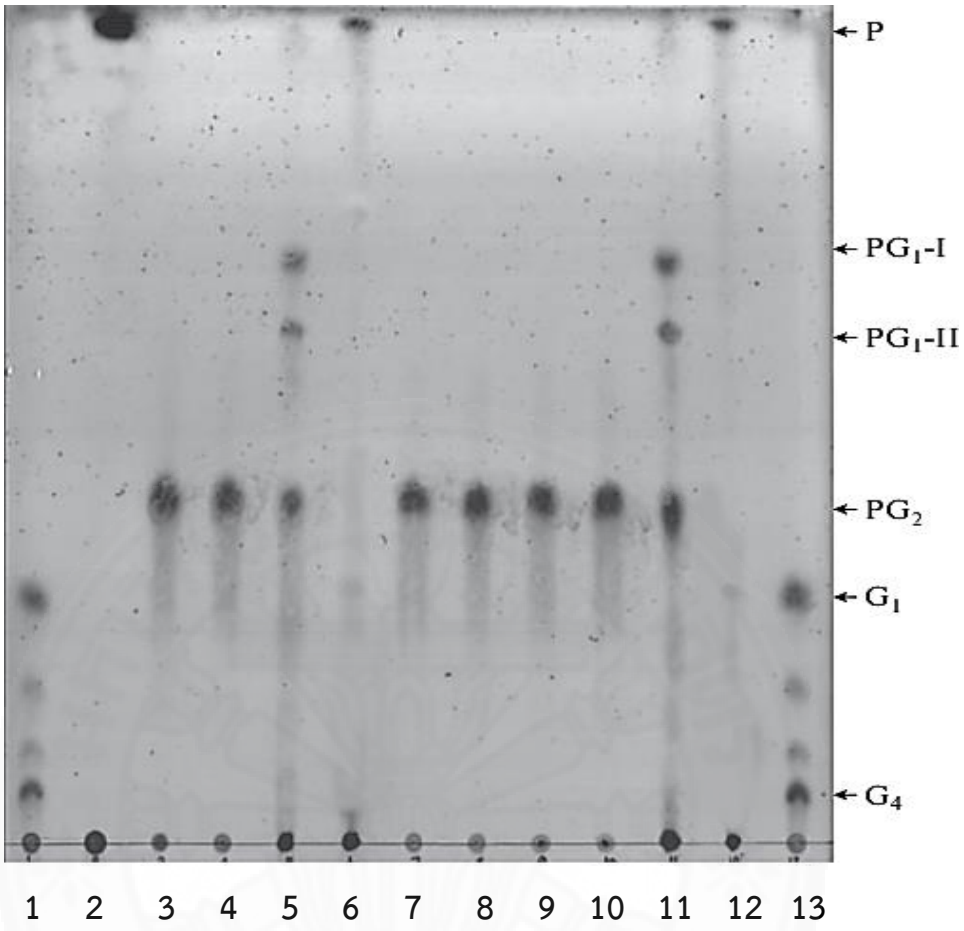


Figure 4.12 TLC analysis of PGs before and after digestion with α-glucosidase, glucoamylase and digestive enzyme. Standards: lanes 1, 13-mixture of G1-G4, lanes 2, 3-mixture of P and PG_2. Lanes 4-6-PG_2 product after digestion with α-glucosidase at 0, 1 and 24 hours, lanes 7–9-PG_2 product after digestion with glucoamylase at 0, 1 and 24 hours, lanes 10–12-PG2 product after digestion with rat digestive enzyme at 0, 1 and 24 hours, respectively.

4.6.2 Mass spectrometry (MS) analysis

To investigate the transglycosylation products by CGTase reaction, mass spectrometry (MS) was employed to determined mass-to-charge ratio of samples. A mass spectrum is a plot of the ion signal. These spectra are used to determine the elemental or isotopic signature of a sample, the mass of particles and of molecules, and to elucidate the chemical structures of molecules such as peptides and other chemical compounds. The molecular weights of the obtained transglucosylation products were analyzed using ESI-TOF mass spectrometer in positive mode. The pseudomolecular ion peak $[M+Na]^+$ of synthesized pinoresinol glycosides appeared at m/z 381.12 (358 plus 23 of sodium molecule), 543.54 (520.54 plus 23 of sodium molecule) and m/z 705.19 (682.19 plus 23 of sodium molecule), respectively. These results correspond to the sizes of pinoresinol (Figure 4.13), pinoresinol monoglycoside (Figure 4.14) and pinoresinol diglycoside (Figure 4.14), respectively.

4.6.3 Nuclear Magnetic Resonance (NMR) analysis

The structures of transferred products were also analyzed by ^{13}C- and ^{1}H-NMR to identify the structure of the pinoresinol glycoside products as showed in Table 4.4 and Figure 4.16; the structure of standard pinoresinol is also shown. When products at R_t = 17.01 minutes and R_t = 6.23 minutes were analyzed, their structures and molecular weights characterised them as pinoresinol monoglycoside (PG_1) and pinoresinol diglycoside (PG_2), respectively. The 400 MHz ^{1}H-NMR was also analyzed to determine the type of glycosidic linkage and was found to be glycosidic linkages of formed pinoresinol mono- and diglycoside were α-configuration type. The ^{1}H-NMR spectrum of PG_1 showed that the anomeric proton of the glucose molecule was a doublet signal at 7.45 ppm with a coupling constant (J) of 3.94 Hz (Figure 4.16). For PG_2, two of doublet signals at 7.08 ppm (J= 4.09 Hz) and 6.85 ppm (J = 4.08 Hz) were assigned to anomeric protons, indicating that PG_2 is composed of two α-linkages (Figure 4.18).

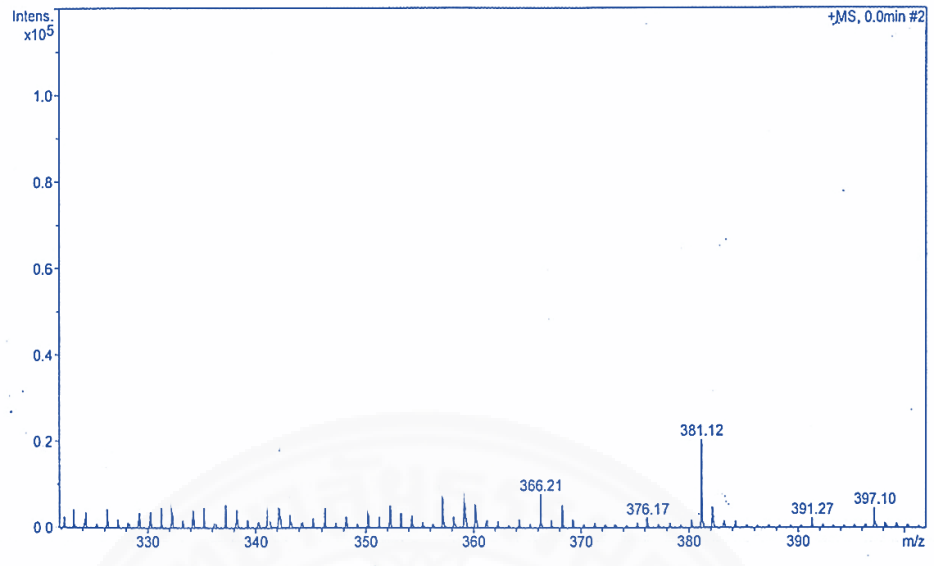


Figure 4.13 Mass spectrum of standard pinoresinol was analyzed by ESI-TOF mass spectrometer.

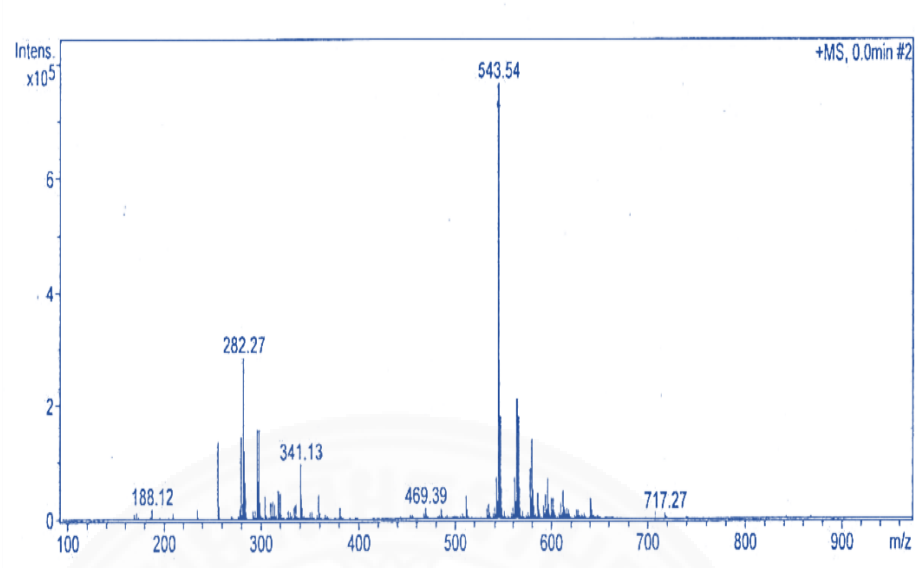


Figure 4.14 Mass spectrum of the formed pinoresinol glycoside (product 1) after the incubation of pinoresinol, β-CD and CGTase.

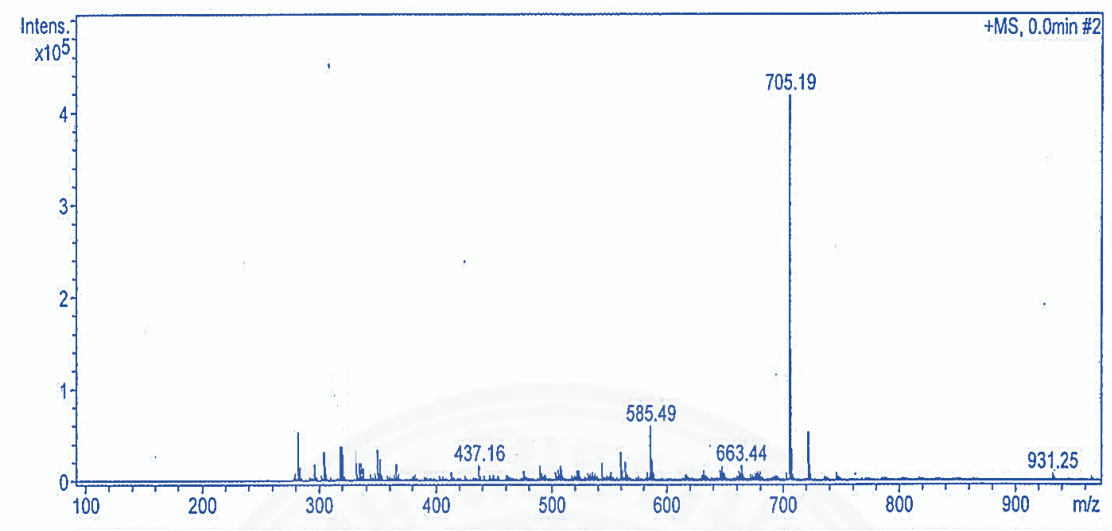


Figure 4.15 Mass spectrum of the formed pinoresinol diglycoside (product 2) after the incubation of pinoresinol, β-CD and CGTase.

Table 4.3 The ESI-TOF-MS analytical masses of pinoresinol, pinoresinol monoglycoside and pinoresinol diglycoside products

Product	Mass number (m/z)
	Determined* $[M+Na]^+$
Pinoresinol (P)	381.12
Pinoresinol monoglycoside (PG_1)	543.54
Pinoresinol diglycoside (PG_2)	705.19

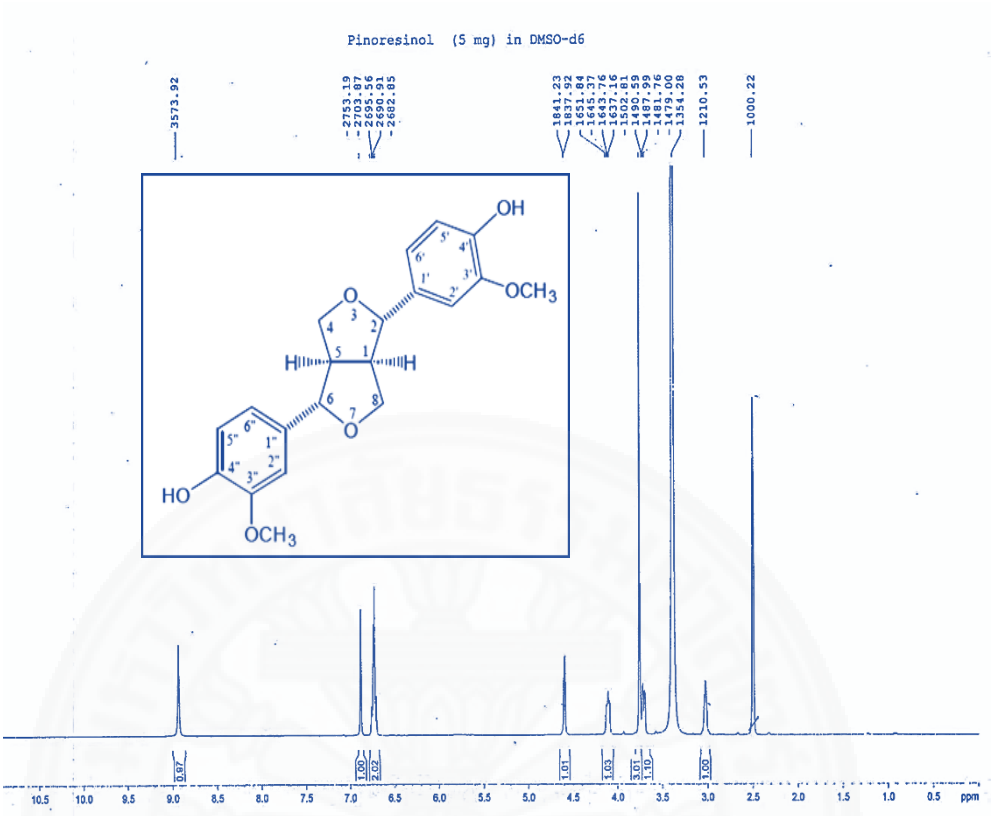


Figure 4.16 The 400 MHz ^{1}H-NMR spectrum of standard pinoresinol. (a.) The structure of pinoresinol with the position of C-atom related to Table 4.4.

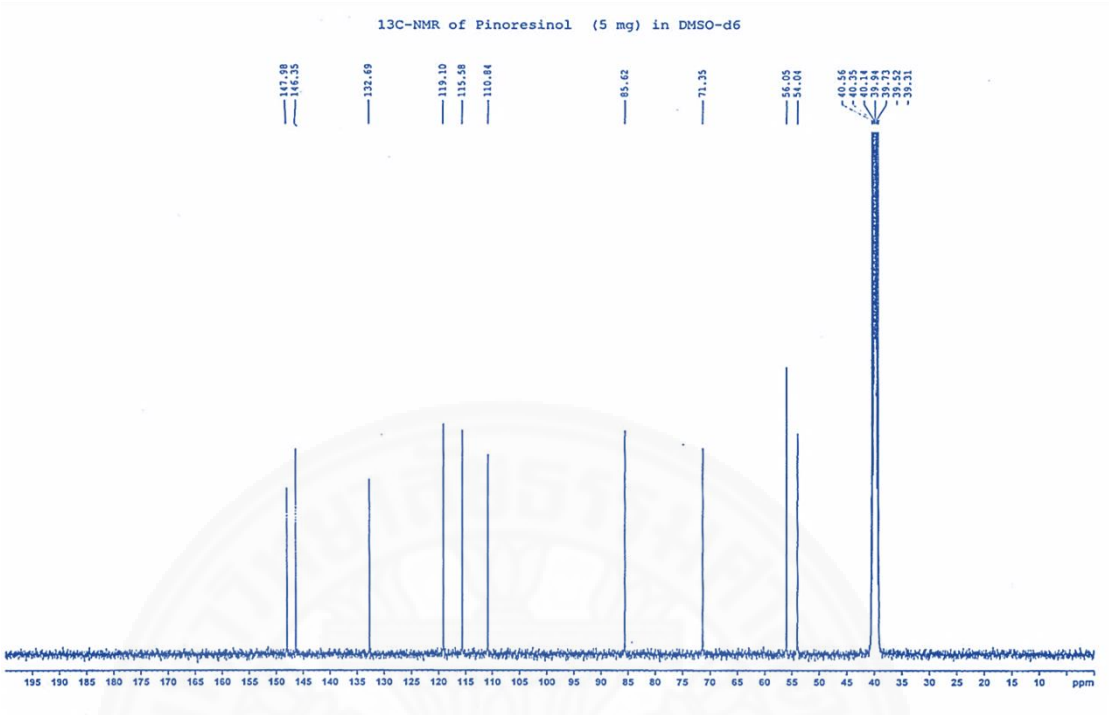


Figure 4.17 The 400 MHz ^{13}C-NMR spectrum of standard pinoresinol.

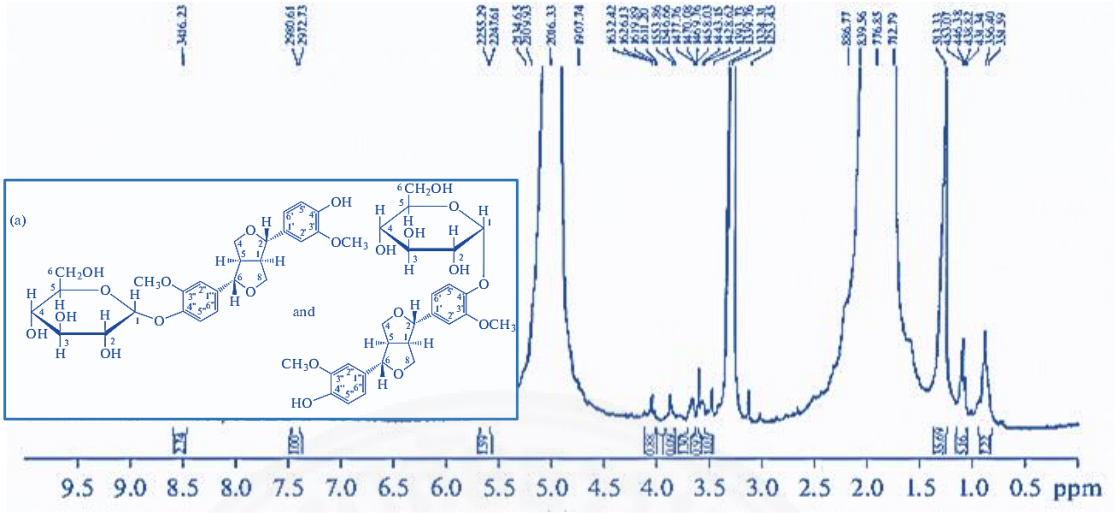


Figure 4.18 The 400 MHz ^{1}H-NMR spectrum of the formed product 1 (PG_1) from reaction of CGTase with **β**-CD and pinoresinol.

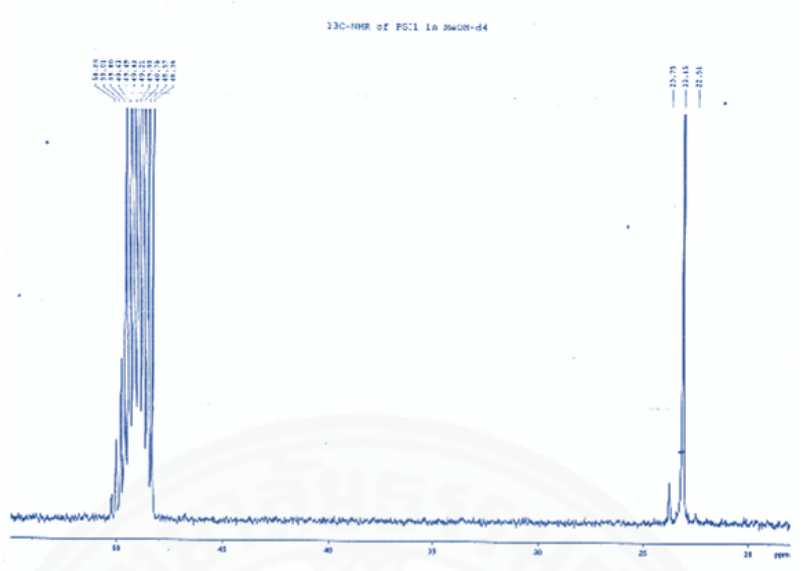


Figure 4.19 The 400 MHz ^{13}C-NMR spectrum of the formed product 1 (PG_1) from reaction of CGTase with **β**-CD and pinoresinol.

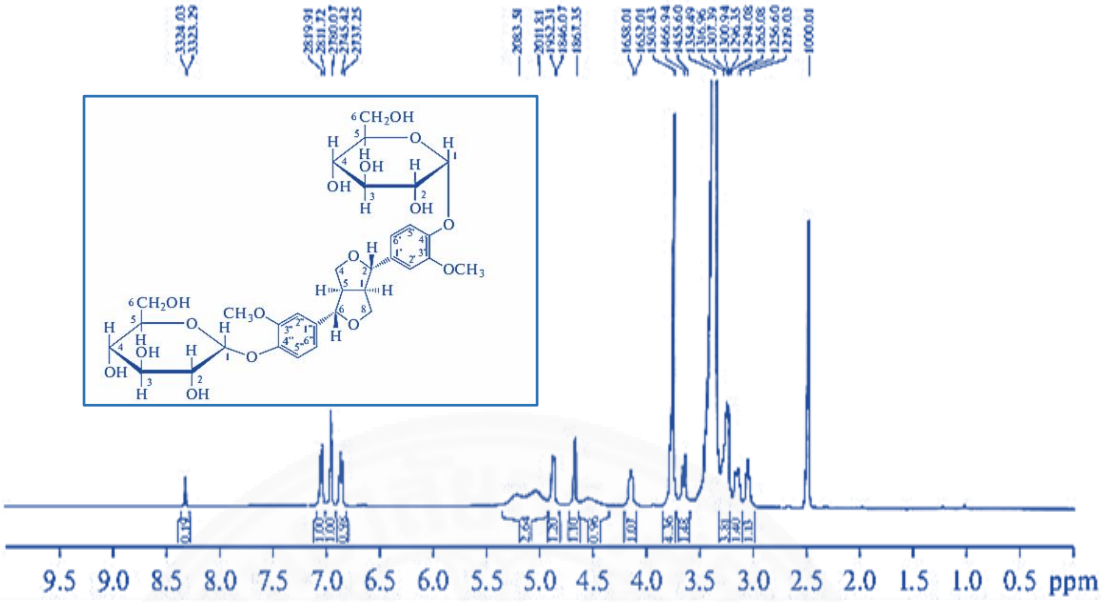


Figure 4.20 The 400 MHz ^{1}H-NMR spectrum of the formed product 2 (PG_2) from reaction of CGTase with β-CD and pinoresinol.

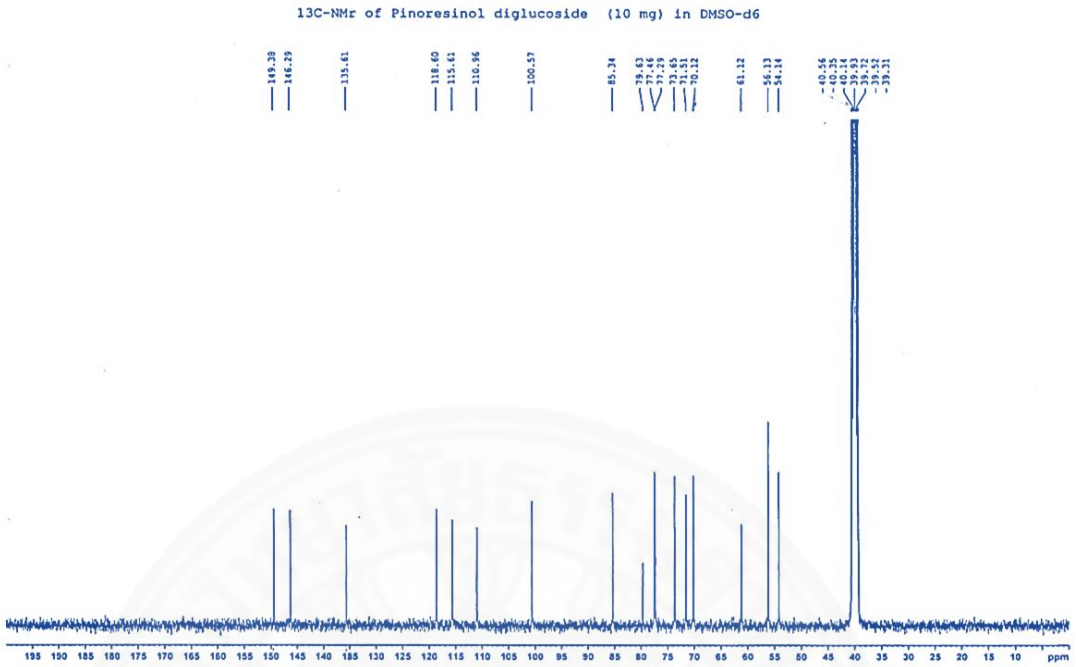


Figure 4.21 The 400 MHz ^{13}C-NMR spectrum of the formed product 2 (PG_2) from reaction of CGTase with **β**-CD and pinoresinol.

Table. 4.4 ^{13}C NMR data of standard pinoresinol, pinoresinol monoglycoside (PG_1) and pinoresinol diglycoside (PG_2) in deuterated DMSO (DMSO-d6)

Carbon atom	^{13}C-NMR[a] (δ, ppm)			
	Glucose	Pinoresinol	PG_1	PG_2
C-Pinoresinol				
C-1		54.04	54.14	54.14
C-2		85.62	85.34	85.34
O-3		-	-	-
C-4		71.35	70.12	70.12
C-5		54.04	54.14	54.14
C-6		85.62	85.34	85.34
O-7		-	-	-
C-8		71.35	70.12	70.12
C-1'		132.61	135.61	135.61
C-2'		110.84	110.96	110.96
C-3'		147.98	149.38	149.38
C-4'		146.35	146.29	146.29
C-5'		115.58	115.61	115.61
C-6'		119.10	118.60	118.60
C-1"		132.61	135.61	135.61
C-2"		110.84	110.96	110.96
C-3"		147.98	149.38	149.38
C-4"		146.35	146.29	146.29
C-5"		115.58	115.61	115.61
C-6"		119.10	118.60	118.60

Table. 4.4 ^{13}C NMR data of standard pinoresinol, pinoresinol monoglycoside (PG_1) and pinoresinol diglycoside (PG_2) in deuterated DMSO (DMSO-d6). (Continued)

Carbon atom	^{13}C-NMR[a] (δ, ppm)			
	Glucose	Pinoresinol	PG_1	PG_2
C-1''''				100.57
C-2''''				77.46
C-3''''				77.29
C-4''''				70.12
C-5''''				79.63
C-6''''				61.12

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

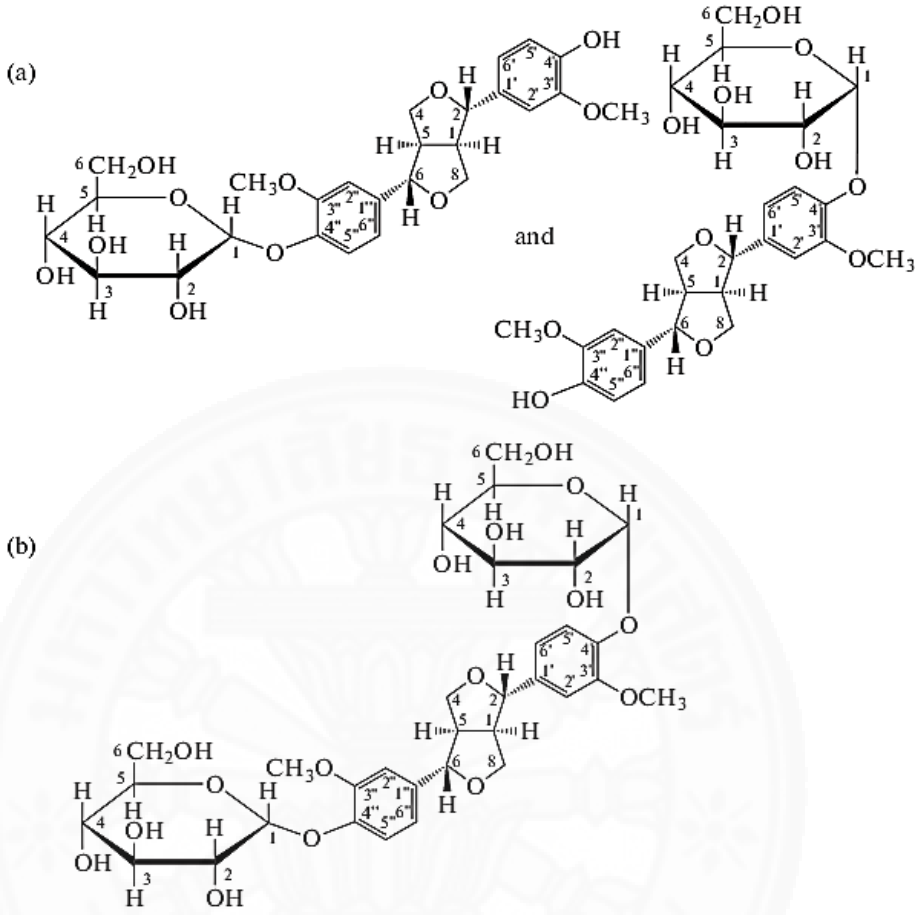


Figure 4.22 Structures of the PG products: PG_1 (**a**) and PG_2 (**b**).

4.7 Physiochemical and biological properties of pinoresinol glycoside products

4.7.1 Solubility test

The water solubility of pinoresinol was compared to that of pinoresinol glycoside products. Pinoresinol and pinoresinol glycoside products were mixed with 200 µl of water in an eppendrof tube and incubated at 25 °C for 15 minutes. The soluble part of the sample was analyzed by HPLC. The results showed that the water solubility of pinoresinol was 5 and 65-fold lower than those of the α-glucosylated derivatives, pinoresinol monoglycoside and pinoresinol diglycoside, respectively.

4.7.2 β-Glucuronidase inhibition assay

Para-nitrophenyl β-D-glucosiduronic acid is proposed as a substrate for the assay of the hydrolytic activity of β-glucuronidase which is related to an inflammation. The reaction mixture was 0.1 ml of 2.5 mM p-nitrophenyl-β-D-glucopyranosiduronic acid in 0.1 M acetate buffer (pH 7.4) was incubated with 1 mg of pinoresinol or its glcoside derivatives for 5 minutes as described in Section 3.13.4 and calculated in Equation 3.4. Anti-inflammation of Salicylic (positive control) was equivalent to 100%. Anti-inflammatory activity of pinoresinol, PG_1 and PG_2 at a concentration of 1 µg/mL had excellent anti-inflammatory properties, showing 95.5, 80.0 and 80.0% inflammatory inhibition compared to 1 mM of salicylic acid (Nile et al., 2012; Rudeekulthamrong et al., 2016), respectively (Figure 4.22). As the results, the increased glucose unit in pinoresinol derivatives showed the decrease of anti-inflammatory activity. This probably due to the increased carbohydrate chain promoted β-glucuronidase activity which did not inhibit the enzyme activity. Moreover, the structure of glucosiduronic acid (glucuronic acid) which is substrate of β-glucuronidase was the same as glucose unit of pinoresinol glycosides. So, that pinoresinol glycosides might be used as substrate which was possibly more than as enzyme inhibitors. Rudeekulthamrong and Kaulpiboon (2016) reported the anti-inflammatory activity of salicin glycosides by using the β-glucuronidase inhibition assay. In contrast to our findings, they found that the long carbohydrate chains of salicin were more effective at inhibiting β-glucuronidase than shorter carbohydrate chains.

Although, lignan glycoside with long carbohydrate chains was less effective at inhibiting β-glucuronidase, but they might be more benefits in other aspects such as solubility, sweetness and antioxidant. Kang et al. (2011) and Ramelet (2001) reported that lignans have been used for anti-inflammatory potential. Various *in vitro* and *in vivo* studies have been established to evaluate pinoresinol about its metabolites, or its synthetic derivatives in the topic of reducing the inflammatory reactions. Differences in the experimental models used can affect the outcomes of the studies and require separate interpretation. In the future, our research group may study the *in vivo* anti-inflammatory activity to identify in detail about inflammatory mechanisms of the pinoresinol glycosides.

4.7.3 DPPH radical scavenging assay

The DPPH radical scavenging assay is commonly used to measure the antioxidant activity. In this study, the free-redical scavenging activities of pinoresinol (P), PG_1 and PG_2 were determined using the stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). The IC_{50} values of P, PG_1, PG_2 and positive control Trolox were 22, 30, 600, and 2 μM, respectively (Figure. 4.23 The IC_{50} values of PG_1, and PG_2 1.4- and 27.3-fold higher than that of the original pinoresinol, suggesting that the free-radical scavenging activity of pinoresinol was reduced when pinoresinol was in the glucosylated form. From the results, Pinoresinol, PG_1 and PG_2 were found to be antioxidants, but they were in a less capacity than Trolox. In addition, the adding of sugar to pinoresinol resulted in decreasing the antioxidant activity. The benefits of pinoresinol glycoside products could be used in the pharmaceutical and food industry. Future work can be established to produce novel drugs from this natural source. Pinoresinol showed their antioxidant properties via two ways: direct radical scavenging and augmenting cellular antioxidant defense. A number of studies revealed that pinoresinol neutralized reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals, peroxynitrite, and nitric oxide radicals (Garg et al., 2001; Kim et al., 2004; Wilmsen et al., 2005). This direct radical scavenging activity of pinoresinol plays an important role in their protection of DNA, proteins, and tissues against damage that is induced by intrinsic (such as oncogenes) and extrinsic (such as radiation, inflammation, and toxins) factors (Parhiz et al., 2015).

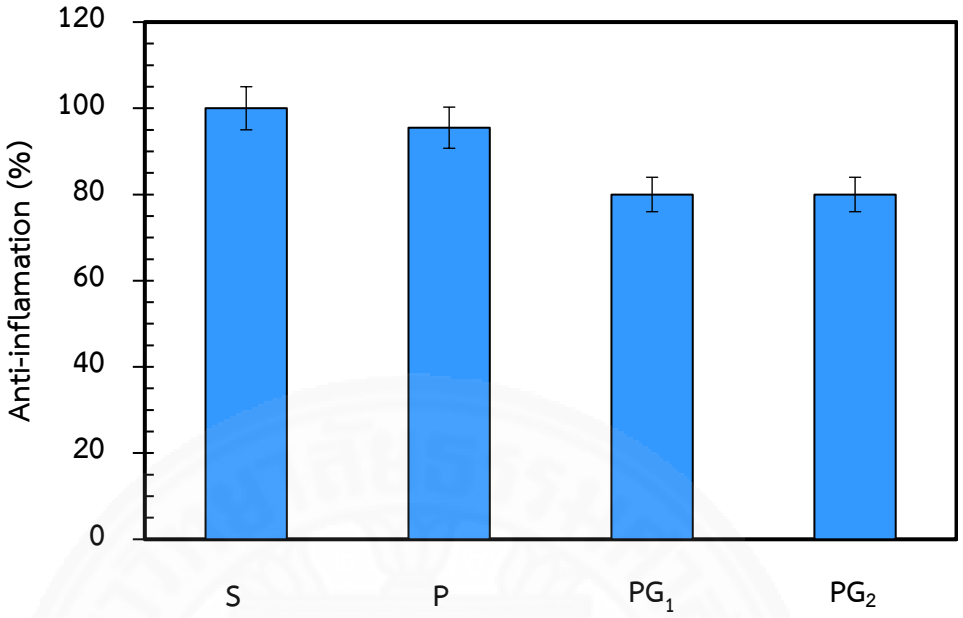


Figure 4.23 β-glucuronidase inhibition assay of Salicylic acid (S), pinoresinol (P), pinoresinol glycoside (PG_1) and pinoresinol diglycoside (PG_2).

2.5 mM *p*-nitrophenyl-β-glucupyranosiduronic acid was incubated with 1 mg of each sample for 5 minutes followed by addition of 0.1 ml of β-glucuronidase solution for 30 minutes and then monitored for its absorbance at 410 nm.

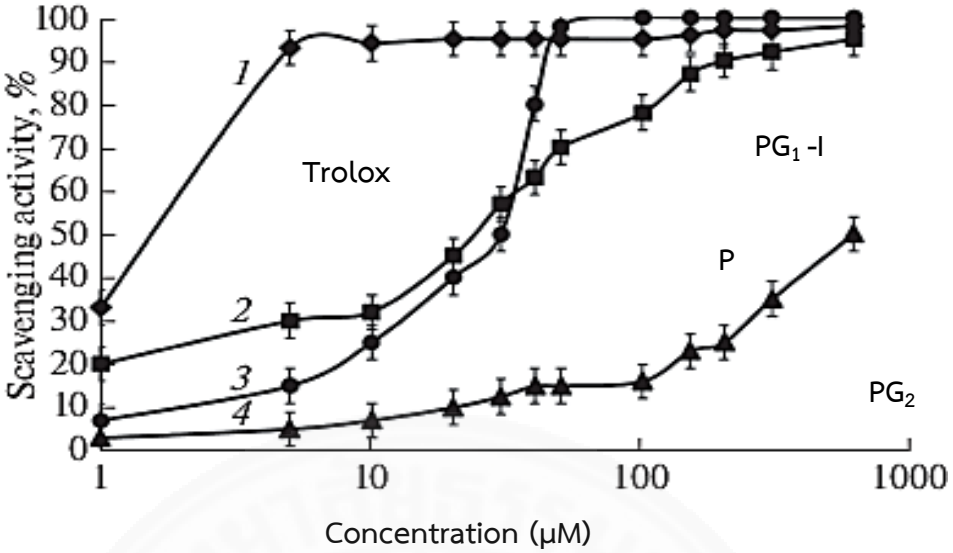


Figure 4.24 DPPH radical-scavenging activities. Trolox (◆), P (■), PG_1-I (●) and PG_2 (▲) at various concentrations were mixed with 100 μM DPPH and left for 30 minutes in the dark. The reaction were then monitored for their absorbance at 520 nm. Data are shown as the mean and are derived from three independent repeats.

4.7.4 Sweetness test

The relative sweetness of 1% (w/v) of the pinoresinol glycosides was determined by measuring the Brix values using a refractometer. The results showed that the sweetness of pinoresinol, pinoresinol glycoside and pinoresinol diglycoside was tested using sucrose as the reference sweetener. The sweetness of pinoresinol was lower than pinoresinol glycoside and pinoresinol diglycoside (Table 4.5), consistent with other studies that sweetness of long chain oligosaccharides is less than monosaccharides (Nakakuki et al., 2005; Patel et al., 2011).

Table 4.5 The relative sweetness of sucrose, pinoresinol, pinoresinol glycosides and maltooligosaccharides at 25 °C

Relative sweetness scale	
Compound	**Relative sweetness (%)**
Sucrose	100
Glucose	73
Maltose	50
Maltotriose	30
Pinoresinol	5
Pinoresinol glycoside (PG_1)	70
Pinoresinol diglycoside (PG_2)	30

PART 2: Synthesis and characterization of functional oligosaccharides

4.8 Bacterial cultivation and enzyme production

Escherichia coli cell harboring the p19bAMY plasmid (Watanasatitarpa et al. 2014) were grown in LB medium containing 100 μl/ml ampicillin at 37 °C for 24 hours. Enzyme expression was induced by adding 0.8 mM IPTG and the intracellular crude amylomaltase fraction was obtained after cell sonication. The enzyme was purified using a Histrap FF™ column, as previously described (section 3.16). The activity fractions were pooled and assayed for enzyme activity using a starch transglucosylation assay (section 3.17). and the protein concentration was determined by the Bradford method (section 3.16) using BSA as the standard.

4.9 Purification and characterization of recombinant AMase

The purified recombinant enzyme showed an activity of 44.58 U/ml (Table 4.6). Crude AMase was 3.2-fold purified with 67.0% yield (Table 4.6) after HisTrap FF™ column. The purity of the recombinant AMase was analyzed by SDS-PAGE. The enzyme revealed one band on protein gel after HisTrap FF™ column with the relative molecular weight of 60 kDa (Figure. 4.24-4.25).

4.10 Characterization of recombinant AMase

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

Purified AMase was determined by native polyacrylamide gel electrophoresis (Native-PAGE) and stained for both protein and dextrinizing activity as described in Section 3.6.1. The degradation of starch substrate by the action of AMase showed distinct clear zones which corresponded to the position or mobility of the active bands (Figure 4.26B). The only 1 active band from the activity attaining were

coincided with those from protein staining (Figure 4.26A). In figure 4.26A, the result showed only one protein band on the native gel of starch adsorption and it was also confirmed on SDS-PAGE with the one band appearance (Figure 4.24, Lane 2). Taken together, this result illustrated that it was desirable even working on only 1 step of purification. So, the HisTrap FF^{TM} column chromatography is an appropriate method for the AMase purification.

Table 4.6. Purification of amylomaltase from p19bAMY recombinant cell

Purification step	Total volume (ml)	Activity (U/ml)	Total activity[c] (U/ml)	Total Protein[b] (mg/ml)	Specific activity (U/mg)	Purification fold	% Yield
Crude AMase[a]	14	28.47	398.58	5,600	0.07	1	100
HisTrapFF^{TM}	6	44.58	267.48	1,200	0.22	3.2	67

[a]Crude enzyme was prepared from 2.1 liters culture (14.82 g wet weight).

[b]Assayed by bradford's method.

[b]Assayed by starch transglycosylation activity.

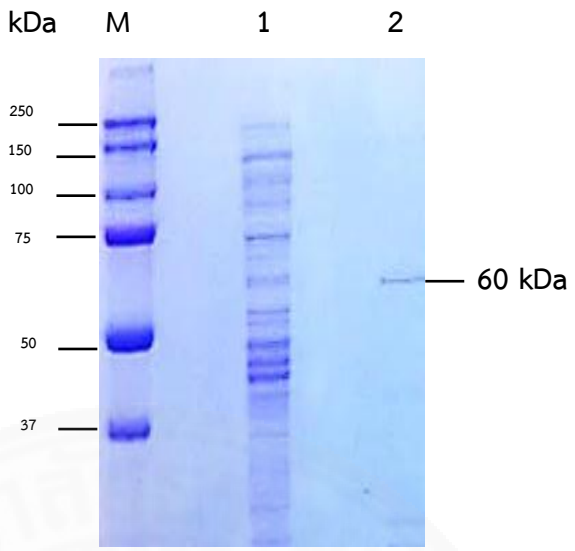


Figure 4.25 SDS-PAGE analysis by Coomassie Brilliant Blue R-250 staining.

Lane M	= Molecular weight marker proteins		
Lane 1	= Crude extract	30	µg
Lane 2	= HisTrapFF^{TM} column	5	µg

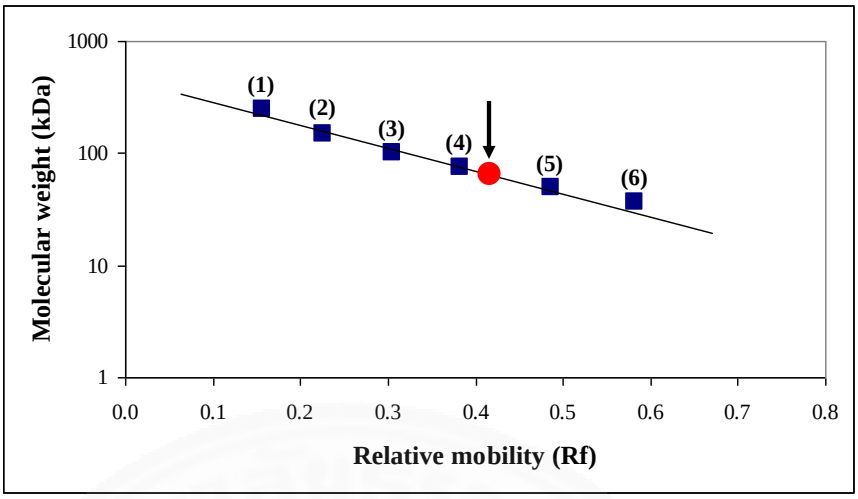


Figure 4.26 Calibration curve for molecular weight of recombinant amylomaltase from p19bAMY containing His-tag by SDS-PAGE.

(1)	=	250,000 Da	(Myosin)
(2)	=	150,000 Da	(Phosphorylase)
(3)	=	100,000 Da	(β-galactosidase)
(4)	=	75,000 Da	(Bovine serum albumin)
(5)	=	50,000 Da	(Ovalbumin)
(6)	=	37,000 Da	(Carbonic anhydrase)

The arrow indicates a determined molecular weight of amylomaltase.

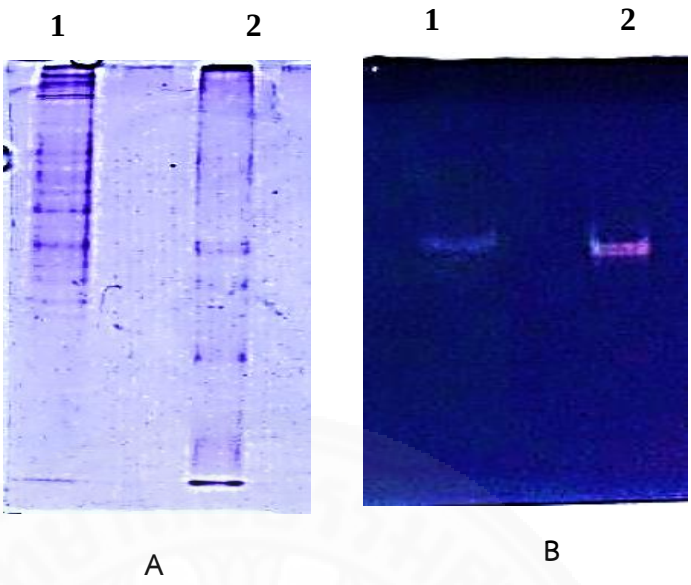


Figure 4.27 10% non-denaturing PAGE of AMase from each purification step.

(A) : Coomassie brilliant blue staining

Lane 1: Crude enzyme 15 µl (2 µg)

Lane 2: HisTrapFF^{TM} colum 30 µl (4 µg)

(B) : Starch transglycosylation activity staining

Lane 1: Crude enzyme 10 µl (0.2 U)

Lane 2: HisTrapFF^{TM} colum 4 µl (0.2 U)

4.11 Donor specifity

In determining donor specificity (Figure 4.27), the recombinant AMase was incubated with cyclodextrins and starches as glycosyl donors and xylose as an acceptor in 20 mM phosphate buffer pH 6.0 at 65 ^{o}C for 24 hours. The obtained xylose glycosides (XG_S) were then run on TLC and analyzed by a densitometer with Quantity One® 1-D analysis program. Their relative intensities (%) of the (XG) products from each donor substrate were reported as shown in Figure 4.27. Tapioca starch (TS) was found to be better donor substrate which was set as 100% relative intensity.

4.12 Acceptor specificity

The acceptor specificity of recombinant amylomaltase was determined from the starch transglucosylation activity assay using soluble tapioca starch as a glucosyl donor and mono- or disaccharides as acceptors (Figure 4.28). The results showed that glucose gave the highest activity so it was set as 100% relative activity, followed by maltose, xylose, palatinose, sucrose, mannose, treharose, fructose, lactose, cellobiose, galactose and ribose (Appendix H), which gave 75, 70, 48, 40, 35, 28, 25, 23, 22, 11 and 10.5% of activity, respectively (Figure 4.28), as compared to glucose. Comparison in monosaccharide between aldose (glucose and mannose) and ketose (fructose) functional group, the result suggested that saccharide with aldose group is usually better acceptor than ketose group. With the positions C of these monosaccharide, the OH configurations of aldose at positions C2, C4 and C6 of glucose were more important than other C positions. Moreover, with the position C4 seems to be more effect on AMase relative activity than the positions C2 and C6. This mention is supported by the lower relative activity when C4 of glucose changing into galactose (a C4 epimer of glucose) than that of C2 of glucose changing into mannose (a C2 epimer of glucose). For C6-monosaccharide (glucose), it showed clearly to be better acceptor than C5–monosaccharide (ribose), so it could imply that the OH configuration at the position C6 was very important for AMase transglycosylation activity. In the disaccharide group, maltose (glucose-α-1,4-glucose) gave the highest activity, followed

by palatinose (glucose-α-1,6-fructose), sucrose (glucose-α-1,2-fructose), trehalose (glucose-α-1,1-glucose), lactose (galactose-α-1,4-glucose), and cellobiose (glucose-β-1,4-glucose). These disaccharides having α-1,4-glycosidic form showed to be better acceptor than having β-1,4-glycosidic form. In addition, α-configuration linked between C1 and C4 was found to be the best acceptor type. In summary, it implied that the AMase preferred the acceptor that consisted of least one glucose residue and/or connected by α-1,4-glycosidic bond. To our knowledge, only few studies on saccharide acceptor specificities of AMase have been reported. In *E. coli* IF03806, the efficient acceptors in transglucosylation reaction were glucose, allose, cellobiose, isomaltose, mannose, and xylose, as determined from intensity of product spots on TLC. This *E. coli* amylomaltase transferred glucosyl residues attached to OH group at C4 of glucose, allose, mannose and xylose producing α-1,4 glycoside products which was confirmed by glucoamylase and β-amylase treatment and NMR (Kitahata et al, 1989). In another study, 4-α-glucanotransferase from *Pyrococcus kodakaraensis* KOD1 showed broad acceptor specificity to various saccharides, G_1-G_3, isomaltose, cellobiose, sucrose and xylose could act as acceptors especially G1 and xylose were preferred. This archaeal enzyme was specific for saccharides with pyranose structure containing OH configuration at C2, C3 and C4 position as glucose which was different from our enzyme that showed the importance of C2 C4 and C6 in catalysis (Tachibana et al., 2000). Recently, Naumthong et al. (2015) found that maltooligosaccharides (G1-G4), mannose, palatinose and sucrose were efficient acceptors for intermolecular transglycosylation reaction of amylomaltase from *Corynebacterium glutamicm*: the best one was glucose. This amylomaltase preferred hexose sugar containing the same configuration of C2-, C4- and C6-hydroxyl groups as glucopyranose (Naumthong et al, 2015).

From all results, here, xylose was chosen as suitable acceptor for the synthesis of xylose glycosides (XGs) to use as prebiotics further.

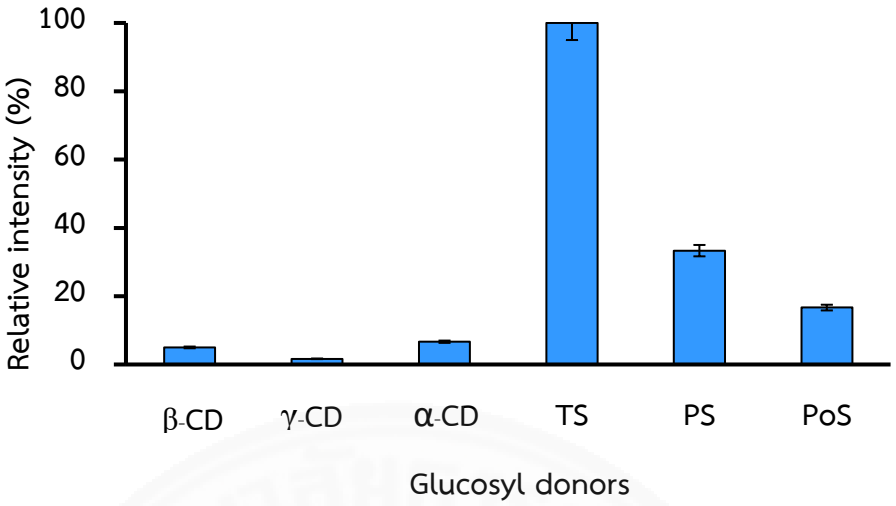


Figure 4.28 Relative intensity of xylose glycosides from transglycosylation reaction of amylomaltase from various 1% (w/v) donors to 1% (w/v) xylose. Glucose spot (1 mg/ml) was set as standard value and determined as 100% relative intensity. [CD = cyclodextrin, TS = tapioca starch, PS = pea starch, PoS = potato starch].

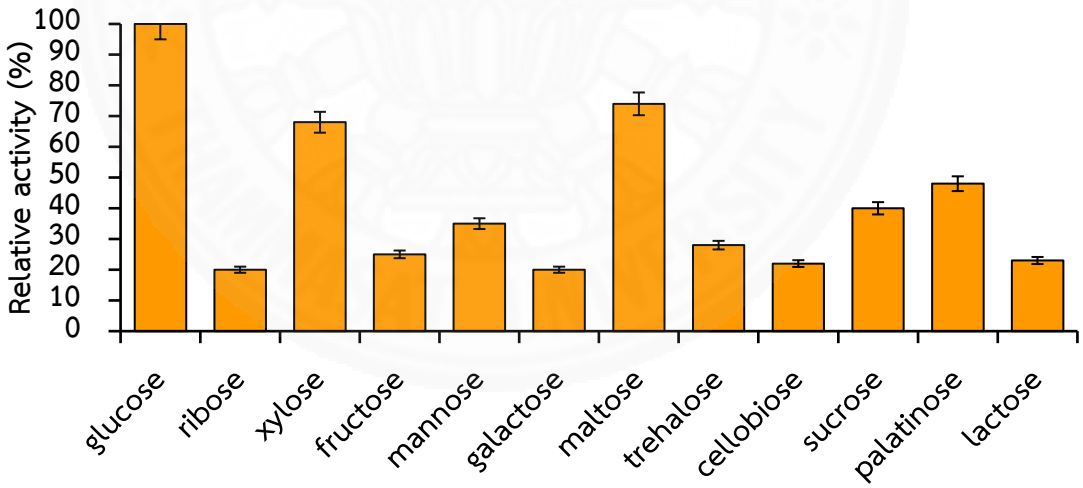


Figure 4.29 Acceptor specificity of amylomaltase. Soluble tapioca starch (1%, w/v) as glucosyl donor was incubated with mono- and disaccharides acceptor (1%, w/v) and enzyme in phosphate buffer, pH 6.0, 65 °C for 24 hours, then starch transglucosylation activity was analyzed as described in Section 3.21.

4.13 Optimization of xylose glycoside production

In order to find the appropriate conditions for the production of xylose glycosides, several parameters involved in the reaction including donor and acceptor concentrations, enzyme concentration, pH, incubation time and temperature were optimized. The reaction was performed as described in Section 3.21 and the reaction products were analyzed by TLC. The optimum conditions were considered from the yield of the highest xylose glycoside product. The amount of xylose glycosides was measured by scanning densitometer.

4.13.1 Effect of tapioca starch concentration

In order to investigate the effect of the concentration of a TS donor on the xylose glycoside production, the reaction mixtures with 0.5-4.0% (w/v) TS in 20 mM phosphate buffer pH 6.0 was mixed with 1% (w/v) xylose and 2 U/ml AMase in 20 mM phosphate buffer pH 6.0. The reaction mixture was incubated at 65 ^{o}C for 24 hours and then stopped by boiling for 5 minutes. The reaction mixture was run on TLC plate as described in Section 3.19.1. The highest production of xylose glycosides was obtained by using 1.5% (w/v) TS donor (Figure 4.29). Due to, the excess quantity of TS might cause a slowdown of transglycosylation reaction. Therefore, this study revealed that the products were decreased when used TS more than 1.5% (w/v).

4.13.2 Effect of xylose concentration

To determine the effect of xylose acceptor on the production of xylose glycosides, the reaction mixture was performed by varying amounts of xylose acceptor in ranging from 0.5-4.0% (w/v) with the fixed concentration of TS donor and incubated with AMase (2 U/ml) at 65 ℃ in 20 mM phosphate buffer, pH 6.0 for 24 hours. After incubation completed, the reaction was stopped by boiling for 5 minutes. The reaction mixture was then run on TLC as described in Section 3.19.1. The optimal xylose concentration was evaluated from the highest yield of the xylose glycoside products. The results found that the optimal reaction conditions were obtained by using 2% (w/v) xylose acceptor (Figure 4.30). Again, in the same manner of the donor, the excess quantity of xylose acceptor might cause a slowdown of transglycosylation

reaction. Therefore, this study showed that the products were decreased when used xylose more than 2.0% (w/v)..

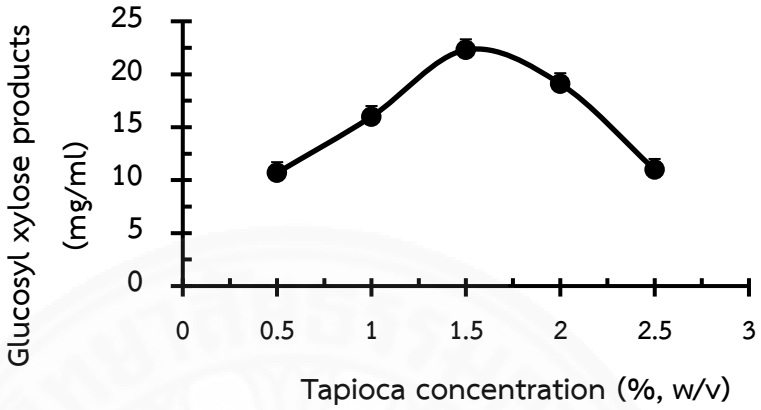


Figure 4.30 Influence of various tapioca starch concentrations on the transfer efficiency (TE) and the products obtained. The reaction conditions containing 1% (w/v) xylose and 0.5, 1, 1.5, 2 and 2.5% (w/v) TS were incubated with AMase at 2 units in 20 mM phosphate buffer (pH 6.0) at 65 °C for 24 hours.

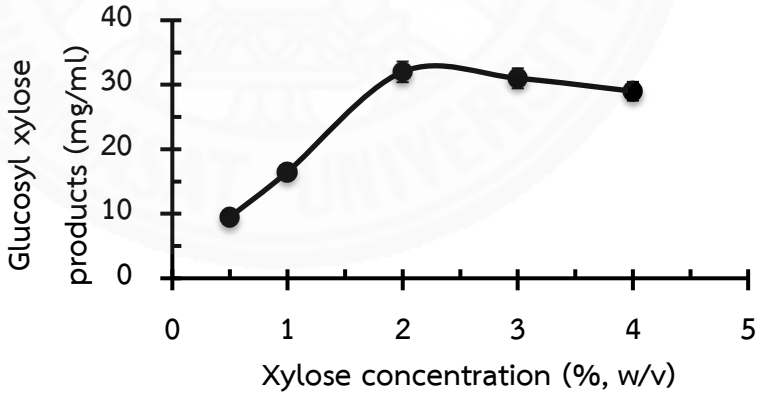


Figure 4.31 Influence of various xylose concentrations on the transfer efficiency (TE) and the obtained products. The reaction conditions containing 1.5% (w/v) TS and 0.5, 1, 2, 3 and 4% (w/v) xylose were incubated with at 2-units AMase in 20 mM phosphate buffer (pH 6.0) at 65 °C for 24 hours.

4.13.3 Effect of enzyme activity

The optimization of the enzyme activity (Unit) for the production of xylose glycosides was performed by using fixed amounts of optimal TS donor concentration (Section 3.21.1) and optimal xylose acceptor concentration (Section 3.21.2). Various amounts of final AMase concentration (2-32 U/ml) in 20 mM phosphate buffer pH 6.0 were added to the reaction and incubated at 65 °C for 24 hours. The optimal enzyme concentration was decided from the amount of xylose glycoside products on TLC analysis as described in Section 3.19.1. The results found that the optimal reaction conditions were 4.0 U/ml of final AMase as shown in Figure 4.31. As resulted, the xylose glycoside products were increased when used enzyme concentration. However, in higher level at enzyme concentration which was 4.0 U/ml higher, it found the yield of xylose glycoside product was signification decreased. This result explained that the AMase is a multiple-function enzyme, consisted of disproportionation, cyclization, coupling and hydrolysis reaction. So, at higher enzyme concentration, the hydrolysis activity might be increased resulting in the decrease of product yield.

4.13.4 Effect of incubation time

The incubation time was varied from 0-144 hours. After the experiment completed, the product yield was analyzed by TLC. The result showed that the production of xylose glycosides was clearly affected by incubation time. The highest yield of xylose glycosides was obtained at an incubation time of 120 hours (Figure 4.32). After 120 hours, it found that incubation time had still offended on product-yield. Trend of yield was decreased with long incubation time. Thus, 120 hours were chosen to be the optimum incubation time.

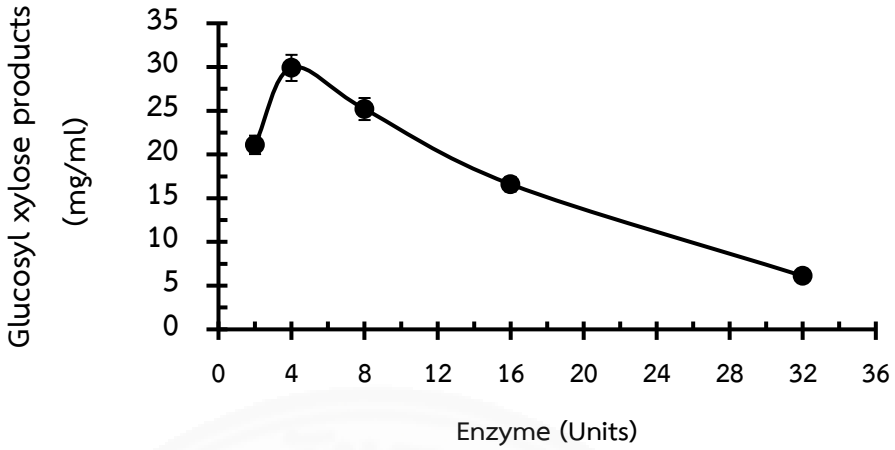


Figure 4.32 Influence of various enzyme activities on the transfer efficiency (TE) and the obtained products. The reaction conditions containing 1.5% (w/v) TS and 2% (w/v) xylose were incubated with AMase at 2, 4, 8, 16 and 32 units in 20 mM phosphate buffer (pH 6.0) at 65 °C for 24 hours.

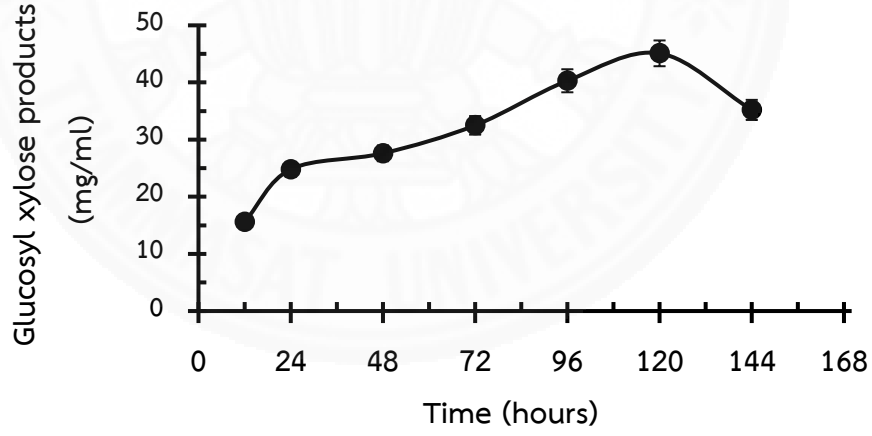


Figure 4.33 Influence of various incubation times on the transfer efficiency (TE) and the products obtained. The reaction conditions containing 1.5% (w/v) TS and 2% (w/v) xylose were incubated with 4 units AMase in 20 mM phosphate buffer (pH 6.0) at 65 °C for 0-144 hours.

4.13.5 Effect of temperature

The catalyzing temperature of AMase in order to transfer glucose from TS donor to xylose acceptor was also checked. The temperature was varied from 50-80 ^{o}C. The reaction mixture of optimized concentration of donor, acceptor, enzyme, pH and incubation time was performed in 20 mM phosphate buffer pH 6.0 for 120 hours. After 120 hours of incubation, the highest yield of xylose glycosides was obtained at 70 ^{o}C (Figure 4.33).

4.13.6 Effect of pH

The effect of pH on AMase reaction for the production of xylose glycosides was investigated. The twelve conditions of different pH values in four kinds of buffer solution were employed. The four diverse buffers consisting of acetate buffer (pH 3.0-5.0), phosphate buffer (pH 5.0-7.0), Tris-HCl buffer (pH 7.0-9.0) and glycine-NaOH buffer (pH 9.0-11.0) with the same concentration of 20 mM were used as the main environment of the reaction mixture. The protocol was done as shown in Section 3.21.4. The maximum xylose product was obtained with the reaction of Tris-HCl buffer pH 7.0 as shown in Figure 4.34. The results revealed that the pH have strongly affected on the products yield. It was found that the product yield was surprisingly decreased when the reaction mixture was performed in 20 mM acetate (pH 3.0-5.0), and glycine-NaOH buffer (pH 9.0-11.0). This effect might be due to low resistance on changing pH of enzyme when strong acids or bases were added to the reaction.

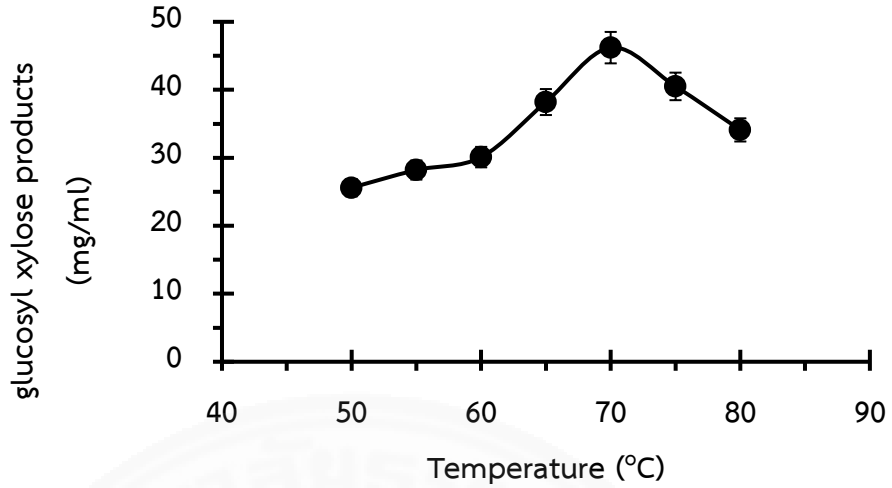


Figure 4.34 Influence of various incubation temperatures on the transfer efficiency (TE) and the products obtained. The reaction conditions containing 1.5% (w/v) TS and 2% (w/v) xylose were incubated with 4 units AMase in 20 mM phosphate buffer (pH 6.0) at 50-80 °C for 120 hours.

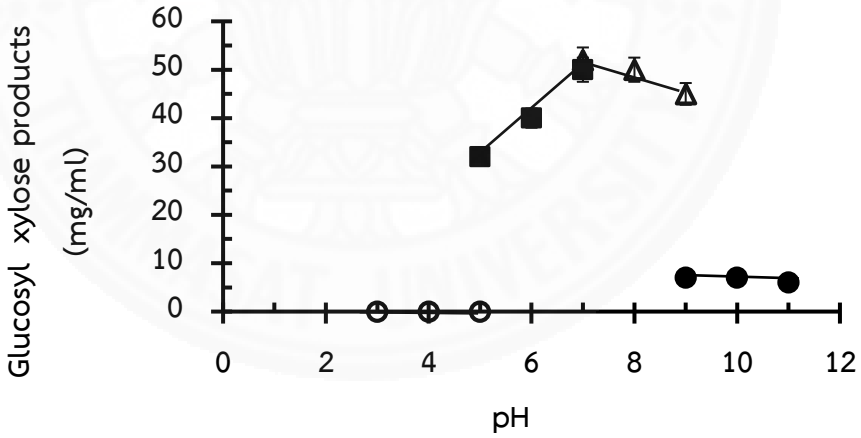


Figure 4.35 Influence of various pHs on the transfer efficiency (TE) and the products obtained. The reaction conditions containing 1.5% (w/v) TS and 2% (w/v) xylose were incubated with AMase 4 units in 20 mM **O**; Acetate buffer, ■; Phosphate buffer, Δ; Tris -HCl buffer, ●; Glycine-NaOH buffer at 70 °C for 120 hours.

4.14 Synthesis of xylose glycoside products at optimal condition

In summary, the previous result showed that the optimum condition for transglycosylation reaction of AMase to produce glucosyl xylose was 1.5% (w/v) tapioca starch (TS) and 2% (w/v) xylose with 4 U/ml of final AMase unit in 20 mM Tris-HCl buffer pH 7.0 at 70 ^{o}C for 120 hours. After optimized under various parameters, the total yield of xylose glycosides was 6-fold increased (60 mg/ml) as compared to the obtained yield before optimaization (10 mg/ml) in Figure 4.35. For before optimizing, the condition was performed by incubation of 1% (w/v) TS and 1% (w/v) xylose with 2 U/ml of final AMase concentration in 20 mM phosphate buffer pH 6.0 at 65 ^{o}C for 24 hours.

4.15 Large-scale production and product purification

The large-scale production was then purified using HPLC. Two main products of each xylose glycoside were observed (Figure 4.35). The yield of xylose glycoside products was increased after optimization as shown Figure 4.36C. Here, HPLC was used to identify xylose glycoside product using an amino column (4.6 x 250 mm). Considerable products were detected with refractive index detector (RID). The column was eluted with 80:20 (acetonitrile: water) at a flow rate 1 ml/min. The result revealed that that two main peaks at retention time (R_t) 11.76 and 26.59 minutes were observed. They were identified as xylose glycoside products, xylose glycoside (XG1) and xylose maltoside XG_2), respectively. The yield of xylose glycoside products (XG_1 and XG_2) increased after optimization were 50.65% and 32.50% product yield, respectively (Figure 4.36). Therefore, these xylose glycosides might be suggested to apply as physiologically functional compounds for food, medicine and cosmetics with potential as nutraceutical compounds for health benefits. So, XGs main product was further characterized for size and structure, and determined for its biological properties.

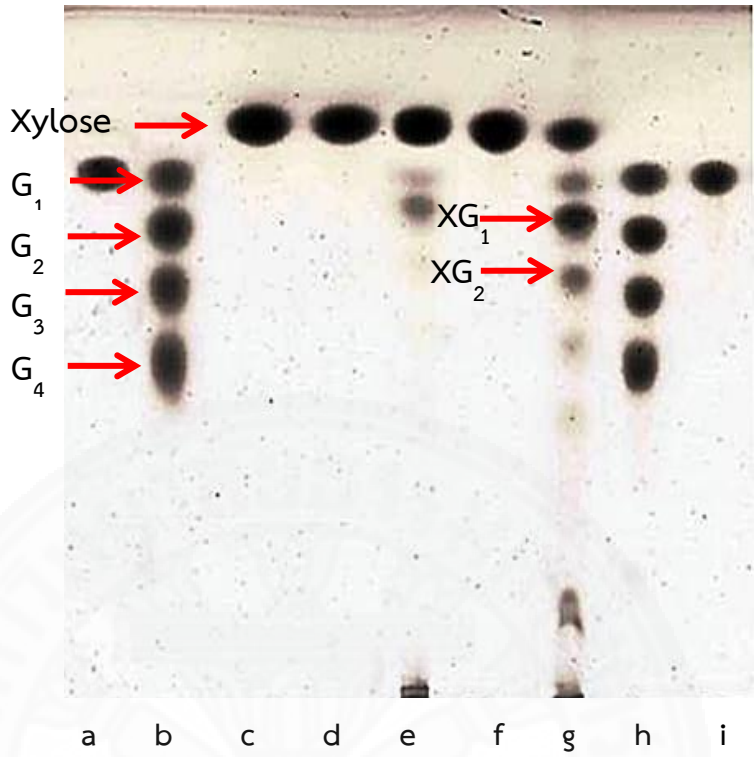


Figure 4.36 TLC analysis of the reaction products from AMase incubation.

The enzyme was incubated with TS (1.5%, w/v) and xylose (2%, w/v) in phosphate buffer, pH 6.0. Standard (lanes a and i) glucose and (lanes b and h) G_1–G_4, (lanes c) xylose, (lanes d and f) control reaction without donor at 0 hour, (e) the reaction products before optimizing (lanes g) the reaction products after optimizing, respectively. XGs = xylose glycosides.

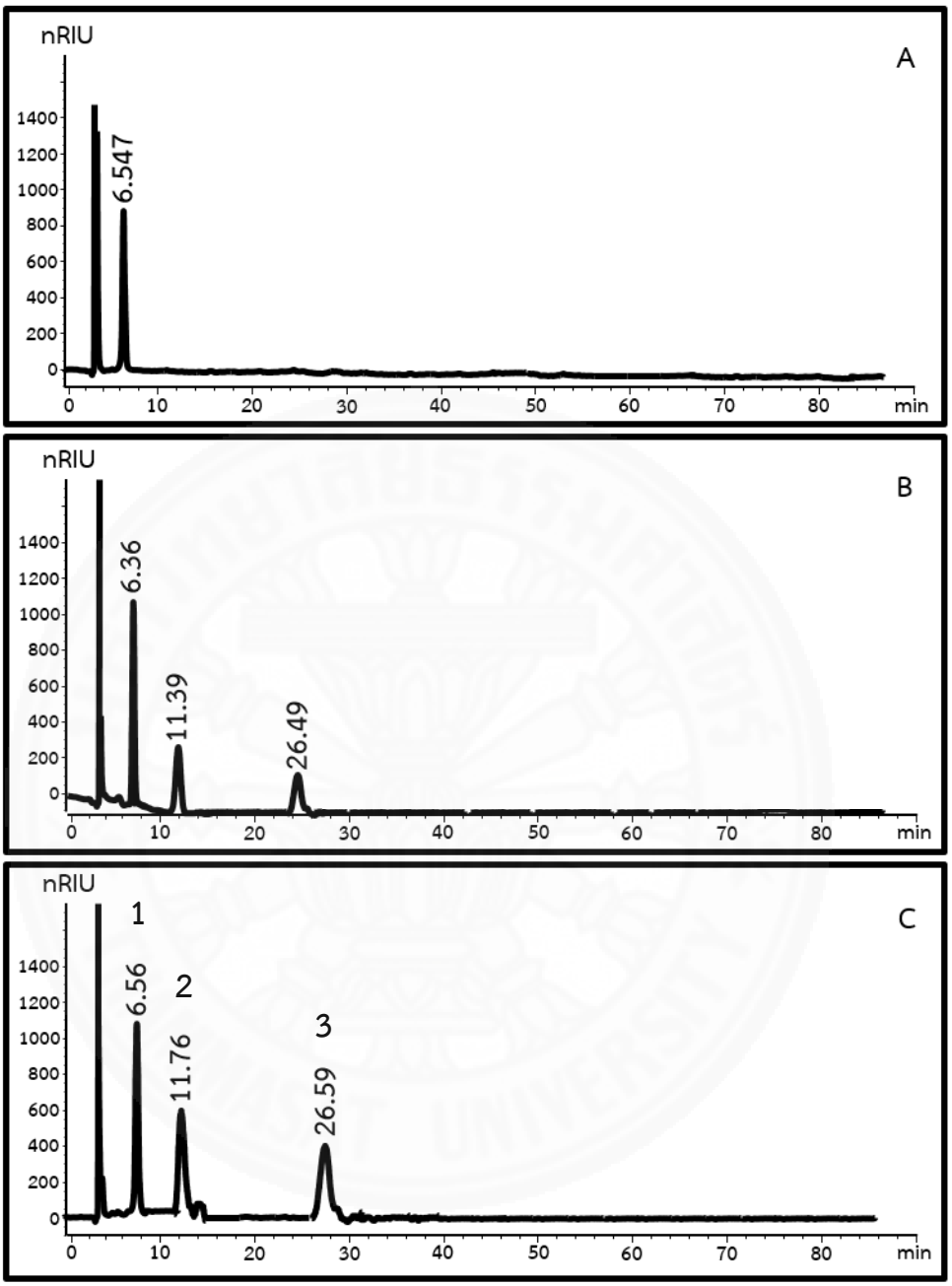


Figure 4.37 HPLC profiles of transglycosylation reaction catalyzed by AMase (A) The reaction without AMase, (B) Before optimization and (C) After optimization conditions. (1) = xylose (X), (2) = xylose monoglycoside (XG_1) and (3) = xylose maltoside (XG_2).

4.16 Structural analysis of xylose glycosides

The molecular weight of xylose glycosides was determined by Mass spectrometry (MS) as described in Section 3.23.1.

4.16.1 Mass spectrometry (MS) analysis

To investigate the molecular weight of transglycosylation products by AMase the reaction, mass spectrometry (MS) was employed to determine mass-to-charge ratio of samples. A mass spectrum is a plot of the ion signal. These spectra are used to determine the elemental or isotopic signature of samples, the mass of particles and of molecules, and to elucidate the chemical structures of molecules. The molecular weights of xylose were determined by ESI-TOF mass spectrometry and found $[M+Na]^+$ at m/z was 173.04 (Figure 4.37) corresponded to the exact site of xylose. Product 1 (XG_1) was determined to be $[M+Na]^+$ at m/z of 335.10 (Figure 4.37, Table 4.7) equivalent to the molecular weight of a single glucose unit attached to xylose Product 2 (XG_2) was determined to be $[M+Na]^+$ at m/z of 497.20 (Figure 4.37, Table 4.7) indicating two glucose units attached to xylose. This result confirmed the ability of AMase in the transglycosylation of glucose from TS to xylose molecule and gave the new xylose glycoside products.

4.16.2 Nuclear magnetic resonance (NMR) analysis

The ^1H NMR and ^{13}C NMR spectram of xylose (X) (Figure 4.38-4.39) and xylose monoglycoside (XG_1) (Figure 4.40-4.41) were performed as described in Section 3.23.2. According to ^1H-NMR (Figure 4.40), a doublet signal at 4.40 (J=3.81 Hz) of XG_1 was found and assigned to the anomeric proton, which suggest that only one glucose unit was linked to xylose molecule with α-1,4 glucosidic bond. The ^{13}C NMR spectram of both xylose and XG_1 were also performed, and showed five and eleven carbon signals, respectively (Table 4.8). From the result, the α-configuration of the D glucose residue in XG_1 (Figure 4.41), was confirmed by anomeric carbon of glucose signal which shifted from 92.77 to 98.94 ppm. Moreover, comparison at position C-4 of xylose and XG_1 was found that has shifted downfield from 69.33 to 76.12 ppm, corresponding to α-1,4 glucosidic form. So, from these results with ESI-TOF MS, the

structures of XG_1 was identified as xylose-α-D-monoglycoside (XG_1) as drawn in (Figure 4.42).

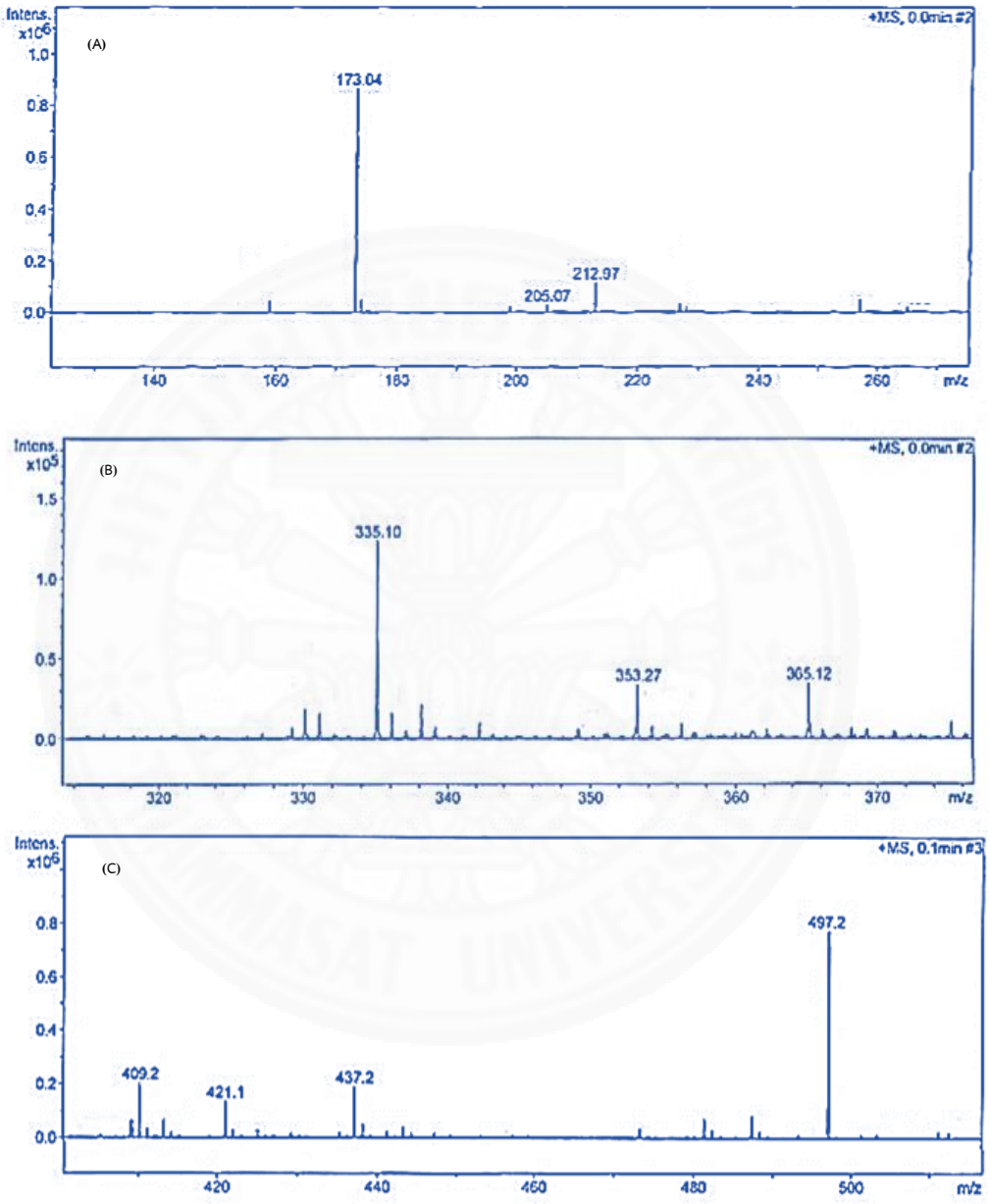


Figure 4.38 ESI-TOF mass spectrum of (A) xylose, (B) XG_1 and (C) XG_2

Table 4.7 The ESI-TOF-MS analytical masses of xylose and xylose glycoside products

Product	Mass number (*m/z*)
	Determined* $[M+Na]^+$
Xylose (X)	173.04
Xylose monoglycoside (XG_1)	335.10
Xylose maltoside (XG_2)	497.20

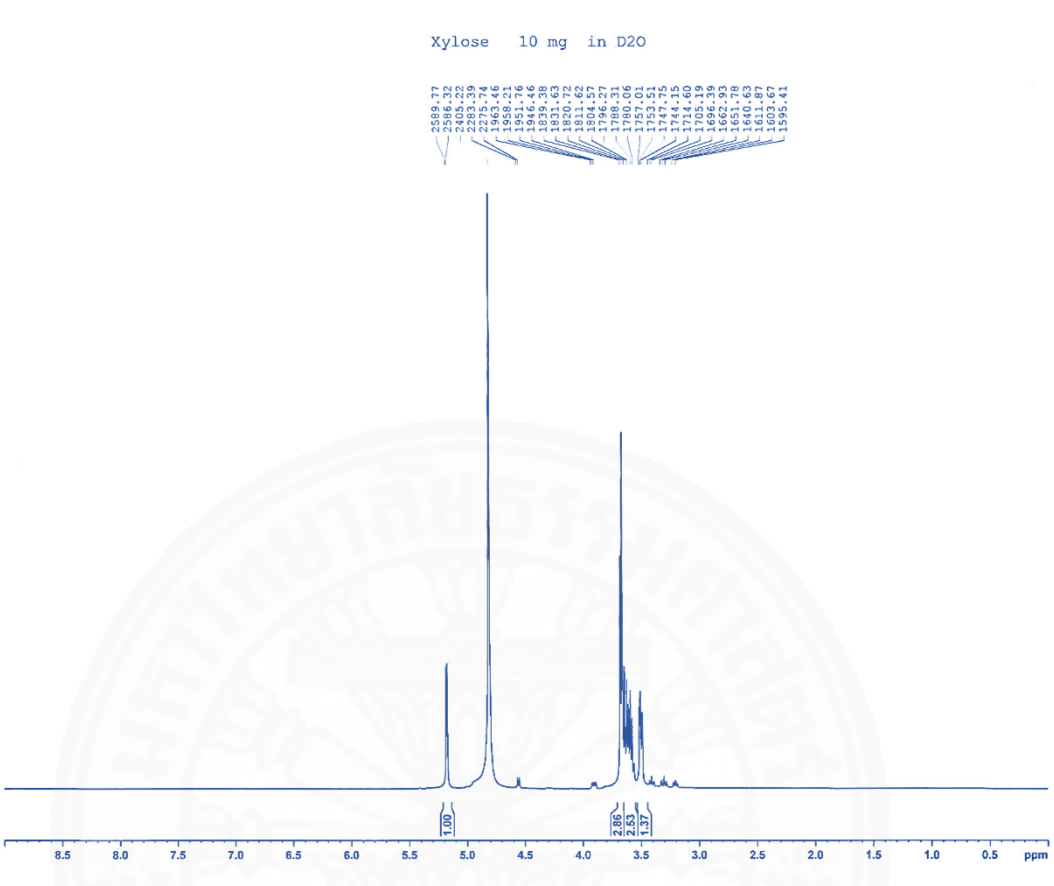


Figure 4.39 The 400 MHz ^{1}H-NMR spectrum of standard xylose.

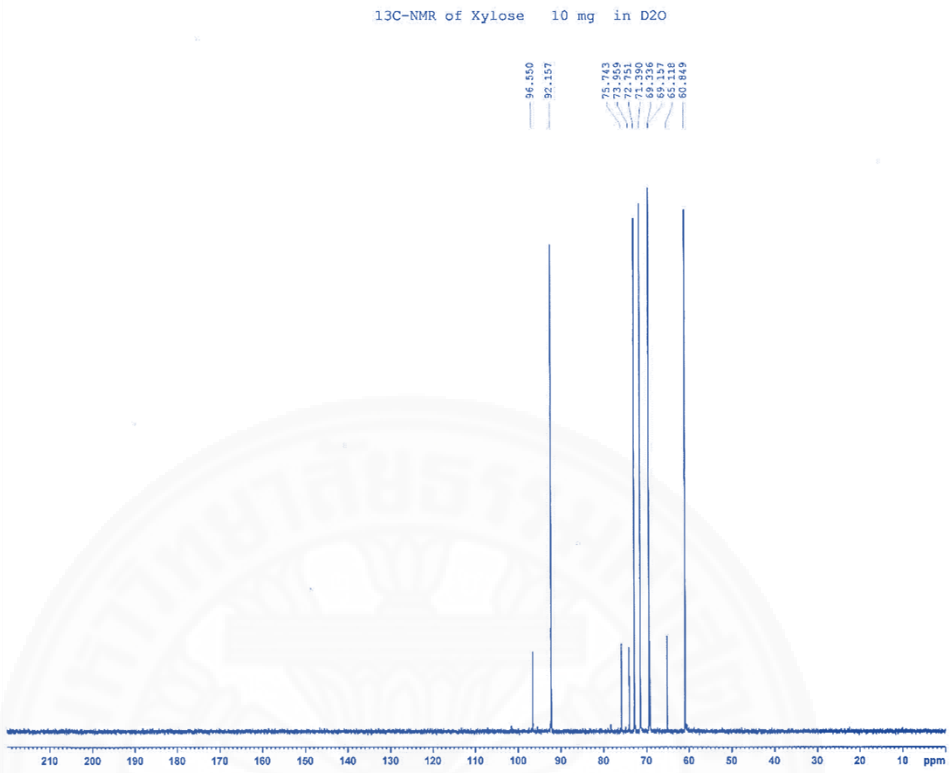


Figure 4.40 The 400 MHz ^{13}C-NMR spectrum of standard xylose.

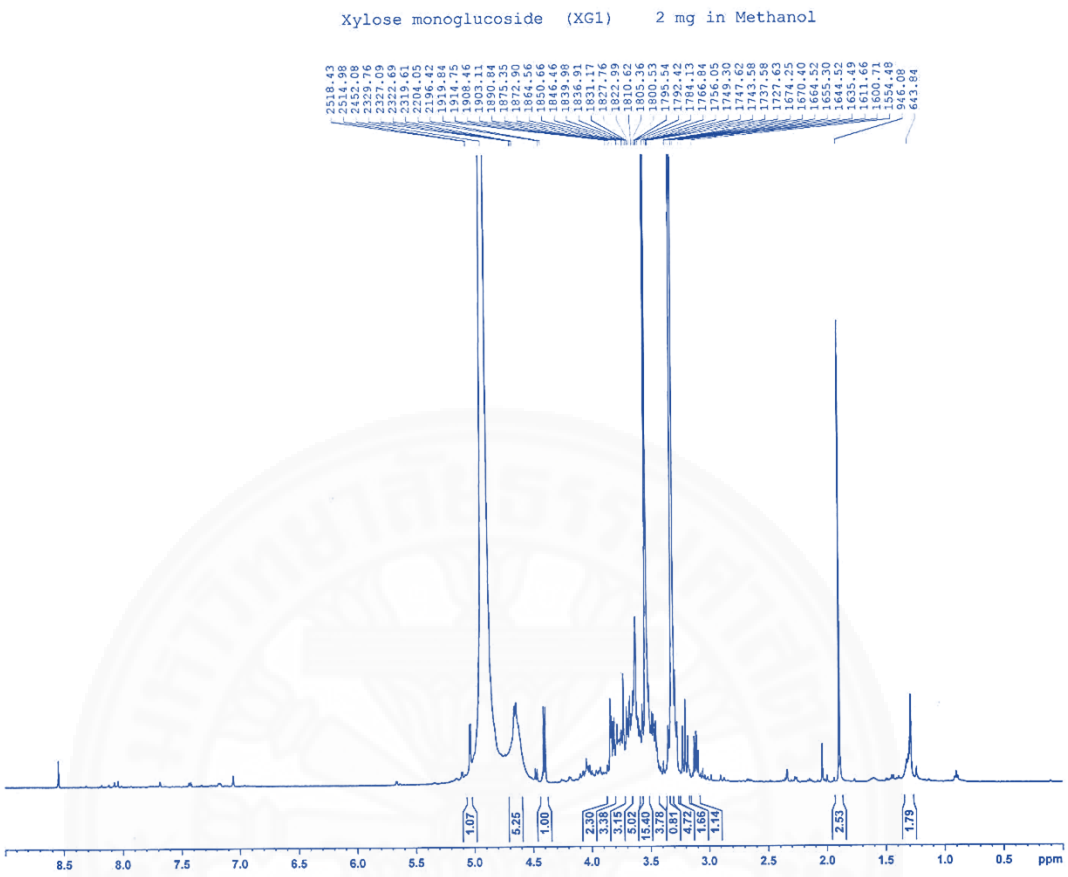


Figure 4.41 The 400 MHz ^{1}H-NMR spectrum of the formed XG_1 product from reaction of AMase with TS and xylose.

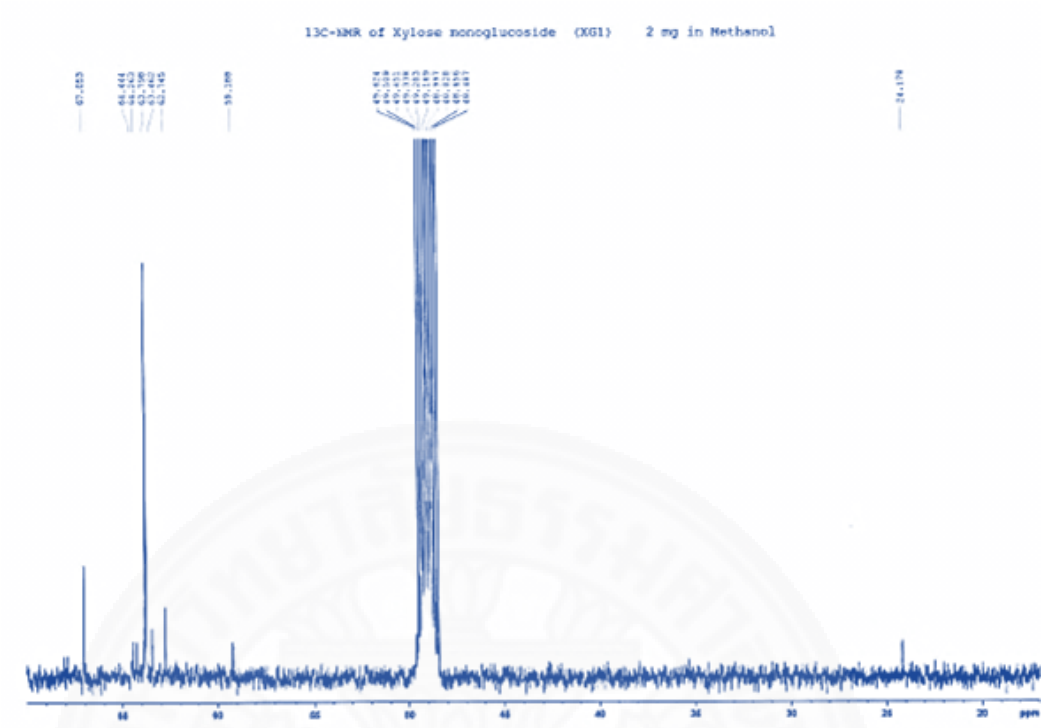


Figure 4.42 The 400 MHz ^{13}C-NMR spectrum of the formed XG_1 product from reaction of AMase with TS and xylose.

Table 4.8 The ^{13}C-NMR data of standard xylose (X), and xylosemonoglycoside (XG_1)

Carbon atom	^{13}C-NMR (δ, ppm)		
	Glucose[a]	Xylose	XG_1
C-1		92.15	94.10
C-2		72.75	71.27
C-3		71.39	71.57
C-4		69.33	76.12
C-5		60.85	62.75
C-1'	92.77		98.94
C-2'	72.15		73.85
C-3'	73.43		77.99
C-4'	70.32		67.05
C-5'	72.10		75.49
C-6'	61.27		63.46

[a]The chemical shift values are taken from (Roslund et al., 2008). Values of chemical shift (δ) are stated in ppm.

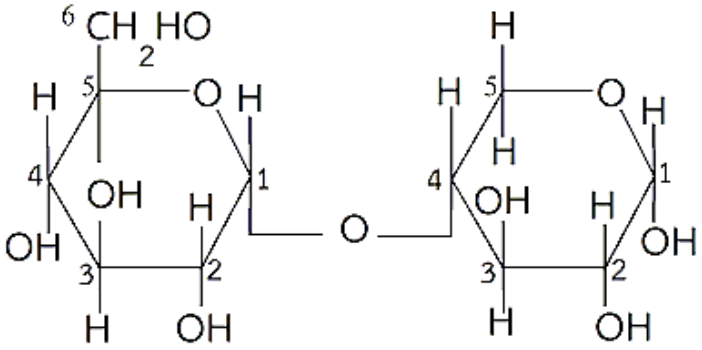


Figure 4.43 Structure of xylose glycosides (XG_1).

4.17 Evaluation of the physiochemical and biological properties of xylose glycosides products

4.17.1 Solubility test

The solubility of each xylose glycosides was compared to that of xylose. The excess xylose and xylose glycoside (XG_1 and XG_2) were mixed with 200 µl of water, vortexes for 5 minutes at room temperature to disperse into solution, and then each sample was incubated at 30 ^{o}C for 15 minutes. The soluble part of the sample was analyzed by HPLC as described in Section 3.24.1. The solubility of xylose glycoside products can be calculated by comparison with standard xylose solubility curve (Appendix H). The solubility of xylose monoglycoside (XG_1) was 280.5 mg/mL whereas the solubility of xylose maltoside (XG_2) was 300.8 mg/ml (Table 4.9). Originally, xylose itself was water soluble at approx. 250 mg/ml. From the solubility value, X and XG_1 were less water soluble than XG_2. This result suggested that increasing glucose was leading to the increasing of hydroxyl group in xylose molecule; consequently, XG_2 effectively improves dissolution. In the same way, Kometani et al. (1994) also reported that the solubility greatly improved when adding more glucose molecules into flavonoid structure.

4.17.2 Sweetness test

The relative sweetness of 1% (w/v) of the xylose glycosides (XG_n) was determined by measuring the Brix values using a refractometer. The results of sweetness were measured by comparison with standard sucrose (Appendix I). The results showed that the sweetness of glucose, maltose, xylose, xylose monoglycoside (XG_1) and xylose maltoside (XG_2) were 0.80, 0.66, 0.53, 0.40 and 0.33 respectively, compared with sucrose which is set to 1.0 (Table 4.10). These results showed that the sweetness decreased as the sugar chain lengths of xylose glycosides increased. Xylose (X) gave sweetness more than the xylose monoglycoside (XG_1) and xylose maltoside (XG_2). The result agrees with previous reports that the sweetness of long chain fructooligosaccharides (Nakakuki, 1993) and isomaltooligosaccharide (Kaulpiboon, et al., 2015) decreased with increasing chain length. A low sweetness property is quite useful in various applications of food products where the use of sucrose is restricted

by its high sweetness property. Normally, the sweetness value of oligosaccharides which is used as prebiotics are approx. 0.3-0.6 times that of sucrose (Crittenden and Playne, 1996).

4.17.3 Maillard reaction test

The Maillard reaction is a chemical reaction between amino acids and reducing sugars under heat which gives browned foods with their desirable flavoours. After 2-hours incubation at 120 °C, xylose (X) and xylose glycosides (XG_1 and XG_2) permitted more coloring through the Maillard reaction than sucrose (Figure 4.43). In addition, it was found that the browning evidently occurred at a high pH, especially short-chain xylose glycoside. Therefore, the xylose glycosides (both XG_1 and XG_2) could aslo be used to alter the intensity of browning caused by the Maillard reaction in heat-processed foods (Ishizu et al., 1999; Kikuchi et al., 2004).

4.17.4 Acidic resistance test

After a 24-hour incubation, both xylose and xylose glycosides showed very low degrading abilities in an acidic buffer, pH of 3.0 at 37 °C (Figure 4.44). Their high stabilities under acidic condition suggestd that the synthesized xylose glycosides (XG_1 and XG_2) could resist the gastric acidity of human. Moreoer, the xylose glycosides in this work might be applied in acidic beverages such as fruit juices, yoghurts, soft drinks, alcoholic beverages, and sparkling water.

Table 4.9 The water solubility of xylose, xylose monoglycoside and xylose diglycoside at 30 °C

Water solubility scale	
Compound	**Water solubility (mg)**
Xylose	250
Xylose monoglycoside (XG_1)	280
Xylose maltoside (XG_2)	300

Table 4.10 Relative sweetness of monosaccharides and xylose glycoside products

Relative sweetness scale	
Compound	**Relative sweetness (%)**
Sucrose	100
Glucose	80
Maltose	66
Xylose	53
XG_1	40
XG_2	33

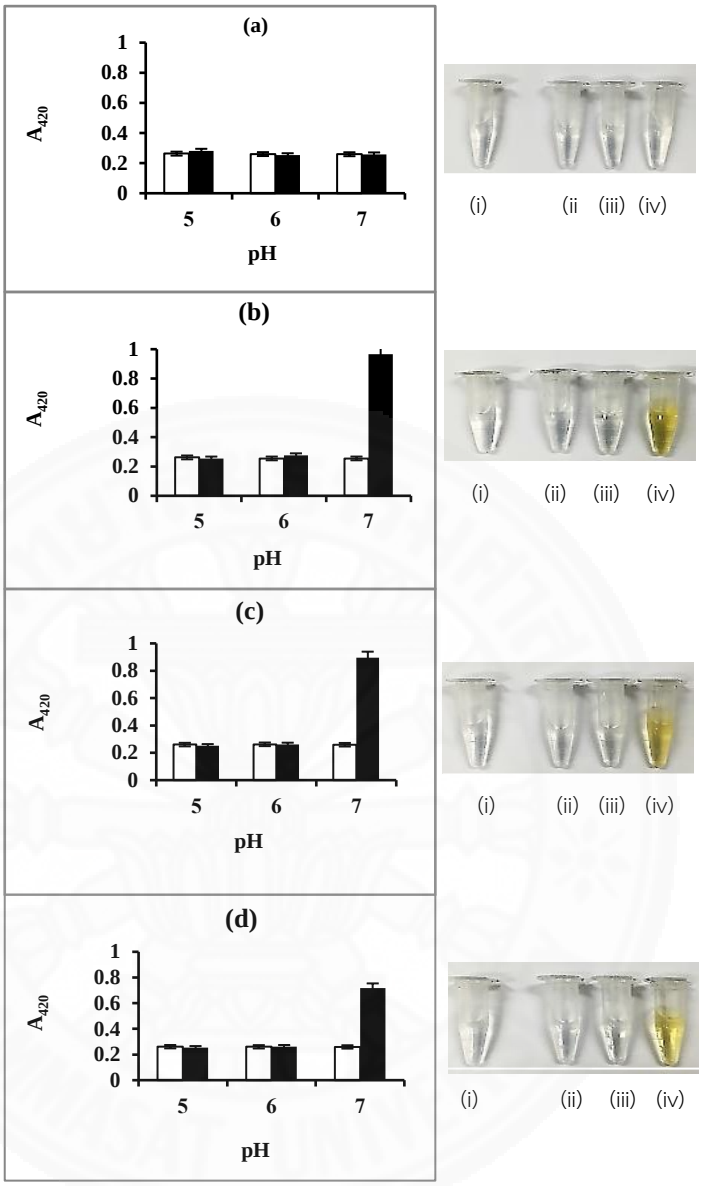


Figure 4.44 Maillard reactions of (a) sucrose, (b) xylose, (c) XG_1 and (c) XG_2. The reaction was incubated for 0 (□, i) and 2 hours (■, ii-iv) in phosphate buffer pH 5, 6 and 7, respectively.

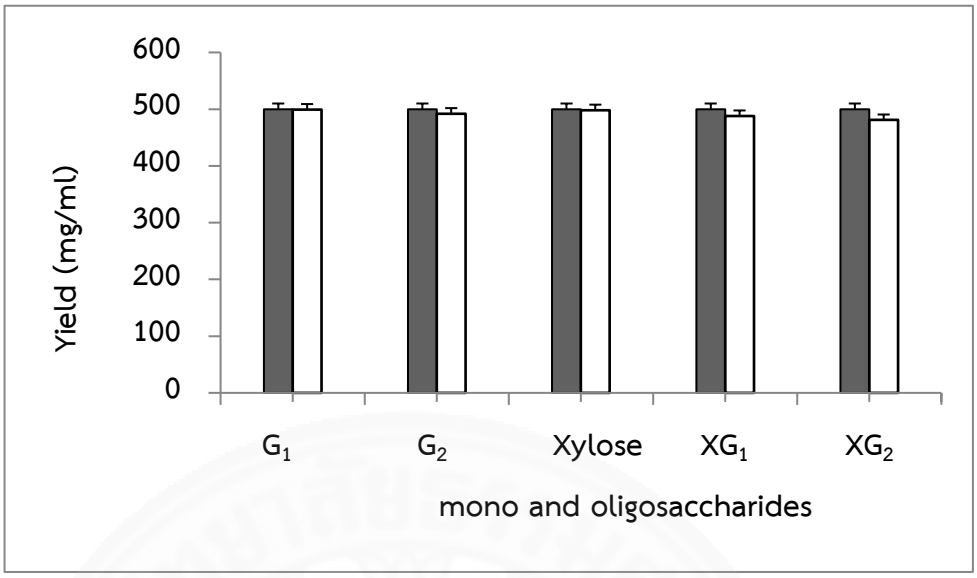


Figure 4.45 Resistance under acidic condition (pH 3.0) of 1% (w/v) glucose (G_1), maltose (G_2), xylose, xylose monoglycoside (XG_1) and xylose maltoside (XG_2) at 37 °C for 24 hours. The products obtained before (■) and after (□) acidic incubation were analyzed by HPLC. In this experiment, each product was tested in a separating tube at three times and reported as mean ± S.D. values

4.17.5 Prebiotic activity test

In vitro utilization of xylose glycoside (XG_1) by probiotic *Lactobacillus brevis* BCC26712 was compared with non-probiotic *Escherichia coli* ATCC® 25922™. As the result, the synthesized XG_1 could be used as a carbon source for the growth of probiotic *L. brevis* BCC26712 and resulted in a lowering of the culture pH (Figure 4.45). The difference in the pH value between the probiotic *L. brevis* and non-probiotic *E. coli* culture for 48 hours was more than one pH unit. This result suggested that *L. brevis* produced extracellular enzyme to metabolize α-1, 4 bonds of XG_1, leading to the production of small organic acids such as acetate, propionate, butyrate, and lactate (Figure 4.46) that caused the decrease in the pH value of the culture (Gänzle et., al; 2007). However, the presence of enzymes for XG_1 metabolism in *L. brevis* and the metabolism behind XG_1 affecting intestinal microbactria are still unclear. But, based on the decrease in pH value, it is possible that XG_1 might be broken down by *L. brevis* α-glucosidase to become glucose and xylose products, and then glucose was further metabolized as proposed by Falony et al. (2009) in Figure 4.46 to produce small organic acids. In addition, the competitiveness of XG_1 -degrading organic acid producers was also investigated through monoculture and co-culture between *L. brevis* and *E. coli* in MRS medium supplemented with XG_1 at 37 $^{\circ}C$ for 48 hours (Figure 4.45). Quantitative analysis of *L. brevis* and *E. coli* growth (serial dilute 1:1,000,000 of starter 0.5 McFarland) in the monoculture was 279 and 179 colonies, respectively. When both bacteria were cultured, measuring bacterial growth on agar plate revealed that total counts of *L. brevis* and *E. coli* were 271 and 105 colonies, respectively. Quantitative comparison of *L. brevis* and *E. coli* colonies in the culture with those in the monoculture found that the growth of non-probiotic *E. coli* significantly decreased in the co-culture. The result was due to the decrease of pH under coculture system, having probiotic *L. brevis*. So, the synthesized XG_1 could be classified as prebiotic because it selectively promotes the growth and metabolic activity of good bacteria, and help improve the health of host.

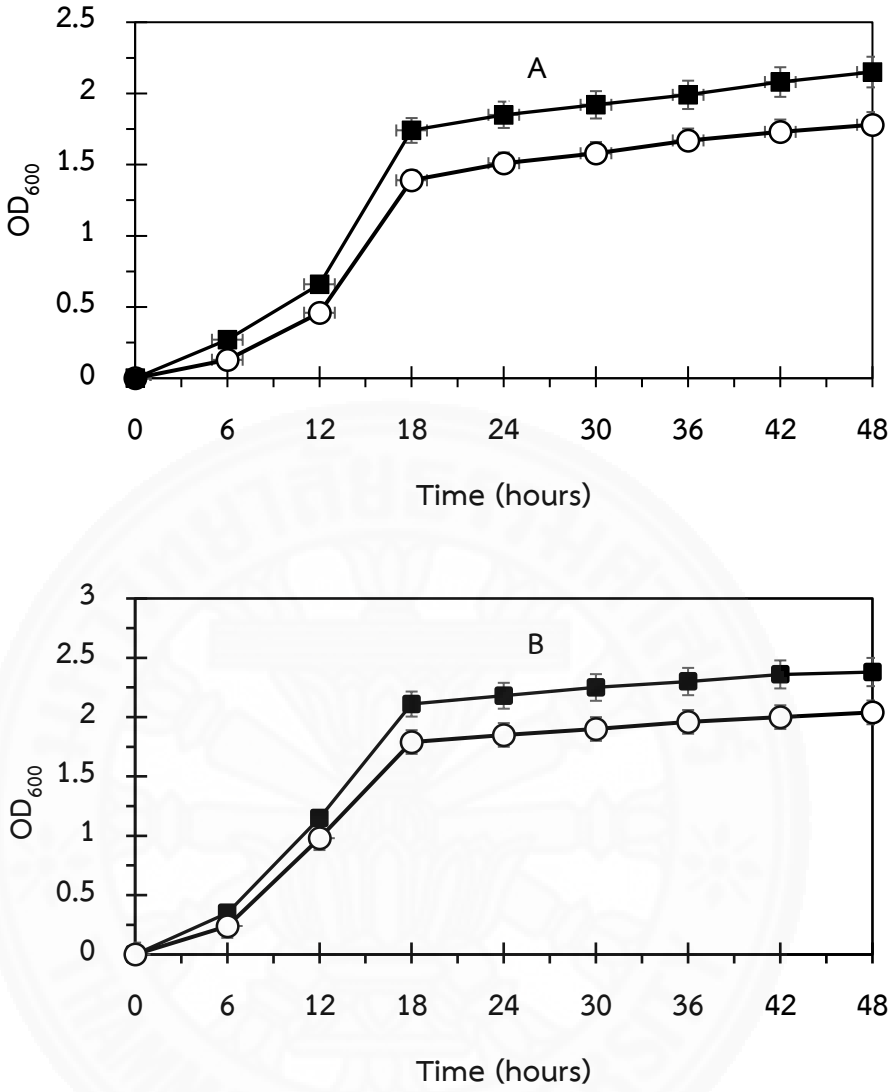


Figure 4.46 The growth curves of L. brevis (■) compared with E. coli (**O**) culture in MRS containing (A) 1% (w/v) xylose, (B) 1% (w/v) XG1. The growth curves were determined by reading the optical densities at 600 nm.

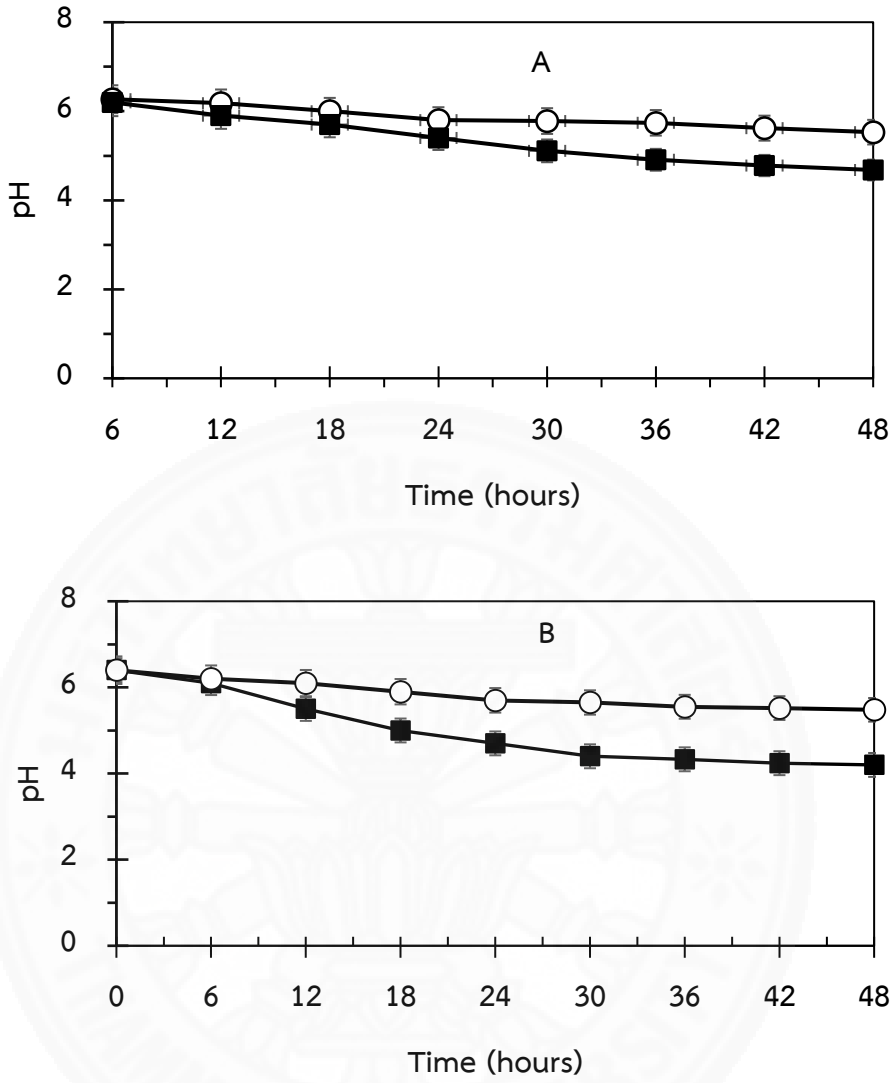


Figure 4.47 The pH profile of *L. brevis* (■) compared with *E. coli* (**O**) culture in MRS containing (A) 1% (w/v) xylose, (B) 1% (w/v) XG_1.

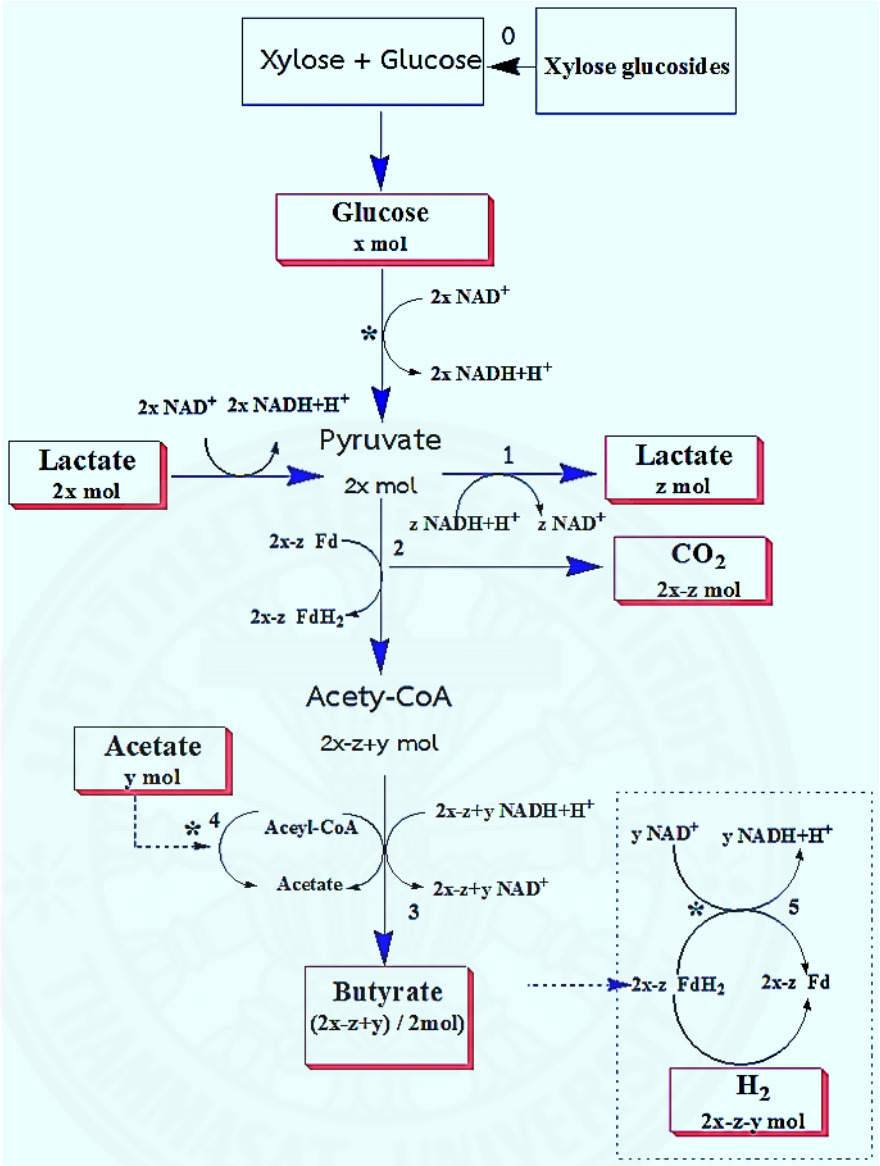


Figure 4.48 Scheme for small organic acids production in probiotic bacteria. 0, *L. brevis* enzyme (α-glucosidase); 1, lactate dehydrogenase; 2, pyruvate: ferredoxin oxidoreductase; 3, butyryl-CoA: acetate CoA transferase; 4, acetate kinase; 5, membrane-associated NADH: ferredoxin oxidoreductase; 6, hydrogenase; *, generation of ATP; x, mM Glucose consumed; 2x, mM lactate consumed; y, mM acetate consumed; z, mM lactate produced. (modified from Falong et al., 2009).

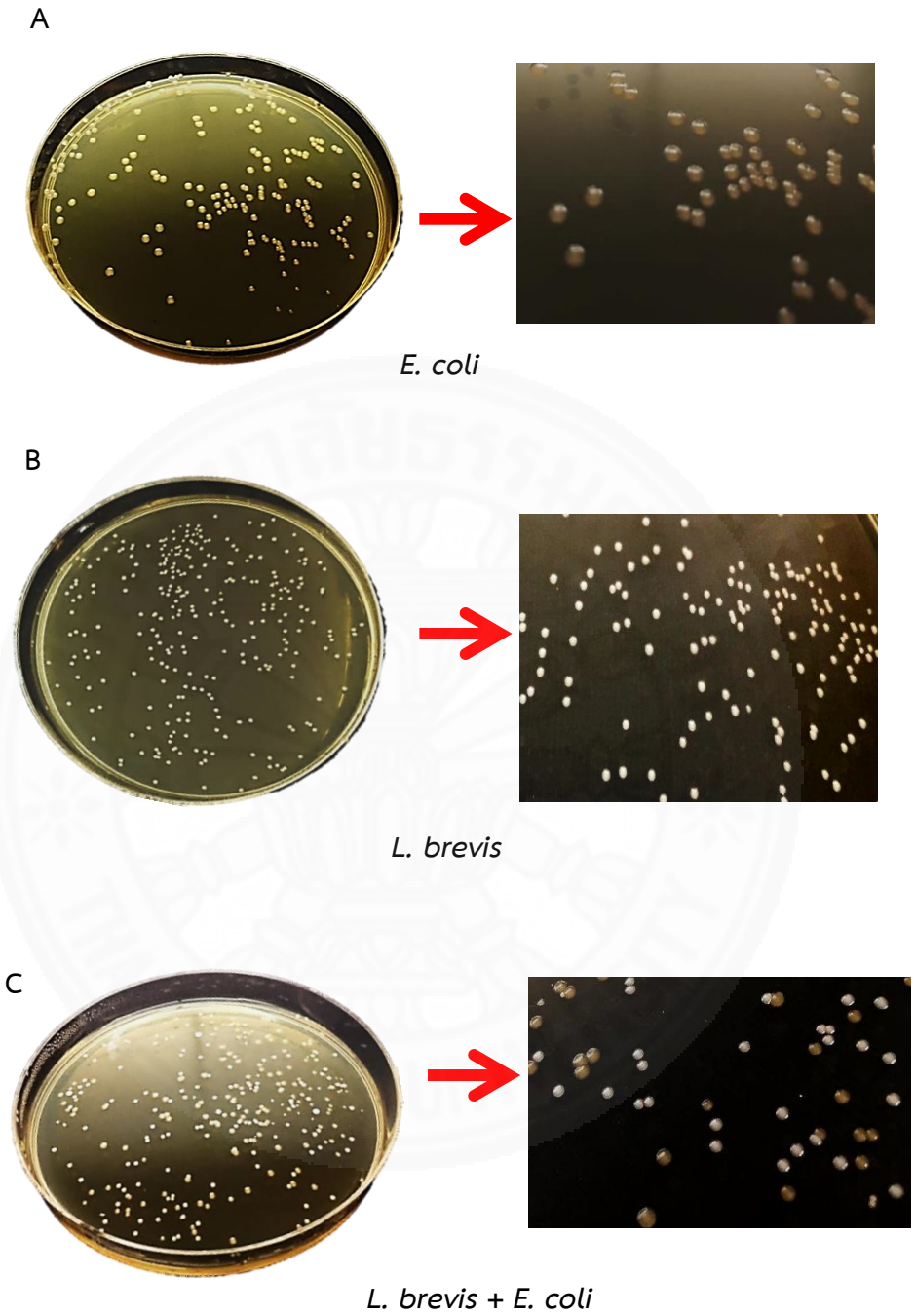


Figure 4.49 The growth of (A.) *L. brevis* compared with (B.) *E. coli* in the monoculture and (C.) co-culture in MRS containing 1% (w/v) xylose monoglycoside (XG_1).

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

PART 1: Synthesis and characterization of functional glycosides

5.1 Preparation of enzyme production

CGTase from the pBC recombinant *E. coli* was used as crude enzyme. The dextrinizing activity and protein were 199 U/ml and 0.25 ml, respectively. In enzyme production, the step of bacterial cultivation should use 1% (v/v) of the starter inoculum to transfer into 200 ml LB medium in 1,000 ml Erlenmeyer flask because large amount of the starter was found to have a significant effect on bacterial growth and enzyme yield.

5.2 Purification of CGTase

From starch adsorption, the activity of enzyme was 2,096 U/ml by dextrinizing activity assay and the recovered proteins were 0.20 mg/ml. So, the specific activity of the enzyme was 10,480.95 units/mg protein. In order to increase the yield and the purity of the enzyme, the purification of CGTase can be done only one step by starch adsorption which was a type of affinity chromatography. In addition, corn starch should be dried in an oven at 60 ^{o}C for 3 hours to increase its ability in enzyme adsorption.

5.3 Enzyme analysis by polyacrylamide gel electrophoresis (PAGE)

CGTase purity was determined by native polyacrylamide gel electrophoresis (Native-PAGE) and stained for both protein and dextrinizing activity. The degradation of starch substrate by the action of CGTase showed distinct clear zones which corresponded to the position of the active bands. In addition, the

molecular mass of CGTase was approximately 72 kDa by SDS-PAGE in comparison to the standard protein. Several results showed that the type of bacteria has affected to molecular weight of enzyme. For example, the molecular mass of *Paenibacillus* sp. RB01 CGTase was approximately 70 kDa (Anurutphan and Prousoontorn, 2014), and *Bacillus firmus* was estimated the molecular weight of 75 kDa (Yim et al., 1997).

5.4 Determination of donor and acceptor specificities

β-CD was reported to be the best donor for transglycosylation reaction of CGTase. Several starches, however, were also reported to be appropriate donor of the enzyme. Here, the appropriate acceptor was pinoresinol. The donor specificity of CGTase depends on the unique property of each CGTase that is used in the catalytic reaction to form a new covalent bond with the acceptor of interest such as pinoresinol. The pinoresinol acceptor was new and interested for this research.

5.5 Determination of transglycosylation

The transglycosylation products of recombinant CGTase were synthesized such as pinoresinol glycoside (PG_1-I and PG_1-II), and pinoresinol diglycoside (PG_2). The intensity of the products can be measured by scanning densitometer with Quantity One® 1-D analysis program. In this study to achieve high production yield, various parameters affecting the yield including substrate ratio, incubation time, enzyme unit, temperature and pH of CGTase were optimized.

5.6 Detection of products

Pinoresinol glycoside products were also analyzed by HPLC, the two separated peaks were detected at R_t 17.01 and 6.23 minutes which were PG_1 and PG_2 respectively. These products were eluted with a mixture of methanol-water (40:75 v/v) at the flow rate of 1.0 ml/min at 25 ^{o}C and detected at A_{210}. As resulted, this technique was high efficiency for detection of PG products, but the cost was also very expensive.

5.7 Optimization of pinoresinol glycoside production

The optimum condition for transglycosylation reaction of CGTase were 0.5% (w/v) β-CD with 1.5% (w/v) pinoresinol and 80 U/ml of final CGTase concentration in 20 mM of Tris-HCl buffer, pH 9.0 containing 20% (v/v) DMSO at 50 ^{o}C for 60 hours. (Table 5.11). The relative quantities of PGs under the optimal condition were determined to be 7.6% of total products. This yield increases almost 14–fold when compared to before optimization (0.56%).

Table 5.11 The optimum condition for transglycosylation reaction of pinoresinol glycoside production.

Characteristic	Optimal conditions
Pinoresinol concentration	1.5% (w/v)
β-CD concentration	0.5% (w/v)
Enzyme unite	80 U/ml
Temperature optimum	50 ^{o}C
Incubation time optimum	60 hours.
pH optimum	Tris-HCl buffer, pH 9.0

5.8 Large scale production of pinoresinol glycosides

The 50 ml of large-scale production was performed under the optimal conditions. The two main products of each pinoresinol glycosides were separated at the R_t 17.01 (PG_2) and 6.23 (PG_1) minutes. The yield of pinoresinol glycoside products was increased after optimization almost 14-fold compared to before optimization. In this study, the large-scale products were then purified using HPLC at 100-µl injection in several times to keep the purified PGs for further experiments. So, it is too difficult and lost in time to do this step for researcher. Therefore, in the future, the large-scale production should be done by using preparative C_{18} column instead of analytical C_{18} column.

5.9 Mass spectrometry (MS) analysis

The molecular weights of pinoresinol determined by ESI-TOF mass spectrometry was $[M+Na]^+$ at m/z of 381.12 (358 plus 23 of sodium molecule). PG_1 was determined to be $[M+Na]^+$ at m/z of 543.54 (520.54 plus 23 of sodium molecule) which was equivalent to the molecular weight of a single glucose unit attached to pinoresinol. PG_2 was determined to be $[M+Na]^+$ at m/z of 705.19 (682.19 plus 23 of sodium molecule). However, the structural feature related to the specific position of the glucose moiety linkage in pinoresinol needs to be further investigated by enzyme treatment and NMR techniques.

5.10 Structural analysis

5.10.1 Enzyme treatment

The structures of the synthesized PGs were elucidated by enzyme treatment with α-glucosidase, glucoamylase and several digestive enzymes from rat. These enzymes help to confirm that the obtained PGs consisted of α-configuration between pinoresinol and first linked-glucosyl unit.

5.10.2 Nuclear Magnetic Resonance (NMR) analysis

The structures of transferred products were also analyzed by ^{13}C- and ^{1}H-NMR. When the products at R_t = 17.01 and 6.23 minutes were analyzed, their structures and molecular weights characterised them as pinoresinol monoglycoside (PG_1) and pinoresinol diglycoside (PG_2), respectively. The 400 MHz ^{1}H-NMR was also analyzed to determine the type of glucosidic linkage belonging to α-configuration type. The ^{1}H-NMR spectrum of PG_1 showed that the anomeric proton of the glucose molecule was a doublet signal at 7.45 ppm with a coupling constant (*J*) of 3.94 Hz. For PG_2, two of doublet signals at 7.08 ppm (*J*= 4.09 Hz) and 6.85 ppm (*J* = 4.08 Hz) were assigned to anomeric protons, indicating that PG_2 is composed of two α-linkages. From ^{13}C-NMR, C-glucose at C-1''' position showed chemical shift from 92.77 to 100.57 ppm of both PG_1 and PG_2. In addition, at C-4' and C-4'' position of pinoresinol had also shifted. Thus, the structure of PG_1 and PG_2 should be as proposed in Figure 4.15.

5.11 Evaluation of the physiochemical and biological properties of pinoresinol glycosides products

5.11.1 Solubility test

The water solubility of pinoresinol was compared to that of pinoresinol glycoside products. Pinoresinol and pinoresinol glycoside products were mixed with 200 µl of water in an eppendrof tube and incubated at 25 °C for 15 minutes. The soluble part of the sample was analyzed by HPLC. The results showed that the water solubility of pinoresinol was 5 and 65-fold lower than those of the α-glucosylated derivatives, pinoresinol monoglycoside and pinoresinol diglycoside, respectively (Table 5.12). Here, we found that the long carbohydrate chains of pinoresinol were more soluble than shorter carbohydrate chains. In this study, the water solubility of pinoresinol glycoside products was greatly improved more than original pinoresinol. In the future, this improved property could be exploited to increase the water solubility and the absorption ability into intestine of PGs in foods and drugs. In addition, more water solubility of PGs could promote their antioxidant and anti-inflammatory activities.

Table 5.12 Water solubility of pinoresinol and pinoresinol glycoside products

Water solubility scale	
Compound	**Water solubility (mg)**
Pinoresinol (P)	-
Pinoresinol glycoside (PG_1)	5
Pinoresinol diglycoside (PG_2)	65

5.11.2 β-Glucuronidase inhibition assay

The anti-inflammation activity of salicylic (positive control) was equivalent to 100%. Anti-inflammatory capacities of pinoresinol (P), pinoresinol glycoside (PG_1) and pinoresinol diglycoside (PG_2) were 95.5, 80.0 and 80.0% respectively. The results showed that the long carbohydrate chains of pinoresinol were less effective in anti-inflammation than shorter carbohydrate chains. Anyway, in further study, *in vivo* experiments or other assays should be performed for the confirmation.

5.11.3 DPPH radical scavenging assay

The antioxidant activities of pinoresinol (P) and pinoresinol glycosides (PG_1 and PG_2) were evaluated using the DPPH radical scavenging reaction in IC_{50} unit. The IC_{50} value of P, PG_1 and PG_2 were 22, 30, and 600 μM, respectively (IC_{50} of Trolox = 2 μM), (Table 5.13). This suggested that the free-radical scavenging activity of P was reduced when these compounds were in the glucosylated form. The loss of antioxidant might result from the addition of glucose in the position at C4-OH of P which had disturbed its electron rotation.

Table 5.13 DPPH radical scavenging activity of IC_{50} value of pinoresinol and pinoresinol glycosides compared with that of the standard trolox.

Compound	IC_{50} (μM)
Trolox	2
Pinoresinol	43
Pinoresinol glycosides	40
Pinoresinol diglycosides	950

5.11.4 Sweetness test

The sweetness of pinoresinol (P), pinoresinol glycoside (PG_1) and pinoresinol diglycoside (PG_2) was tested using sucrose as the reference sweetener. The sweetness of P was lower than PG_1 and PG_2 (Table 5.14). In the future, the developed pinoresinol glycosides which, having more sweetness than pinoresinol can help to reduce their bitter taste in drugs.

Table 5.14 The relative sweetness of sucrose, pinoresinol, pinoresinol glycosides and maltooligosaccharides at 25 °C

Relative sweetness scale	
Compound	**Relative sweetness (%)**
Sucrose	100
Glucose	73
Maltose	50
Pinoresinol	5
Pinoresinol glycosides	70
Pinoresinol diglycosides	30

PART 2: Synthesis and characterization of functional oligosaccharides

5.12 Preparation of enzyme production

Amylomaltase was first found in *Escherichia coli* as a maltose-inducible enzyme, which is essential for the metabolism of maltose (Monod and Torriani, 1950). The amylomaltase gene has been cloned from several bacteria and archaea e.g. *Streptococcus pneumoniae* (Stassi, 1981), *E. coli* (Pugsley and Dubreuil, 1988), *Thermococcus litoralis* (Jeon, et al., 1997), *Thermus aquaticus* ATCC 33923 (Terada, et al., 1999), *Aquifex aeolicus* (Bhuiyan, et al., 2003), *Thermus brockianus* (Bang, et al., 2006) and *Corynebacterium glutamicum* (Srisimarat, et al., 2011). In this study, AMase was prepared from p19bAMY recombinant cells which consisted of AMase gene from *Thermus* sp. (Watanasatitarpa et al., 2014).

5.13 Purification of amylomaltase (AMase)

The AMase from p19bAMY recombinant cells was used to produce enzyme containing His-tag residues at N-terminal to make it easy for purification. So, the enzyme was continuously purified by HisTrap FF^{TM} column which is a prepacked column containing 90 µm highly cross-linked agarose bead with an immobilized chelating group. This chelator is complexed with a polyvalent Ni^{2+} ion. This column binds selectively histidine-tag protein which has specific affinity on Ni^{2+} ion. In this study, the purified recombinant showed a specific activity of 0.22 U/mg. Moreover, crude AMase was 3.2-fold purified with 67.0% yield after HisTrap FF^{TM} column.

5.14 AMase analysis by polyacrylamide gel electrophoresis (PAGE)

AMase purity was determined by native polyacrylamide gel electrophoresis (Native-PAGE) and stained for both protein and starch transglycosylation activity. The degradation of starch substrate by the action of AMase showed distinct clear zones

which corresponded to the position of the active bands. In addition, the molecular mass of AMase was approximately 60 kDa by SDS-PAGE in comparison to the standard protein.

5.15 Optimization of xylose glycoside (XG) production

The optimum condition for transglycosylation of AMase was 1.5% (w/v) TS and 2% (w/v) xylose with 4 U/ml of final AMase concentration in 20 mM Tris-HCl buffer pH 7.0 at 70 ^{o}C for 120 hours (Table 5.15). In this study, at the optimal condition, the total yield of XGs was increased as compared to the obtained yield before optimization.

Table 5.15 The optimum condition for transglycosylation reaction of xyloseglycoside production

Characteristic	Optimal conditions
Xylose concentration	2.0% (w/v)
Tapioca starch concentration	1.5% (w/v)
Enzyme unite	4U/ml
Temperature optimum	70 ^{o}C
Incubation time optimum	120 hours.
pH optimum	Tris-HCl buffer, pH 7.0

5.16 Large scale production and HPLC analysis of xylose glycosides

The large-scale productions were performed and then purified using HPLC. The two main products of xylose glycosides were observed R_t 11.76 (XG_1) and 26.59 (XG_2) minutes. The yield of xylose glycoside products was increased after optimization almost 14-fold compared to before optimization. In this study, XG main product was kept to further study.

5.17 Structural analysis of xylose glycosides

5.17.1 Mass spectrometry (MS) analysis

The molecular weights of xylose (X) and xylose glycoside (XG_1 and XG_2) were determined by ESI-TOF mass spectrometry. The molecular weight of X was $[M+Na]^+$ at *m/z* of 173.04. The molecular weight of XG_1 was determined to be $[M+Na]^+$ at m/z of 335.10 equivalent to the molecular weight of a single glucose unit attached to xylose. The molecular weight of XG_2 was determined to be $[M+Na]^+$ at *m/z* of 497.20 indicating two glucose units attached to xylose. From this work, structure of xylose glycoside products would be further confirmed by NMR techniques. When combined the results between MS and NMR, it could confirm the structure of XG_1 as shown in Figure 4.42, and named as xylose-α-D-glycoside, or xylose-α-D-monoglycoside.

5.17.2 Nuclear magnetic resonance (NMR) analysis

The linkage of XGs product was confirmed by ^{1}H NMR compared to parent xylose. The peak of anomericproton of parent xylose shown as doublet signal at 4.40 ppm (*J*=3.81 Hz) corresponded to the α-1,4 linkage, so the result confirmed that glucose connected to xylose by α-1,4 glucosidic bond. By ^{13}C-NMR, the X and XG_1 showed 5 and 11 carbon signals, respectively. The position of anomeric carbon of glucose, forming α-configuration, have shifted carbon signal from 92.77 to 98.94 ppm. Moreover, the position at C-4 of xylose of X and XG_1 has also shifted downfield. So, it could be identified XG_1 structure as 4-*O*-α-D-glucopyranosyl-D-xylose.

5.18 Evaluation of the physiochemical and biological properties of xylose glycosides products

5.18.1 Solubility

The solubility of xylose glycoside (XG_1) was 280.5 mg/ml whereas the solubility of xylose maltoside (XG_2) was 300.8 mg/ml (Table 5.16). The results showed that the long carbohydrate chains of xylose were more soluble than shorter carbohydrate chains. In this study, the water solubility of xylose glycoside products

was greatly improved more than original xylose. In future, it can use xylose glycoside products, having the property of high-water solubility in various applications i.e. intestinal absorption test and prebiotic test.

Table 5.16 The water solubility of xylose, XG_1 and XG_2 at 30 ^{o}C

Water solubility scale	
Compound	**Water solubility (mg)**
Xylose	250
Xylose monoglycoside (XG_1)	280
Xylose maltoside (XG_2)	300

5.18.2 Sweetness test

The sweetness of xylose (X), xylose glycoside (XG_1) and xylose maltoside (XG_2) were 0.53, 0.40 and 0.33, compared with sucrose (relative sweetness scale = 1 (Table 5.17). In the future, the developed xylose glycoside has less sweetness than xylose and sucrose that can be used as low-calorie sweeteners for diabetics or as prebiotic in health-promoting foods.

Table 5.17 Relative sweetness of monosaccharides and xylose glycoside products

Relative sweetness scale	
Compound	**Relative sweetness (%)**
Sucrose	100
Xylose	53
XG_1	40
XG_2	33

5.18.3 Maillard reaction test

The browning resistance test to the Maillard reaction, sucrose was higher resistance than XG_2, XG_1 and X. It means that sucrose was more stable than xylose and its derivatives when tested via the Maillard reaction. Recently, the maillard reaction has played an important role in improving the appearance and taste of foods. It has been a central and major challenge in food industry, since the Maillard reaction is related to aroma, colour and taste. Moreover, during the Maillard reaction is formed; the nutritional value of food does not be decreased (Martins et al., 2001). Here, we found that at pH 7 or below, xylose underwent to become browning after incubation for 2 hours at 120 ^{o}C. In addition, we also found that the glucosylated xylose i.e XG_1 and XG_2, showed lower complexity between sugar and amino groups (the Maillard reaction products) than original xylose.

5.18.4 Acidic resistance test

For acidic resistance test, both synthesized XG products were almost undegraded at 37 ^{o}C for 24 hours under acidic buffer (pH 3.0) system, compared to commercial xylose. The high stability under acidic conditions suggests that the synthesized XG products can resist to gastric acidity of human and can be added to acidic beverages such as fruit juices, yoghurt, alcoholic beverages, soft drinks, and sparkling water.

5.18.5 Prebiotic activity test

In vitro utilization of the XG_1 was compared under mono-culture and co-culture conditins of probiotic *L. brevis* BCC26712 and non-probiotic *E. coli* ATCC® 25922. Briefly, a test organism suspension was inoculated into MRS broth (Himedia, India) containing 1% (w/v) XG_1. The inoculated media were incubated at 37 ^{o}C with rotary shaking at 100 rpm for 48 hours. The degree of utilization was appreciated by reading the optical density at 600 nm and determined the value of decreased pH of the medium. The results found that XG_1 could be used as carbon source of two bacteria but *L. brevis* showed the ability in the utilization higher than non-probiotic *E. coli*. Moreover, *L. brevis* led to more acidic condition than *E. coli* in which inhibited the growth of other pathogenic bacteria. With co-culture condition, it found that the

growth of non-probiotic *E. coli* was significantly reduced, due to higher growth rate of probiotic *L. brevis* and its acidic producing.

REFERENCES

Aramsangtienchai, P., Chavasiri, W., Ito, K. & Pongsawasdi, P. (2011). Synthesis of epicatechin glucosides by a β-cyclodextrin glycosyltransferase, *J Mol Catal B-Enzym*, 73, 27-34.

Bar, R. (1989). Cyclodextrin-aided bioconversions and fermentations. *Trends biotechnol,* 7(1), 2-4.

Biwer, A., Antranikian, G., & Heinzle, E. (2002). Enzymatic production of cyclodextrins, *Appl Microb Biot*, 59, 609–617.

Bousquet, M.P., Willemot, R.M., Monsan, P. and Boures, E. (1998). Production, purification, and characterization of thermostable α-transglucosidase from *Talaromyces duponti*-application to α-glucosin synthesis. *Enzyme Micrib. Technol*. 23: 83-90.

Bollge, M.D., and Edelstein, J.S. (1996). Protein methods. John Wiley & Sons, Inc. New York. 95-142.

Bhuiyan, SH., Kitaoka, M., Hayashi, K. (2003). Cycloamylose-forming hyperthermostable 4-α-glucanotransferase of *Aquifex aeolicusexpressed* in *Escherichia colil.* J. Mol Catal. B: Enzyme. 22: 45-53.

Bradford, M.M. 1976. A rapid and sensitive method for the qualitatively of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248-54.

Buschmann, H. J., Knittel, D., & Schollmeyer, E. (2001). New textile applications of cyclodextrins, *J Incl Phenom Macro*, 40(3), 169-172.

Challa, R., Ahuja, A., Ali J., & Khar, R. K. (2005). Cyclodextrins in drug delivery: An updated review. *Aaps Pharmscitech*. 6, 1522-1528.

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

Charoensapyanan, R., Takahashi, Y., Murakami, S., Ito, K., Rudeekulthamrong, P., & Kaulpiboon, J. (2017). Synthesis, structural characterization, and biological

properties of pentyl- and isopentyl-α-D-glucosides. *Appl Biochem Micro+,* 53(4), 410-420.

Chotipanang, K., Bhunthumnavin, W., & Prousoontorn, M. H. (2011). Synthesis of alkyl glycosides from cyclodextrin using cyclodextrin glycosyltransferase from *Paenibacillus* sp. RB01. *J Incl Phenom Macro,* 70(3), 359-368. small cell lung cancer cells. *Cell Oncol,* 38, 195-204.

Doudoroff, M., Hassid, WZ., Putman, EW., Potter, AL. (1949). Direct utilization of maltose by Escherichia coli, *J. Biol. Chem*. 179: 921-934.

Fujii, K., inagawa, H., Terda, Y., Takaha, T., Kuriki, T., Shimada, J. and Kaneko, H. (2005). Use of random and saturation mutageness to improve the properties of Thermus aquaticus amylomaltase for efficient production of cycloamyloses. *Appl Environ Microbiol* 71: 5823-5827.

Fuwa, H. (1954). A new method for microdeterrmination of amylase activity by the use of amylose as the substrate. *J. Biochem.* 41: 583-603.

Goda ,S.K, Eissa O., Akhtar, M., Minto, NP. (1997). Molecular analysis of a *Clostridium butyricum* NCIMB 7423 gene encoding 4-α-glucanotransferase and characterization of the recombinant enzyme produced in *Escherichia coli*, Microbiology. 143: 3287-3294.

Harata, K. (1996). X-ray structure of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011. Comparison of two independent molecules at 1.8 A resolution. *Acta Cryst,* 52, 1136-1145.

Hashimoto, H. (1998). Production and application of cyclodextrins. In hanbook of amylase and related enzyme. The amylase research society of Japan, Tokyo. Pergamon Press: 233-238.

Hiroshi, K., Hitoshi, N., Yuichi, F., Sunju, Y. and Yuriko, K. 1985. Application of synthetic alkyl glycoside vesicles as drug carriers. I. Preparation and physical properties. *Chem. Pharm. Bull.* 33(2): 753-759.

Itoh, H. and Kamiyama, Y. (1995). Synthesis of alkyl β-mannosides from mannobiose by *Aspergillus niger* β-mannosidase. *J. Ferment. Bioeng*. 80(5)**:** 510-512.

Jeon, B., Taguchi, H., Sakai, H., Ohshima, T., Wakagi, T., Matsuzawa, H. (1997). 4-α-glucanotransferase from the hyperthermophilic archaeon Thermococcus litoralis, *Eur. J. Biochem*., 248: 171-178.

Jung, J.H., Seo, D.H., Ha, S.J., Song, M.C., Cha, J., Yoo, S.H., Kim, T.J., Baek, N.I., Baik, M.Y. and Park, C.S. (2009). Enzymatic synthesis of salicin glycosides through transglycosylation catalyzed by amylosucrases from *Deinococcus geothermalis* and *Neisseria polysaccharea*. *Carbohydrate Research.* 344(1): 1612–1619.

Kato, K. and Horikoshi, Y. (1984). Immobilized cyclodextrin gkucanotransferase of an alkalophilic *Bacillus* sp. No. 38-2. *Biotechnol. Bioeng*. 26: 295-598.

Kaulpiboon, J., prasong, W., Rimphanitchayakit, V., Murakami, S., Aoki, K. and Pongsawasdi, P. (2010). Expression and characterization of a fusion protein-containing cyclodextrin glysyltransferase from *Paenibacillus* sp. A11. *J. Basic Microb*. 50: 427-435.

Kikuchi, H., Nagura, T., Inoue, M., Kishida, T., Sakurai, H., Yokota, A., Asano, K., Tomita, Kitahata S, Murakami H, Sone Y. Misaki A. The acceptor specificity of amylo-maltase from Escherichia coli IFO 3806. Agric Biol Chem 1989; 53 :2661-6.

Kitahata, S., Tsuyama, N., & Okada, S. (1974). Purification and some properties of cyclodextrin glycosyltransferase from a strain of Bacillus species, *Agr Biol Chem Tokyo*, 38, 387-93.

Kitahata, S. and Okada, S. (1976). Studies on cyclodextrin glycosyltransferase IV. Enzymatic synthesis 3-O-α-D-glucopyranosyl-L-sorbose and 4-O-α-D-glucopyranosyl-D-xylose using cyclodextrin glycosyltransferase. *J Biochem* 79: 641-648.

Kitao, S. and Sekine, H. (1994). α-D-Glucosyl transfer to phenolic compounds by sucrose phosphorylase from *Leuconostoc mesenteroides* and production of α-arbutin. *Biosci. Biotech. Biochem*. 58: 38-42.

Klein, C., & Schulz, G. E. (1991). Structure of cyclodextrin glycosyltransferase refined at 2.0 Å resolution. *Journal of Molecular Biology,* 217(4), 737-750.

Klibanov, A. M. (1997). Why are enzymes less active in organic solvents than in water? *Trends Biotechnol*, 15(3), 97-101.

Kometani, T., Terada, Y., Nishimura, T., & Okada, S. (1994). Transglycosylation to

hesperidin by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* 96 species in alkaline pH and properties of hesperidin glycosides. *Biosci Biotech Bioch*, 58, 1990-1994.

Kometani, T., Yoshinobu, T., Nishimura, T., Takashi, N., Hiroshi, T., & Shigetaka, O. (1996). Acceptor specificity of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species and synthesis of glucosyl rhamnose. *Biosci. Biotech Bioch*, 60, 1176-1178.

Kometani, T., Nishimura, T., Nakae, T., Takii, H., & Okada, S. (1996). Synthesis of Neohesperidin Glycosides and Naringin Glycosides by Cyclodextrin Glucano-transferase from an Alkalophilic Bacillus Species. *Biosci Biotech Bioch*, 60(4), 645-649.

Kouptsova, O.S., Klyachko, N.L. and Levashov, A.V. (2001). Synthesis of alkyl glycosides catalyzed by β-glucosidases in a system of reverse micelles. *Russ. J. Biooranic Chem*. 27: 380-384.

Kren, V. and Martínková, L. (2001). Glycosides in medicine: The role of glycosidic residue in biologicalactivity". *Current Medicinal Chemistry.* 8: 1303-1328.

Kurimoto, M. (1997). Synthesis of glycosyl-trehalose by cyclodextrin glycosyltransferase through the transglycosylation reaction. *Biosci Biotech Biochem* 61: 1146-1149.

Lawson, C. L., van Montfort, R., Strokopytov, B., Rozeboom, H. J., Kalk, K. H., de Vries, G. E., & Dijkstra, B. W. (1994). Nucleotide Sequence and X-ray Structure of Cyclodextrin Glycosyltransferase from *Bacillus circulans* Strain 251 in a Maltose-dependent Crystal Form. *J Mol Biol*, 236(2), 590-600.

Larsson, J., Svensson, D. and Adlercreutz, P. (2005). α-Amylase-catalysed synthesis of alkyl glycosides. *J. Mol. Catal. B: Enzym.* 37: 84-85.

Lee, H., Auh, J., Yoon, H., Kim, M., Park, J., Hong, S., Kang, M., Kim, T., Moon, T., Kim, J., Park, K. (2002). Cooperative action of α-glucanotransferase and maltogenic amylase for an improved process of isomaltooligosaccharide (IMO) production. J. Agric. Food Chem. 50: 2812-2817.

Liebl, W., Feil, R., Gabelsberger, J., Kellermann, J., Schleifer, KH. (1992). Purification and characterization of a novel thermostable 4-α-glucanotransferase of Thermotoga maritima cloned in Escherichia coli. Eur. J. Biochem. 207: 81-88.

Mabuchi, N., & Ngoa, M. (2001). Controlled release powered flavor preparations and confectioneries preparations. *Jpn Patent JP*, 128, 638-641. 97.

Machida, S., Ogawa, S., Xiaohua, S., Takaha, T., Fujii, K., & Hayashi, K. (2000). Cycloamylose as an efficient artificial chaperone for protein refolding. *Febs Lett*, 486, 131-135.

Malsagov, M., Tugan, T., Timur, S. and Varuzhan, H.A. (2007). US Patent Application, Publication, US2007/0082102 A1.

Moon, Y.H., Kim, G., Lee, J.H., Jin, X.J., Kim, D.W. and Mol, J. (2009). A ORAC and TEAC assays comparison to measure the antioxidant capacity of food product. *Food Chemistry*. 114 (1): 310-316.

Martin, M. T., Alcalde, M., Plou, F. J., Dijkhuizen, L., & Ballesteros, A. (2001). Synthesis of maltooligosaccharides via the acceptor reaction catalyzed by cyclodextrin glycosyltransferases. *Biocatal Biotransfor*, 19, 21-35.

Naumthonh, W., Ito, K., & Pongsawasdi P. (2015). Aceptor specifity of amylomaltase from *Corynebacterium glutamicum* and transglucosylation reaction to synthesize palatinose glucosides. *Process Biochemitry*, 1359-5113.

Nia, R., Paper, D.H., Essien, E.E., Oladimeji, O.H., Iyadi, K.C., & Franz, G. (2003). Investigation into in-vitro radical scavenging and in-vivo anti-inflammatory potential of Tridax procumbens. *J Physiol Sci*, 18, 39-43. 98.

Nakakuki, T. (2002). Present status and future of function oligosaccharides delopment in Japan. *Pure and applied chemistry* 74: 1245-1251.

Nakano, H. and Kitahata, S. (2005). Application of cyclodextrin glucanotransferase to the synthesis of useful oligosaccharides and glycosides. *Handbook of Industrial Catalysis.* 90: 1-17.

Naumthong, W., Ito, K. and Pongsawasdi, P. (2015). Acceptor specificity of amylomaltase from Corynebacterium glutamicum and transglucoslylation reaction to synthesize palatinose glucosidase. *Process Biochemistry*. 1359-5113.

Nishimura, T., Kometani, T., Takii, H., Terada, Y. and Okada, S. (1994). Purification and some properties of α-amylase from *Bacillus subtilis* X-23 that glucosylates phenolic compounds such as hydroquinone. *J. Ferment. Bioeng.* 78**:** 31-36.

Paloheimo, M., Haglund, D., & Aho, S. (1992). Production of cyclomaltodextrin glucanotransferase of *Bacillus circulans* var. alkalophilus ATCC21783 in *B. subtilis*. *Appl Microbiol Biot*, 36, 584.

Park, K. H., Kim, M. J., Lee, H. S., Han, N. S., Kim, D., & Robyt, J. F. (1998). Transglycosylation reactions of *Bacillus stearothermophilus* maltogenic amylase with acarbose and various acceptors. *Carbohyd Res*. 313, 235–240.

Park, J.H., Kim, H.J., Kim, Y.H., Cha, H., Kim, Y.W., Kim, T.J., Kim, Y.R. and Park, K.H. (2007). The action mode of *Thermus aquaticus* YT-1 4-α-glucanotransferase and its chimeric enzymes introduced with starch-binding domain on amylose and amylopectin. *Carbohydr. Polym.* 67: 164–173.

Penninga, D., Veen, B. A. V. D., Knegtel, R. M. A., Hijum, S. A. F. T. V., Rozeboom, H. J., Kalk, K. H., & Dijkhuizen, L. (1994). The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. *Biol Chem*, 271(51), 32777-32784.

Qi, Q., & Zimmermann, W. (2005). Cyclodextrin glucanotransferase: from gene to applications. *Appl Microbiol Biot*, 66, 475-485.

Ramelet, A. A. (2001). Clinical Benefits of Daflon 500 mg in the Most Severe Stages of Chronic Venous Insufficiency. *Angiology*, 52(1), 49-56.

Rosen, M. J. 1989. Surfactants and Interfacial Phenomena. 2nd ed. John Wiley & Sons, Inc. USA, pp.1-32.

Rudeekulthamrong, P., & Kaulpiboon, J. (2016). Application of amylomaltase for the synthesis of salicin-α-glucosides as efficient anticoagulant and anti-inflammatory agents. *Carbohyd Res*, 432, 55-61.

Saehu, S., Srisimarat, W., Prousoontorn, M.H. and Pongsawasdi, P. (2013). Transglucosylation reaction of amylomaltase for the synthesis of anticariogenic oligosaccharides. *J. Mol. Catal. B: Enzym.* 88: 77- 83.

Salva, T.J.G., Lima, V.B. and Pagan, A.P. (1997). Screening of alkaphilic bacteria for cyclodextringlycosyltransferase production. *Microbiol* 28: 157–164.

Sarney, D. and Valfson, E. (1995). Application of enzymes to the synthesis of surfactants. *Trends Biotechnol*. 13(5): 164-172.

Seo, N., Roh, S., Auh, J., Park, J., Kim, Y., Park, K. (2007). Structural characterization of rice starch in rice cake modified by Thermus scotoductus 4-α-glucanotransferase (TS α-GTase). J. Food Sci. 72: 331-33.

Shimoda K., Akagi M. and Hamada, H. (2009). Production of beta maltooligosaccharides of alpha-and delta-tocopherols by *Klebsiella pneuniae* and cyclodextrin glucanotransferase as anti-allergic agents. *Molecules*. 14(8):3106-3114.

Shimoda, K. and Hamada, H. (2010). Synthesis of beta-maltooligosaccharides of glycitein and daidzein and their anti-oxidant and anti-allergic activities. *Molecules*. 15(8):5153-61.

Shimoda, K., Kubota, Na. and Akagi, M. (2012). Synthesis of capsaicin oligosaccharides and Their anti-allergic activity. *Scientific Research*. 2: 45-49.

Srisimarat, W., Kaulpiboon, J., Zimmerman, W., Powviriyakul, A., Krusong, K. and Pongsawasdi, P. (2011). A novel amylomaltase from *Corynebactrerium glutamicum* and analysis of the large-ring cyclodextrin products. *J Incl Phenom Macrocyl Chem* 70: 369-575.

Sugimoto, K., Nishimura, T., Nomura, K., Sugimoto, K. and Kuriki, T. (2003). Synthesis of arbutin-α-glycosides and comparison of their inhibitory effects with those of α-arbutin and arbutin on human tyrosinase. *Chem. Pharm. Bull.* 51**:** 798-801.

Svensson, D., Ulvenlund, S. and Adlercreutz. (2009). Efficient synthesis of a long carbohydrate chain alkyl glycoside catalyzed by cyclodextrin glycosyltransferase (CGTase). *Biotechnol. Bioeng*. 104(5): 854-861.

Szejtli, J. (2004). Introduction and General Overview of Cyclodextrin Chemistry. *Chem Rev*, 98(5), 1743-1754.

Takaha, T. and Smith, S.M. (1999). The functions of 4-α-glucanotransferases and their use for the production of cyclic glucans. *Biotechnol Genet Eng Rev.* 16**:** 257-280.

Takaha, T., Yanase, M., Okada, S. and Smith, S.M. (1993). Disproportionating enzyme (4-alpha-glucanotransferase; EC 2.4.1.25) of potato: Purification, molecular

cloning, and potential role in starch metabolism. *J. Biol. Chem.* 268(2): 1391-1396.

Tantanarat, K., O'Neill, E., Rejzek, M., Field, R., Limpaseni, T. (2014). Expression and characterization of 4-α-glucanotransferase genes from Manihot esculenta Crantz and Arabidopsis thaliana and their use for the production of cycloamylases. Process Biochem. 49: 84-89.

Terada, Y., Fujii, K., Takaha, T. and Okada, S. (1999). *Thermus aquaticus* ATCC 33923 amylomaltase gene cloning and expression and enzyme characterization: production of cycloamylose. *Appl. Environ. Microbiol.* 65(3): 910-915.

Tomita, K., Fukuda, M. and Kawasaki, K. (1990). Mechanism of arbutin inhibitory effect on melanogenesis and effect on the human skin with cosmetic use. *Fragrance Journal*. 18: 72–77.

Tonkova, A. (1998). Bacterial cyclodextrin glucanotransferase. *Enzyme Microb Tech*, 22, 678-686.

Turner, P., Svensson, D., Adlercreutz, P. and Karlsson, E. (2007). A novel variant of *Thermotoga neapolitana* β-glucosidase B is an efficient catalyst for the synthesis of alkyl glucosides by transglycosylation. *J. Biotechnol.* 130**:** 67-74.

Uitdehaag, J. C. M., van der Veen, B. A., Dijkhuizen, L., & Dijkstra, B. W. (2002). Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-amylase family. *Enzyme Microb Tech, 30*(3), 295-304.

Van der Veen, B. A., Uitdehaag, J. C. M., Penninga, D., van Alebeek, G. W. M., Smith, L. M., Dijkstra, B. W., & Dijkhuizen, L. (2000). Rational design of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 to increase α- cyclodextrin production11Edited by G. Von Heijne. *J Mol Biol*, 296, 1027- 1038.

Vulfson, E.N., Patel, R., Beecher, J.E., Andrews, A.T. and Law, B.A. (1990). Glucosides in organic solvents: I. alkyl-β-glucoside synthesis in water-organic two-phase system. *Enzyme Microb. Technol*. 12: 950-954.

Wannapa, W. (2000). Specificity of glycosyl acceptor in coupling and transglycosylation reaction of cyclodextrin glycosyltransferase from *Bacillus circulans* A11. Master's Thesis (Biochemistry), Chulalongkorn University.

Weber, K. and Osborn, M. (1975). Proteins and SDS: molecular weight determination on polyacrylamide gels and related procedures. In Neurath, H., Hill, R. L., and Border, C. (eds.), *the proteins*, vol. 1. Academic Press, New York. pp. 179-233.

APPENDICES

APPENDIX A

PREPARATION FOR CULTURE MEDIUM

LB broth	(50 ml)
Tryptone	0.5 g
Yeast extract	0.25 g
Sodium chloride (NaCl)	0.5 g
Adjust volume to 50 ml with distilled water	
LB broth (300 ml)	
Tryptone	3.0 g
Yeast extract	1.5 g
Sodium chloride (NaCl)	3.0 g
Adjust volume to 300 ml with distilled water	
IPTG	
1 M IPTG (MW = 238.3 g/mol)	0.238 g
Adjust volume to 1 ml with distilled water	

APPENDIX B

PREPARATION FOR STARCH TRANSGLYCOSYLATION ASSAY

Reagent and buffer

1. 0.2 % (w/v) Soluble starch

-Soluble starch 0.2 g

Added the distilled H_2O to make a final volume of 100 ml

2. 1% (w/v) maltose

- Maltose 1 g

Added the distilled H2O to make a final volume of 100 ml

3. Iodine solution (stock solution) (0.2 % (w/v) I in 2 % (w/v) KI)

- Potassium iodide 2 g

- Iodine 0.2 g

Added the distilled H_2O to make a final volume of 100 ml

4. Iodine solution (working solution) (0.02 % (w /v) 12 in 0.2 % (w/v) KI)

- Iodine solution (stock solution) 10 ml

- Distilled water 90 ml

5. 1M sodium phosphate buffer, pH 7.4 (stock solution)

1M Na_2HPO_4 (100 ml) 7 g

- Na_2HPO_4 14.2 g

Added the distilled H_2O to make a final volume of 100 ml

APPENDIX C

PREPARATION FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

1) Stock reagents

30% Acrylamide,	100 ml
acrylamide	29.2 g
N, N'- methylene-bis-acrylamide	0.8 g
Adjusted volume to with distilled water	100 ml

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 g
Adjusted pH to 8.8 with 1 M HCl and Adjusted volume to distilled water	100 ml

2 M Tris-HCl pH 8.8

Tris (hydrox ymethyl)-aminomethane	24.2 g
Adjusted pH to 8.8 with 1 M HCl and Adjusted volume to distilled water	100 ml

0.5 M Tris-HCl pH 6.8

ris (hydroxymethyl)-aminomethane	6.06 g
Adjusted pH to 6.8 with 1 M HCl and Adjusted volume to distilled water	100 ml

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1 g
Adjusted pH to 6.8 with 1 M HCl and Adjusted volume to distilled water	100 ml

Solution B (SDS-PAGE

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (SDS-PAGE)

1 M Tris-HCl pH 6.8	50 ml

10% SDS	4 ml
Distilled water	46 ml

2) Denaturing PAGE (SDS-PAGE)

10.0 % separating gel

30 % Acrylamide solution	2.50 ml
Solution B (SDS-PAGE)	2.50 ml
Distilled water	2.39 ml
10 % $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

5.0 % stacking gel

30 % Acrylamide solution	0.84 ml
Solution B (SDS-PAGE)	1.0 ml
Distilled water	3.1 ml
10 % $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

Sample buffer	1 M
Tris-HCl pH 6.8	0.6 ml
50% glycerol	5.0 ml
10% SDS	2.0 ml
1% Bromophenol blue	1.0 ml
Distilled water	0.9 ml

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

Electrophoresis buffer, 1 litre

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

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

APPENDIX D

PREPARATION FOR BUFFER SOLUTION

0.2 M sodium acetate pH 4.0, 5.0 and 6.0

CH_3COONa 1.21g

adjusted volume to 100 ml. with distilled water. Adjusted to pH 4.5 and 6 by 0.2 M acetaic acid

0.2 M phosphast pH 6.0

KH_2PO_4 3.28 g

K_2HPO_4 0.16 g

distilled water 100 ml

0.2 M phosphast pH 7.0

KH_2PO_4 1.35 g

K_2HPO_4 0.67 g

distilled water 100 ml

0.2 M phosphast pH 8.0

KH_2PO_4 0.48 g

K_2HPO_4 2.34 g

distilled water 100 ml

0.2 M Tris-glycin NaOH pH 8.0, 9.0 and 10.0

Glycin 1.5g

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

APPENDIX E

HISTRAP FFTM AFFINITY COLUMN CHROMATOGRAPHY

Reagents and buffer

1. 2M Imidazole

- Imidazole 136.16 g

Added distilled water to 1,000 ml

2. 0.4M EDTA - EDTA 145.7 g

Added distilled water to 1,000 ml

3. 0.1M $NISO_4$

- NISO 15.48 g

Added distilled water to 1,000 ml

4. 8x stock solution, pH 7.4

(160 mM sodium phosphate buffer, 4 M NaCl)

5. Binding buffer

(20 mM sodium phosphate buffer, 0.5 M NaCl, 20 mM Imidazole)

8x stock solution, pH 7.4 6.25 ml

2 M Imidazole 0.5 ml

Added distilled water to 50 ml

6. Elution buffer

(20 mM sodium phosphate buffer, 0.5 M NaCl, 500 mM Imidazole)

8x stock solution, pH 7.4 6.25 ml

2 M Imidazole 12.5 ml

Added distilled water to 50 ml

Buffer

7. Stripping buffer

(20 mM sodium phosphate buffer, 0.5 M NaCl, 50 mM EDTA)

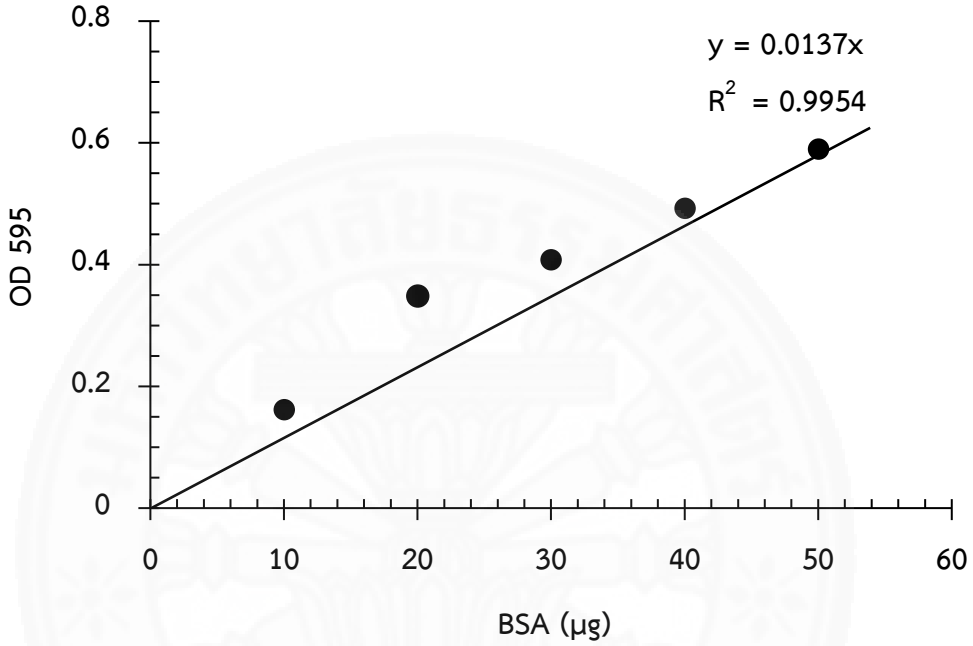
- 8x stock solution, pH 7.4 6.25 ml

-0.4 M EDTA 6.25 ml

Added distilled water to 50 ml

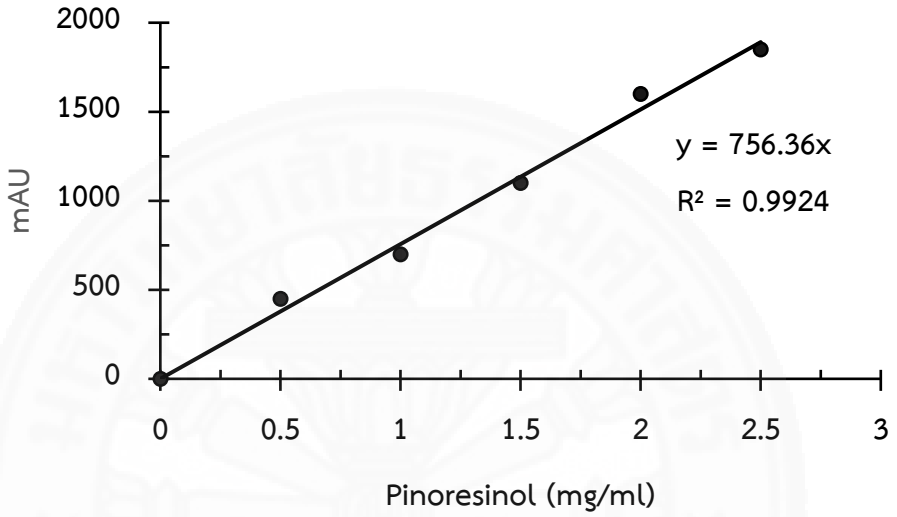
APPENDIX F

STANDARD CURVE FOR PROTEIN DETERMINATION BY BRADFORD'S METHOD



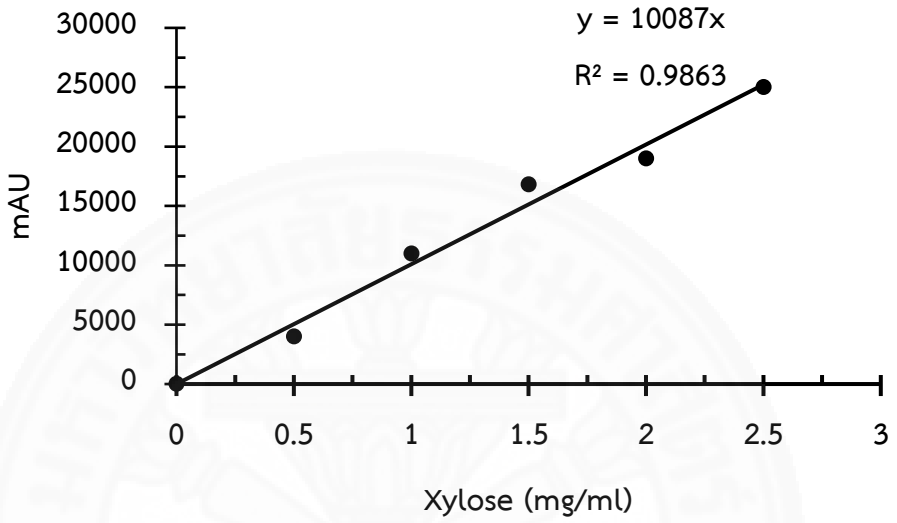
APPENDIX G

STANDARD CURVE FOR PINORESINOL SOLUBILITY BY HPLC



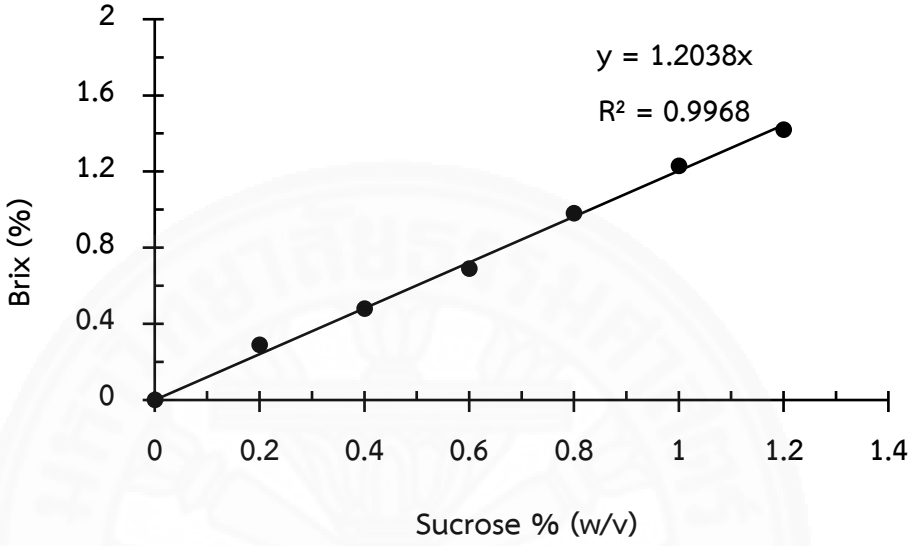
APPENDIX H

STANDARD CURVE FOR XYLOSE SOLUBILITY BY HPLC



APPENDIX I

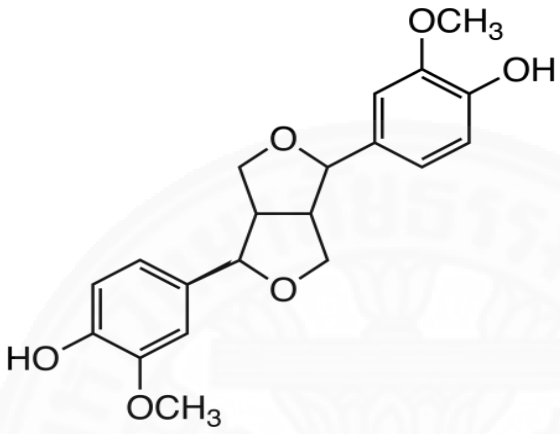
STANDARD CURVE FOR SWEETNESS OF SUCROSE BY REFRACTOMETER



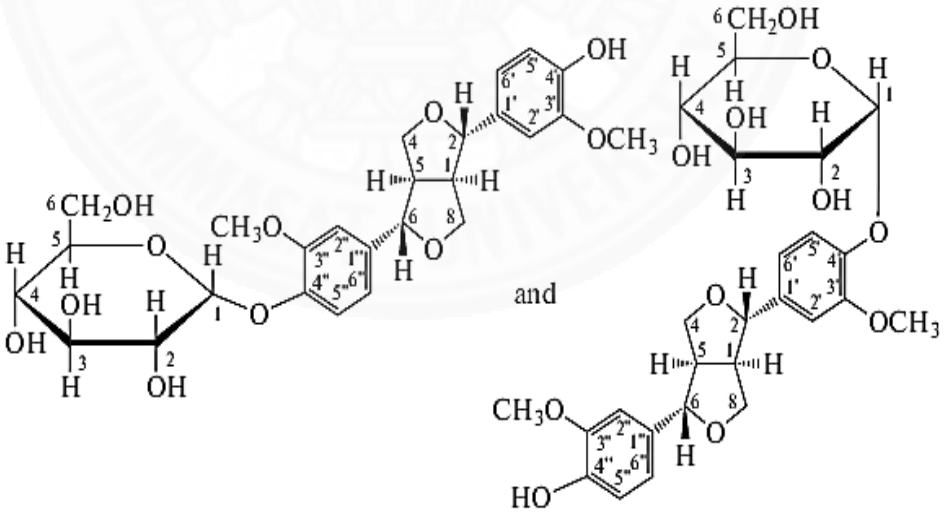
APPENDIX J

STRUCTURE OF ACCEPTOR

1. pinoresinol

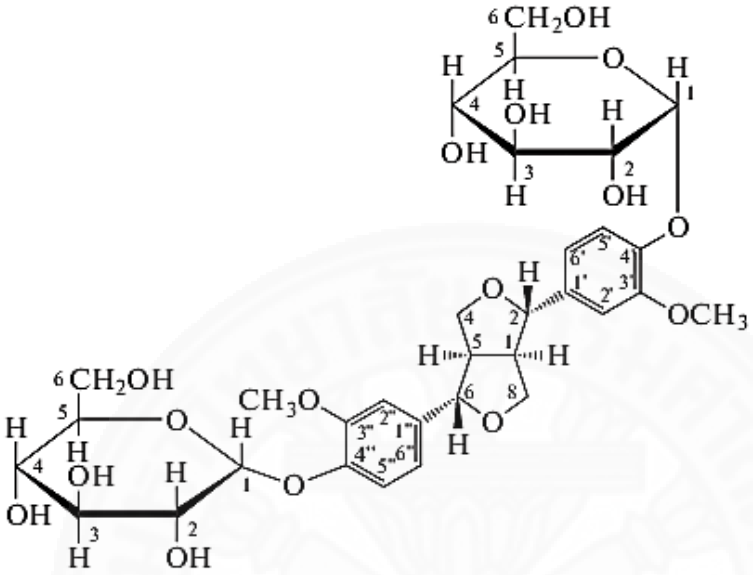


2. pinoresinol glycoside

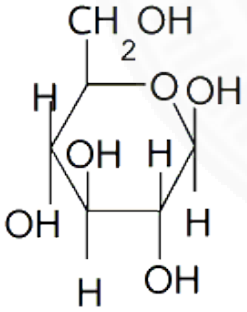


3.

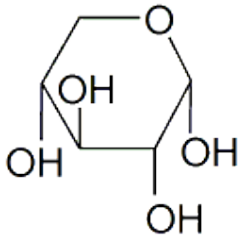
3. pinoresinol diglycoside



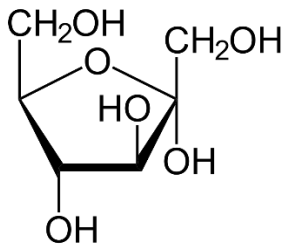
4. Glucose



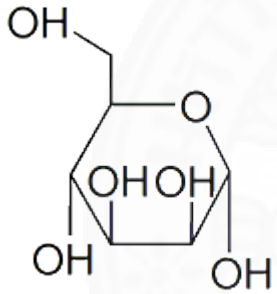
5. Xylose



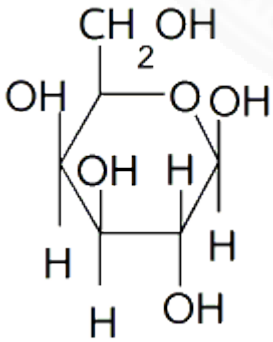
6. Fructose



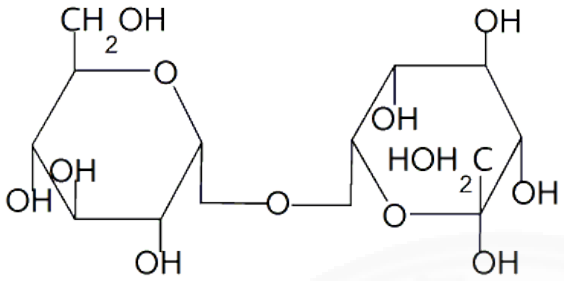
7. Mannose



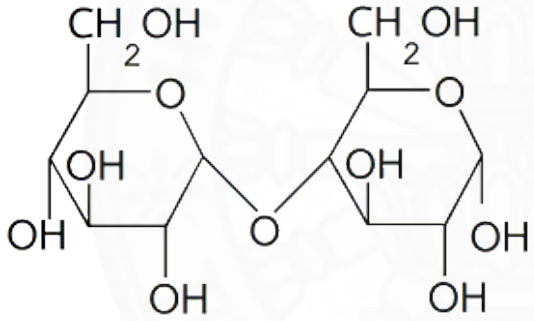
8. Galactose



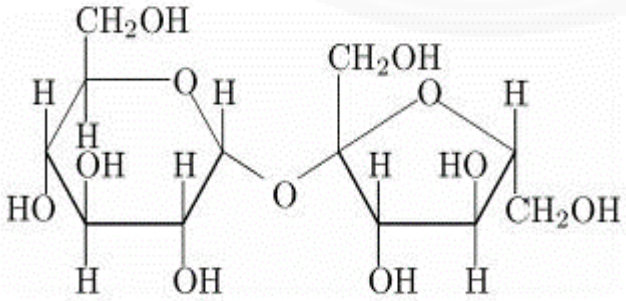
9. Trehalose



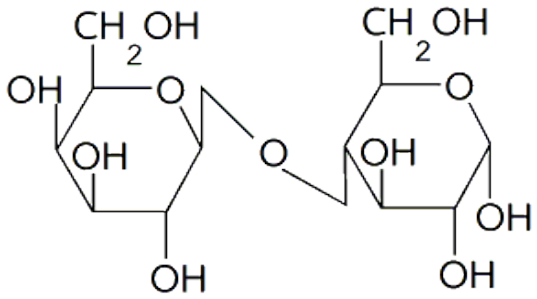
10. Maltose



11. Sucrose



12. Lactose



BIOGRAPHY

Name	Miss Nattida Khammanee
Date of Birth	October 23, 1991
Educational Attainment	2013: Bachelor Degree of Science (Biomedical Sciences), Rangsit University, Thailand
Work Position	Faculty of medicine Thammasat University
Scholarship	2015: Teaching Assistant Scholarship of Thammasat University

Publications

Khummanee, N., Rudeekulthamrong, P. and Kaulpiboon. J., Cyclodextrin Glycosyltransferase-Catalyzed Synthesis of Pinoresinol-α-D-glucoside Having Antioxidant and Anti-Inflammatory. Activities Applied Biochemistry and Microbiology volume 55, pages 360–370 (2019).

Presentations

Khummanee, N. and Kaulpiboon, J., 2018, Cyclodextrin glycosyltransferase-catalyzed synthesis of pinoresinol-α-D-glucoside with antioxidant and anti-inflammatory activities. Poster session presented at: 24th IUBMB and 15th FAOBMB Congress, 4-8 June 2018, COEX, Seoul, Korea.