การสังเคราะห์ไฟซีทินไกลโคไซด์โดยไซโคลเด็กซ์ทรินไกลโคซิลแทรนส์เฟอเรส
จาก *Paenibacillus* sp. RB01

นางสาวณัฐวดี ล้อทองพานิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SYNTHESIS OF FISETIN GLYCOSIDES BY CYCLODEXTRIN GLYCOSYL TRANSFERASE FROM *Paenibacillus* sp. RB01

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Thesis Title	SYNTHESIS OF FISETIN GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01
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ณัฐวดี ล้อทองพานิชย์ : การสังเคราะห์ไฟซีทินไกลโคไซด์โดยไซโคลเด็กซ์ทรินไกลโคซิลแทรนส์เฟอเรสจาก *Paenibacillus* sp. RB01 (SYNTHESIS OF FISETIN GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. มัญชุมาส เพราะสุนทร, 131 หน้า.

ไฟซีทินเป็นสารฟลาโวนอยด์ที่ได้รับความนิยมอย่างกว้างขวางในอุตสาหกรรมเภสัชวิทยา แต่อย่างไรก็ตามไฟซีทินมีความสามารถในการละลายน้ำต่ำและไม่เสถียรในสารละลายที่มีน้ำ ทำให้การนำไฟซีทินไปใช้จึงมีข้อจำกัด ในการศึกษานี้มีวัตถุประสงค์เพื่อเพิ่มความสามารถในการละลายน้ำของไฟซีทินโดยอาศัยปฏิกิริยาแทรนส์ไกลโคซิเลชันโดยใช้เอนไซม์ไซโคลเด็กซ์ทรินไกลโคซิลแทรนส์เฟอเรส (cyclodextrin glycosyltransferase, CGTase) จากแบคทีเรีย *Paenibacillus* sp. RB01 ซึ่งในปฏิกิริยานี้จำเป็นต้องใช้ระบบสารละลายอินทรีย์ร่วมด้วย และพบว่า DMSO ที่ร้อยละ 40 (v/v) เป็นสารละลายอินทรีย์ร่วมที่เหมาะสมที่สุด เนื่องจากให้ความคงตัวของเอนไซม์สูงและให้ผลผลิตสูง นอกจากนี้ยังศึกษาชนิดของตัวให้หมู่ไกลโคซิลและพบว่าบีตาไซโคลเด็กซ์ทรินเป็นตัวให้หมู่ไกลโคซิลที่เหมาะสม จากนั้นทำการศึกษาผลของความเข้มข้นของไฟซีทิน, บีตาไซโคลเด็กซ์ทรินและเอนไซม์ และระยะเวลาในการบ่มต่อการสังเคราะห์ไฟซีทินไกลโคไซด์ พบว่าสภาวะที่เหมาะสมคือการบ่ม 0.25% (w/v) ของไฟซีทินและ 1.0% (w/v) ของบีตาไซโคลเด็กซ์ทรินกับ 200 U/mL ของ CGTase ที่ 40 องศาเซลเซียสเป็นเวลา 24 ชั่วโมง ซึ่งภายใต้สภาวะที่เหมาะสมในการผลิตนี้มีเปอร์เซ็นต์การเปลี่ยนแปลงของไฟซีทิน (17.1% ± 4.21) สูงกว่าเมื่อเปรียบเทียบกับสภาวะที่ไม่เหมาะสม (10.1% ± 1.15) อย่างมีนัยสำคัญ ในการสังเคราะห์ผลิตภัณฑ์ไฟซีทินไกลโคไซด์โดย CGTase พบผลิตภัณฑ์ 5 ชนิด เมื่อวิเคราะห์ด้วย HPLC การวิเคราะห์โครงสร้างด้วยกลูโคแอมิเลสและแอลฟากลูโคซิเดสพบว่าไฟซีทินเชื่อมต่อกับกลูโคสด้วยพันธะแอลฟา-ไกลโคไซด์ ขณะที่กลูโคสเองต่อกันด้วยพันธะ α-1, 4 ไกลโคซิดิส จากนั้นทำบริสุทธิ์ผลิตภัณฑ์ไฟซีทินไกลโคไซด์ที่ได้โดยการสกัดด้วย n-butyl acetate และแยกต่อด้วย HPLC เมื่อวิเคราะห์ด้วย LC-MS/MS พบว่า CGTase สามารถสังเคราะห์ไฟซีทินมอนอกลูโคไซด์ได้ 2 ชนิด (m/z ที่ 447), ไฟซีทินไดกลูโคไซด์ 2 ชนิด (m/z ที่ 609) 2 ชนิด และไฟซีทินไตรกลูโคไซด์ 1 ชนิด (m/z ที่ 771) และรูปแบบการกระจายตัวบ่งชี้ว่าโครงสร้างของผลิตภัณฑ์ไฟซีทินมอนอกลูโคไซด์มีอยู่ 2 รูปแบบ จากการศึกษาความสามารถในการละลายน้ำของผลิตภัณฑ์บริสุทธิ์ทั้งสามชนิด พบว่าความสามารถในการละลายน้ำของไฟซีทินไกลโคไซด์เพิ่มขึ้นอย่างน้อย 500 เท่าเมื่อเทียบกับไฟซีทิน การวิเคราะห์โดย DPPH แสดงให้เห็นถึงกิจกรรมการต้านอนุมูลอิสระของไฟซีทินไกลโคไซด์ที่สังเคราะห์ได้นั้นไม่แตกต่างกันเมื่อเปรียบเทียบกับไฟซีทิน

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

5771981323 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
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NATTAWADEE LORTHONGPANICH: SYNTHESIS OF FISETIN GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01. ADVISOR: ASST. PROF. MANCHUMAS PROUSOONTORN, Ph.D., 131 pp.

Fisetin is a flavonoid which was widely exerted in pharmacological industries. However, fisetin has low water solubility and unstable in aqueous solution. So the uses of fisetin have been limited. The purpose of this study was to increase water solubility of fisetin by transglycosylation reaction using cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus sp.* RB01. The reaction had to perform in co-organic solvent system and DMSO at 40% (v/v) was proved to be the most suitable co-organic solvent since it provided the high enzyme stability and high production yield. Moreover, the types of glycosyl donor were also determined and β-cyclodextrin was found to be an appropriate glycosyl donor. The effects of fisetin, β-cyclodextrin, and enzyme concentration and incubation time on the fisetin glycoside synthesis were also determined. It was found that the optimum condition was to incubate 0.25% (w/v) of fisetin and 1.0% (w/v) of β-CD with 200 U/mL of CGTase at 40ºC for 24 hours. Under optimum condition, the percent conversion of fisetin (17.1% ± 4.21%) was significantly increased when compared to that of non-optimized condition (10.1% ± 1.15%). HPLC analysis showed that at least 5 fisetin glycosides were synthesized by CGTase. Structural analysis by glucoamylase and α-glucosidase revealed that fisetin linked with glucose through alpha-glycosidic bonds, while glucose molecules joined together by α-1, 4 glycosidic bond. The obtained fisetin glycoside products were purified by n-butyl acetate extraction and further refined by HPLC. LC-MS/MS revealed that CGTase synthesized two types of fisetin monoglucosides (m/z of 447), two types of fisetin diglucosides (m/z of 609) and one type of fisetin triglucoside (m/z of 771). Fragmentation patterns suggested two possible structures of fisetin monoglucoside products. The water solubility of three purified products were investigated. It was found that water solubility of fisetin glycosides increased at least 500 times higher than the parent fisetin. DPPH assay showed that the antioxidant activity of synthesized fisetin glycosides was not different when compared with fisetin.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

A	: Absorbance
BSA	: bovine serum albumin
CDs	: Cyclodextrin
CGTase	: Cyclodextrin glycosyltransferase
cm	: centimeter
°C	: degree celcius
ESI-TOF-MS	: Electrospray Ionization-Time of Flight Mass spectrometry
et al.	: Et. Alii (latin), and others
g	: gram
h	: hour(s)
HPLC	: High Performance Liquid Chromatography
kDa	: kiloDalton
L	: Liter
µg	: Microgram
µL	: Microliter
M	: Molar
min	: minute(s)

mg	: Milligram
ml	: Milliliter
mM	: Millimolar
MS	: Mass Spectrometer
MS/MS	: Tandem Mass Spectrometry
nm	: nanometer
PAGE	: Polycrylamide gel electrophoresis
rpm	: revolution per minute
Rf	: Relative mobility
Rt	: Relation time
RSA	: Radical scavenging activities
SDS	: Sodium dodecyl sulfate
TLC	: Thin Layer Chromatography
U	: Unit(s)
v/v	: Volume by volume
w/v	: Weight by volume

CHEPTER I
INTRODUCTION

1.1 Flavonoids

Flavonoids are a group of natural compounds which contain several phenolic molecules. They are found in a variety of fruits and vegetables including, grains, bark, roots, stems, flowers, tea and wine. Therefore, flavonoids are part of the human diet. Moreover, they are now considered as an indispensable component in many of nutraceutical, pharmaceutical and medicinal applications. Scientific studies reported that flavonoids exhibited various biological activities, such as anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic, coupled with their capacity to modulate key cellular enzyme function (Kumar & Pandey, 2013; Wang, Li, & Bi, 2018). Flavonoids can be classified to various subgroups, and it is important to keep in mind that the biological and chemical properties of flavonoids belonging to different subgroups can be quite different (Heim, Tagliaferro, & Bobilya, 2002).

1.1.2 Chemistry and classification of flavonoids

The main structure of flavonoids is polyphenol based on flavan nucleus. Flavonoids can be divided into various groups dependent on the substitution on the aromatic rings. The generic structure of flavonoids and numbering system which was used to distinguish the carbon position around the molecule is shown in Figure 1. The three phenolic rings are defined as the A, B and C (or pyrane) (Heim et al., 2002; Wang et al., 2018). The group of flavoniod in which the B ring is linked in position 3 of the C ring is called isoflavones. Those in which the B ring is linked in position 4 are called neoflavonoids while those in which the B ring is linked in position 2 can be further

subdivided into several subgroups on the basis of the structural features of the C ring. These subgroups are: flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins and chalocones (Figure 1) (Panche, Diwan, & Chandra, 2016).

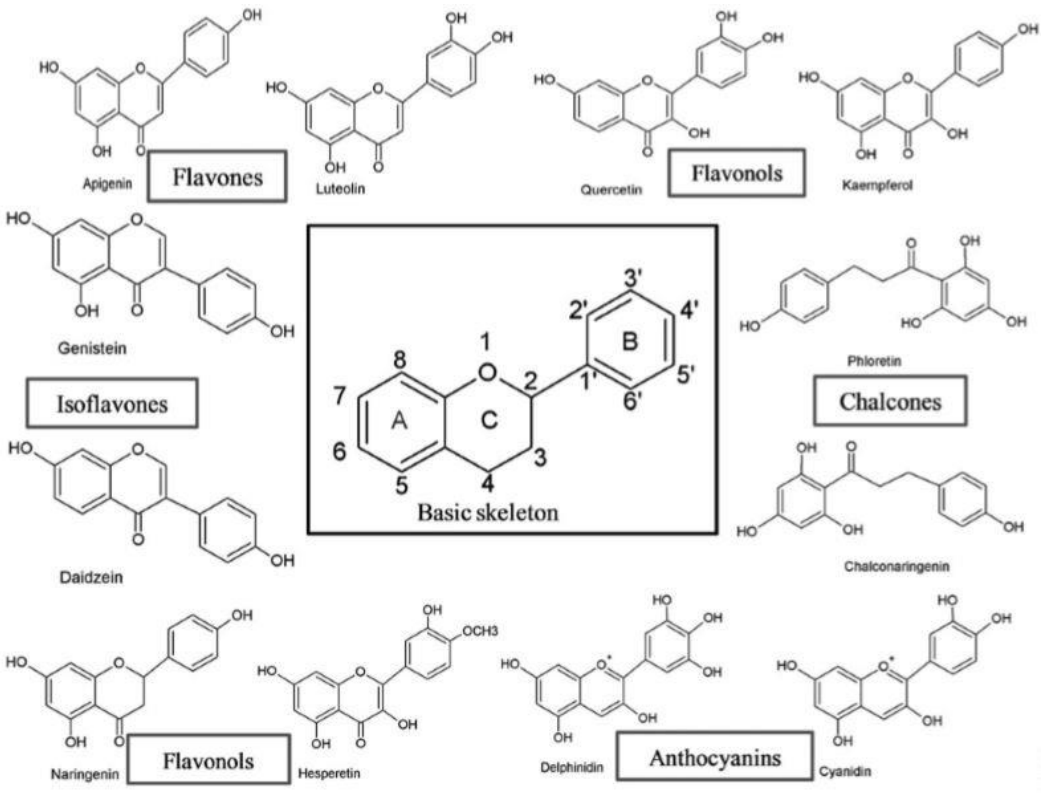


Figure 1 Basic skeleton structure of flavonoids and their classes (Panche et al., 2016).

1.2 Fisetin

1.2.1 General information of fisetin

Fisetin (3, 3', 4', 7-tetrahydroxyflavone, Figure 2) is one of the polyphenols in the flavonoid group which belongs to flavonol subgroup. It has very interesting spectroscopic and pharmacological properties, and has been widely used in many applications. Fisetin can be found in many plants including fruits and vegetables, For

example, strawberries, apples, persimmons, grapes, onions and cucumbers where they contain fisetin at concentration in the range of 2-160 µg/g. The highest concentration of fisetin was found in strawberries (160 µg/g wet food) followed by apples (26.9 µg/g wet food) and persimmons (10.5 µg/g wet food). Moreover, other dietary sources of fisetin and their concentration which were measured in freeze-dried fruits and vegetables after hydrolysis of the parent glycosides are shown in Figure 3 (Khan, Syed, Ahmad, & Mukhtar, 2013). Although fisetin has been used in many applications, the uses of fisetin still showed some limitations because of its poor aqueous solubility (<1 mg/ml) (Sowa, Ślepokura, & Matczak-Jon, 2014). On the other hand, it can be dissolved well in organic solvents such as ethanol (to 10 mM) and DMSO (100 mM). Other physical and chemical properties of fisetin are showed in the Table 1.

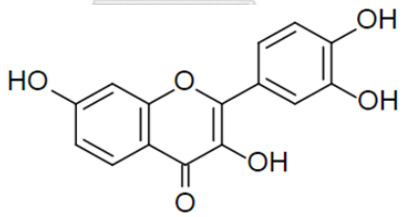


Figure 2 Structure of fisetin.

Strawberry  160 µg/g	Apple  26.9 µg/g	Persimmon  10.6 µg/g
Lotus root  5.8 µg/g	Onion  4.8 µg/g	Grape  3.9 µg/g
Kiwi  2.0 µg/g	Peach  0.6 µg/g	Cucumber  0.1 µg/g

Figure 3 Dietary sources of fisetin (Khan et al., 2013).

Table 1 Physical and chemical properties of fisetin.

Properties	Characteristics
Synonyms	Natural brown 1 5-Deoxyquercetin 3, 3', 4', 7-Tetrahydroxyflavone
Formula	$C_{15}H_{10}O_6$
Molecular weight	286.24 g/mol
Melting point	330ºC (626ºF; 603 K)
Density	1.688 g/ml
Solubility	DMSO to 100 mM In ethanol to 10 mM In water to < 3.5 mM
Solubility (color)	Yellow to green
Solubility (turbidity) 5 mg/ml of 95% ethanol	Clear to slightly hazy
Toxicity	No data available

1.2.1 Biological activities of fisetin

Antioxidant activity

The flavonol fisetin has been reported to display high antioxidant properties in membrane, suggesting its potential as a therapeutic agent against various diseases such as atherosclerosis, ischemia, neural degeneration, cardiovascular ailments including cancer, tumors, allergies, AIDS and etc (Chaudhuri, Banerjee, Basu, Sengupta, & Sengupta, 2007; Sengupta, Banerjee, & Sengupta, 2005). Reactive oxygen species (ROS), such as oxygen free radicals possibly damage almost all types of important biolomolecules, including lipids, amino acids, carbohydrates and nucleic acids via peroxidation or mutation. Antioxidant molecules help to maintain a balance between the formation and elimination of ROS in the body. Two possible mechanisms of scavenging free radicals by flavonoids were proposed. (i) Rapid donation of the H atom to a radical forming a new radical which is more stable than the previous one [ROH + $HO^{\bullet} \rightarrow RO^{\bullet} + H_2O$] and (ii) the chain breaking mechanism by which the antioxidant donates an electron to the free radical (such as lipid), leading to indirect H-abstraction [$ROH + HO^{\bullet} \rightarrow ROH^{+\bullet} + HO^{-} \rightarrow RO^{\bullet} + H_2O$]. Due to specific structural features, fisetin is capable of effective scavenging of free radicals (Marković, Mentus, & Dimitrić Marković, 2009). Antioxidant activities of fisetin are mainly resulted from its hydroxyl groups at C-3, C-3′, C-4′ and C-7 and carbonyl group at C-4. The presence of double bond between C-2 and C-3 conjugated with the 4-oxo group also enables higher electron delocalization.

Anticancer activity

Even today conventional therapeutic and surgical methods of treatment of cancers have failed to check the increased incidence of most cancers. It is therefore very urgent to establish sound action plans for reducing or preventing the incidence of cancers. It is now fully established that consumption of an appropriate diet rich in polyphenols can prevent development of cancer. Polyphenols constitute an important group of chemopreventive agents by virtue of their ability to inhibit the different stages of cancer development i.e., tumor initiation, promotion and progression, which could be achieved through inactivation of carcinogen or inhibition of its formation, growth inhibition, cell cycle arrest, induction of apoptosis and differentiation, angiogenesis inhibition, and antioxidation or combination of these effects (Abubakar, Abdullah, Sulaiman, & Suen, 2012).

1.2.1 Bioavailability of fisetin

Due to the fact that fisetin possesses low water solubility and poor bioavailability, its *in vivo* uptake is a challenge of nowadays research. In order to improve bioavailability of fisetin and increase its antitumor activity, Seguin *et al.* prepared liposomal capsule of fisetin (Seguin et al., 2013). Owing to amphiphilic surface of phospholipids, liposomes form an aqueous core surrounded by a lipid bilayer. This structure allows it to transport both hydrophilic and lipophilic species, which enables this pharmaceutical preparation to be used as drug carrier of several drug classes including antibiotics, antifungals as well as anticancer agents in many clinical studies (Allen & Cullis, 2004). The bioavailability of liposomal fisetin after administration was evaluated for the anticancer effects in tumor bearing mice (Seguin et al., 2013). Experimental results demonstrated that these formulations can

significantly increase fisetin bioavailability after administration and improve its anticancer activity in Lewis lung carcinoma bearing mice.

Jung and co-workers studied the complexation of barely soluble fisetin with cationic dimer of cyclosophoraose (Cys), an exopolysaccharide (such as cyclic β-(1, 2)-glucan) to improve the solubility of fisetin (Jeong et al., 2013). It was found that Cys possesses the ability to form complexes with drugs or hydrophobic molecules, causing the increase of water solubility (E. Cho, Jeong, Choi, & Jung, 2016). Moreover, the enhanced bioavailability of the complex was evaluated by the measurement of cytotoxic effect of fisetin and the fisetin-Cys dimer complex at concentrations ranging from 0 to 100 μM toward HeLa cells. The results of this assay indicated that the Cys dimer was non-toxic to HeLa cells even upto 1000 μM and the fisetin-Cys dimer complex was found to exhibit higher cytotoxicity than free fisetin at concentrations more than 40 μM. Thus, it was concluded that Cys dimer not only enhances the solubility of the drug fisetin but also increases its bioavailability.

The formation of inclusion complex with cyclodextrin has been used to increase the bioavailability of many drugs (Figure 4). Due to the fact that cyclodextrin is a molecule that contain a hydrophilic outer surface and a lipophilic inner cavity, it can accommodate a wide variety of lipophilic drugs. Zhang and co-workers evaluated the cytotoxicity of free fisetin and fisetin contained inclusion complexes against Hela and MCF-7 cells using the MTT assay (Zhang et al., 2015). It was found that fisetin/CDs complexes displayed better cytotoxicity than free fisetin against Hela and MCF-7 cells, indicating that the increase anticancer activity of fiseitn was completed by inclusion complexes formation.

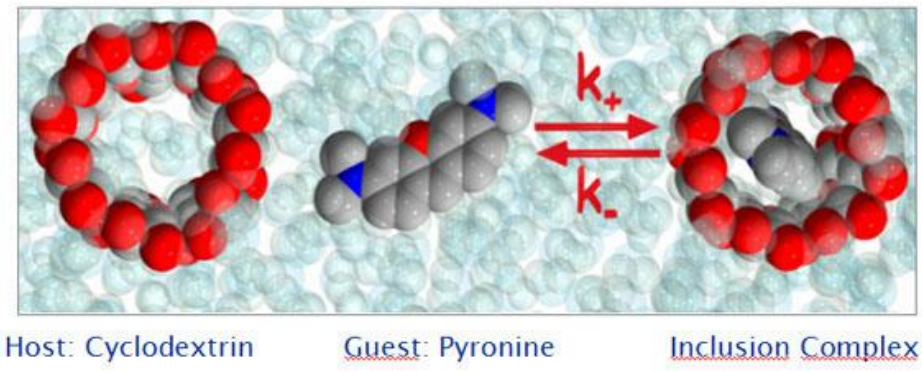


Figure 4 Formation of inclusion complex between cyclodextrin (host) and guest molecules.

1.3 Glycoside and tranglycosylation

Glycosides are the compound that comprised of two independent parts; the aglycone and the glycone (saccharide) parts. In a glycoside, the saccharide part (X) is linked to the aglycone (A) part via glycosidic linkage as shown below:

$$A\text{-}OH + X\text{-}OH \leftrightarrow A\text{-}O\text{-}X + H_2O$$

The glycosidic bond is mostly unstable and susceptible to hydrolysis. Accordingly, the types of glycosidic linkages are classified as:

- O-glycosides (linkage via an oxygen)
- C-glycosides (linkage via a carbon); this type of linkage is resistant to hydrolysis.
- S-glycosides (linkage via a sulfur; aglycone must have —SH group) present in glucosinolates (thioglycosides).
- N-glycosides (linkage via a nitrogen; aglycone must have —NH group) present in nucleosides.

The glycone is most frequently found as monosaccharides and glucose (called a glucoside) is commonly found. Moreover, other glycones, such as L-rhamnose, L-fructose, L-arabinose, and D-xylose, were found. The sugar unit can also be a di-, tri-, or tetrasaccharide. The configuration of the anomeric carbon of the glycone can happen as α or β diastereoisomer. The number of saccharide units/chains attached to the algycone might be one (monodesmoside), two (bidesmoside), or three (tridesmoside), which are commonly seen in saponin glycosides. The main groups of glycosides are terpene, sterol, phenol, or phenylopropanoid glycosides. Furthermore, glycosides also

may be classified (due to the nature of the attached sugar) as galactosides, apiosides, rhamnosides, xylosides, rutinosides and etc.

The overall effect of glycoside biological activity is dependent on their two components; the aglycone portion primarily influences the therapeutic direction while the saccharide part (sugar chain/s) increases water solubility and bioavailability. The glycosidic linkage (normally β-linked in active plant glycosides) is resistant to human digestive enzymes and hence glycosides are often poorly absorbed from the digestive tract. They usually travel to the distal ileum or large bowel and the aglycone is formed by the microbial activity which is less polar and can be absorbed into the bloodstream (Bartnik & Facey, 2017).

Transglycosylation is the reaction that involves in the transfer of a sugar residue from one glycoside to another. Enzymatic tranglycosylations have the advantage over chemical reaction because of their specific and mild reaction condition. Transglycosylation is normally used to increase the solubility of many organic compounds including flavonoids. Various enzymes have been reported for flavonoid glycoside synthesis. The examples of enzymatic synthesis of flavonoid- and stilbenoid glycosides are listed in Table 2. Glycosylatransferase is a group of enzyme that catalyzes the transfer of sugar moieties from an activated sugar (also known as the "glycosyl donor") to a glycosyl acceptor molecule, the nucleophile of which can be oxygen-, carbon-, nitrogen-, or sulfur-based (Williams & Thorson, 2009). The mechanism of glycosyltransferase is involved in the formation of glycosyl-enzyme intermediate, and then the nucleophilic substitution. The mechanism of glycosylatransferase can be divided into 2 groups: retaining and inverting. Inverting mechanism requires a single nucleophilic attack from the accepting atom to invert

stereochemistry (Figure 5A) while retaining mechanism has been a matter of debate, but there exists strong evidence against a double displacement mechanism (which would cause two inversions for a net retention of stereochemistry) or a dissociative mechanism (Figure 5B) (Vasella, Davies, & Böhm, 2002). Many of glycosyltransferases have been found. The different glycosyltransferase might utilize different substrates and then produce different products. For instance, levansucrase (EC 2.4.1.10) is a fructosyltransferase which catalyzes the transfer of fructose from sucrose to an acceptor and the main product is levan (Lu, Lu, & Xiao, 2014). Inulosucrase (EC 2.4.1.9) is a sister of levansucrase but it prefers to synthesize inulin (Anwar, Kralj, van der Maarel, & Dijkhuizen, 2008). Dextransucrase also utilizes the sucrose as a glycosyl donor but it transfers the glucose moieties instead (Naessens, Cerdobbel, Soetaert, & Vandamme, 2005). Moreover, these enzymes also showed difference in acceptor specificity, and thus different enzymes can be used to synthesize a vast variety of glycosides.

Enzyme	Gene size (bp)	Strains	Acceptor/donor	Products	MW (kDa)	pH	Tem (°C)	Yield of product (mg/L) or conversion rate (%)	Synthetic methods
Dextransucrase		*Leuconostocmesenteroides* B-512FMCM	Kaempferol/sucrose	Kaempferol-3-*O*-β-D-nigeroside, kaempferol-3-*O*-β-D-isomaltoside		5.2	28	21.8 mg	In vitro
Dextransucrase	8,511	*Leuconostoc mesenteroides* B-1299CB4	Ampelopsi/sucrose	Ampelopsin-4′-*O*-α-D-glucopyranoside	313.3	5.2	28	3,400 mg	In vitro
Dextransucrase (LLDexT, 512FDexT, SMDexT)	4,503	*Leuconostoc lactis* EG001, *Leuconostoc mesenteroides*B-512F DexT, *Streptococcus* mutans DexT (SMDexT)	Puerarin/sucrose	α-D-glucosyl (1 → 6)-puerarin, α-D-isomaltosyl (1 → 6)-puerarin	165	5.0–5.2	28–30	14,500 mg	In vitro
α-Amylase	1,389	*Trichoderma viride* JCM22452	(+)-catechin and (−)-epigallocatechingallate (EGCG)/dextrin	s (+)-catechin 5-*O*-α-D-glucopyranoside, (+)-catechin 5-α-D-maltoside, (+)-catechin 4′-*O*-α-D-maltoside, EGCG 5-*O*-α-D-glucopyranoside, EGCG 7-*O*-α-D-maltoside		5.0	45–55		In vitro
UGT78D1	1,362	*Arabidopsis thaliana*	Quercetin, kaempferol/TDP-rhamnose	Kaempferol 3-*O*-rhamnoside (1), quercetin 3-*O*-rhamnoside (2)			30	150 mg for (2) and 200 mg for (1)	In vivo
YjiC	1,179	*Bacillus lincheniformis*	Phloretin/UDP-glucose	Phloretin 4′,4-*O*-diglucoside (1), phloretin 4,6′-*O*-diglucoside (2) and phloretin 2′,4′,4-*O*-triglucoside (3)	43.67	7.5	25	23.3 %	In vitro & in vivo
OleD	1.248	*Streptomyces antibioticus*	Daidzein, flavopiridol, resveratrol, 10-hydroxycamptothecin, 2-methoxyestradiol/UDP-glucose	Flavopiridol-(1), 2-methoxyestradiol-(2), resveratrol-(3), daidzein-(4), 10-hydroxycamptothecin (5) glucosides		8.0	25	4.9 mg of (1); 1.2–2.2 mg of (3); 6 mg of (5); 0.1–4 mg of (2); 1–5 mg of (4) in died form	In vitro

Table 2 Enzymatic synthesis of flavonoid-and stilbenoid glycosides (Thuan & Sohng, 2013).

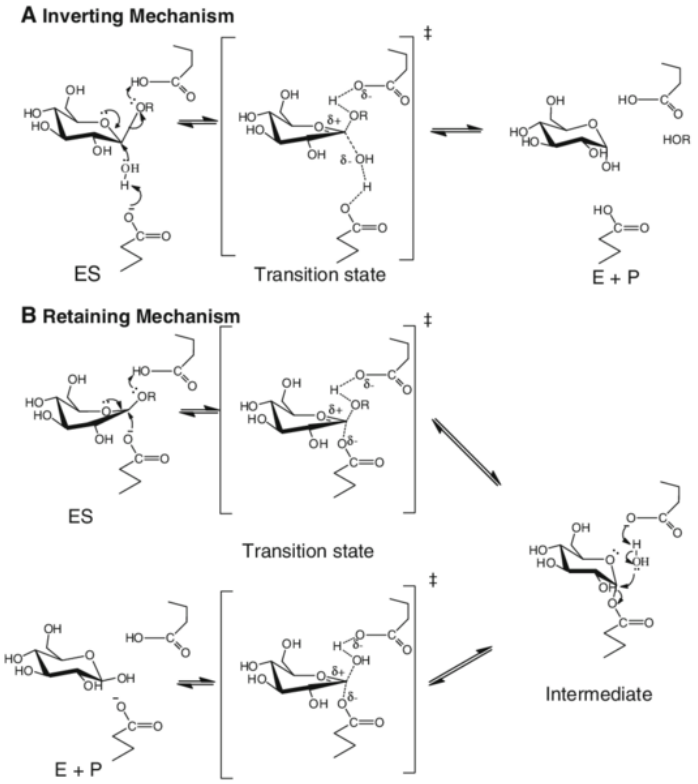


Figure 5 Mechanism of glycosyltransferase.

1.4 Cyclodextrin glycosyltransferase (CGTase)

1.4.1 General information of CGTase

Cyclodextrin glycosyltransferase or cyclodextrin gluconotransferase, (CGTase, E.C.2.1.4.19) is a member of the α-amylase family. α-Amylases (E.C.3.2.1.1) are enzymes that catalyze the hydrolysis of the internal α-1,4-glycosidic linkages in starch, converting starch into low-molecular-weight products such as glucose, maltose, and maltotriose units (Mikawlrawng, 2016).

CGTase is typically 75 kDa proteins consisting of five domains, labeled A to E (Figure 6) (Uitdehaag, van der Veen, Dijkhuizen, & Dijkstra, 2002). Domain A is the catalytic $(\alpha/\beta)_8$ domain which CGTase has in common with other α-amylase family members. Domain B is an extended loop region inserted after β-strand 3 of domain A. It contributes to substrate binding by provide several amino acid chains alongside a long groove on the surface of the enzyme that interact with the substrate (Uitdehaag et al., 2002). Domains C and E have a -sheet structure, and are specialized in binding to raw starch granules (Penninga et al., 1996). The function of domain D is unknown (Kelly, Dijkhuizen, & Leemhuis, 2009).

1.4.2 The reaction and product of CGTase

CGTase is an extracellular enzyme that can catalyze four different reactions: hydrolysis, disproportionation, coupling and cyclization (Figure 7). Also, CGTase has a weak hydrolyzing activity which transfers the covalently bound oligosaccharide to a water molecule. Disproportionation is the transfer of the covalently bound oligosaccharide to a second sugar molecule. Coupling reaction is the breakdown of cyclodextrin (Kelly et al., 2009). In addition, CGTase has unique ability to form circular α(1→4)-linked oligoglucosides as cyclodextrins. (Uitdehaag, Kalk, van der Veen,

Dijkhuizen, & Dijkstra, 1999). Cyclodextrins are cyclic oligosaccharides mainly consisting of 6, 7, or 8 glucose residues (α-, β-, or γ-cyclodextrin, respectively) (Figure 8a). The glucose residues in the cyclodextrin ring are arranged in such manner that the secondary hydroxyl-groups (C2-C3) are located on one edge of the ring and the primary hydroxyl-groups (C6) on the other edge, resulting in torus shaped molecules (Figure 8b). The properties of cyclodextrin is shown in Table 3 (van der Veen, Uitdehaag, Dijkstra, & Dijkhuizen, 2000).

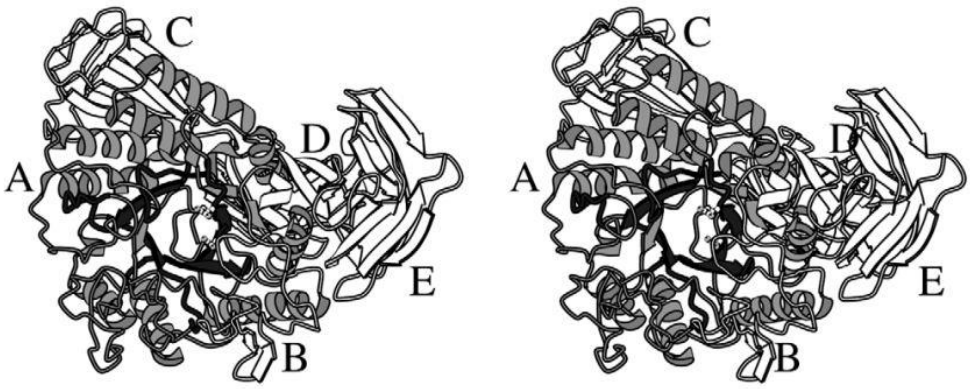


Figure 6 Stereo-view of the structure of CGTase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form (Uitdehaag et al., 2002).

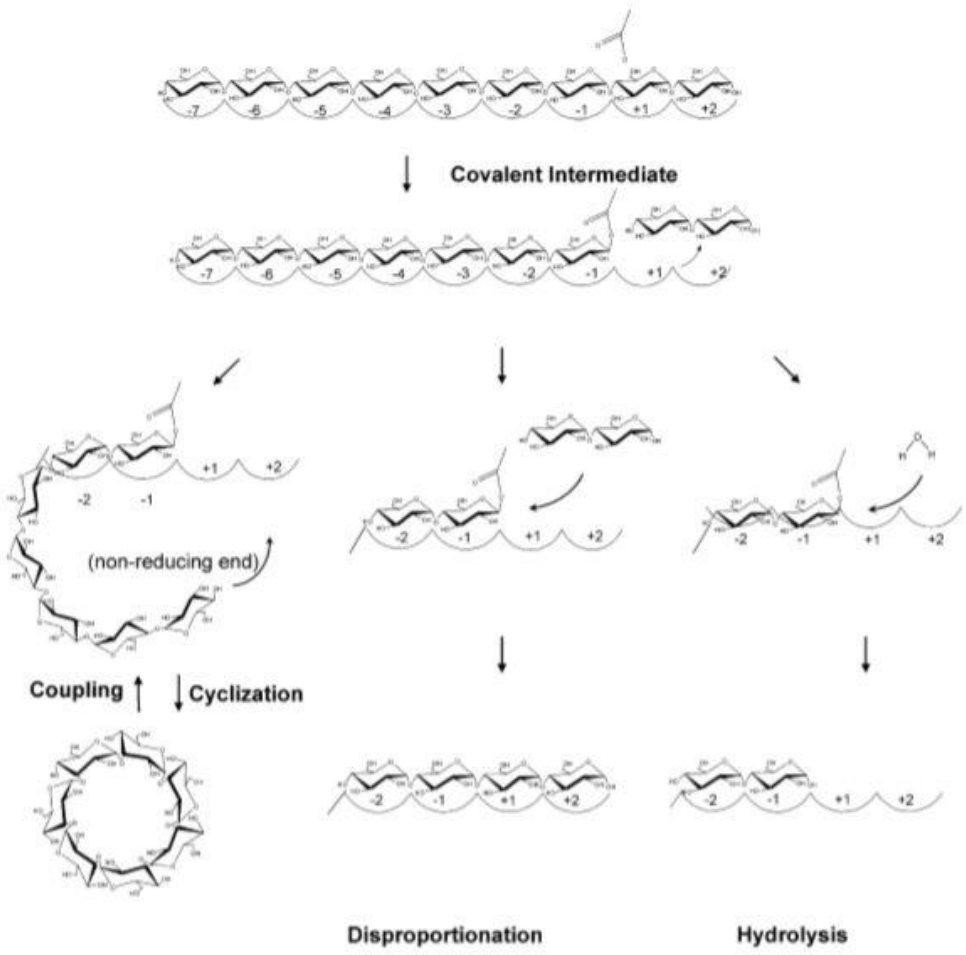


Figure 7 Schematic representation of the reactions catalyzed by CGTase. A linear glucan chain binds to the substrate binding subsites of CGTase followed by bond cleavage to yield a covalent glycosyl–enzyme intermediate. The nature of the acceptor molecule in the second step of the reaction, to which the covalently bound oligosaccharide is transferred, determines the enzyme reaction specificity (Kelly et al., 2009).

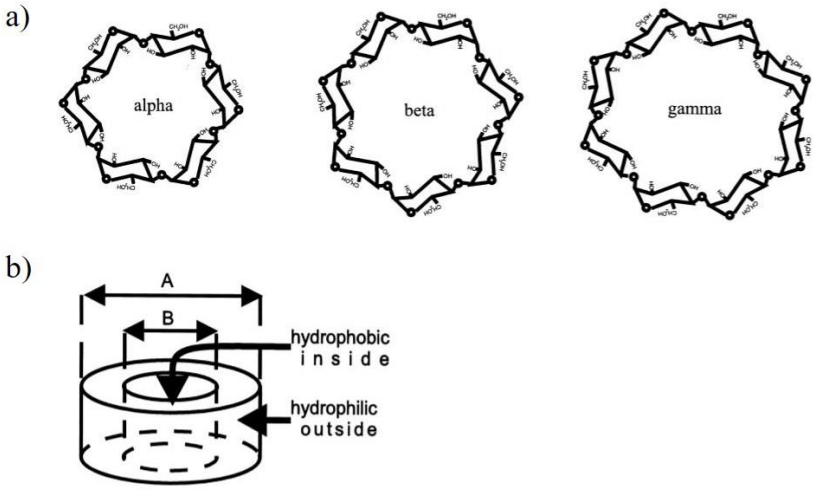


Figure 8 Structure of cyclodextrins. (a) α-, β-, and γ-cyclodextrin; (b) three-dimensional form and properties of cyclodextrin (for size of A and B, see Table 3).

Table 3 The properties of cyclodextrin (van der Veen et al., 2000).

Properties	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25ºC (%w/w)	14.5	1.85	23.2
Outer diameter (A) (Å)	14.6	15.4	17.5
Inner diameter (B) (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	174	262	427

1.4.3 Applications of CGTase

Normally, CGTase is employed for the production of cyclodextrin from glycosyl donor via cyclization reaction. Although starch is generally used as glycosyl donor, other saccharides such as dextrins and oligosaccharides can also be used. In addition, another application of CGTase is that it can synthesize the derivatives of some organic compounds in order to improve some properties, such as solubility and stability, via disproportionation reaction. Many scientific researchers have explored the transglycosylation reactions of CGTase.

Kometani and his co-workers studied the enzymatic synthesis of hesperidin mono-glucoside and oligoglucosides using starch as a donor. It was found that the water solubility of both hesperidin mono- and diglucosides increased about 300 times higher than that of starting hesperidin (Kometani, Terada, Nishimura, Takii, & Okada, 1994). After that, Kometani *et al*. also synthesized naringin monoglucoside by using CGTase and showed that the solubility of naringin monoglucoside increased at least 1,000 times higher than that of naringin (Kometani, Nishimura, Nakae, Takii, & Okada, 1996). Thanadolsathien synthesized the novel vitamin E (Trolox) glycosides by two-step enzymatic synthesis of α-glucosidase and CGTase. It was found that the water solubility of glycoside derivative was improved by $7x10^4$ times (Thanadolsathien, 2007). Epicatechin glucosides (EC) were obtained through the transglycosylation reaction of CGTase, using β-CD as a glycosyl donor. These glycoside derivatives showed higher water solubility and stability towards UV radiation (Aramsangtienchai, Chavasiri, Ito, & Pongsawasdi, 2011a). Li *et al*. (2013) reported the production of stevioside derivative to improve the edulcorant quality by lower substitution using corn starch hydrolysate and CGTase. The product consisted of mono- and di-glucosylated stevioside and the

highest stevioside conversion reached to 77.11% (Li, Li, Xiao, & Xia, 2013). In 2011, Torres and co-worker reported the glycosylation at 3-OH, 4‟-OH or both of resveratrol by CGTase from *Thermoanaerobacter* using starch as donor. It was found that the water solubility was improved by 65 times greater than that of the unglycosylated one (Pamela et al., 2011). Moreover, the synthesis of resveratrol glycosides was also achieved by CGTase from *Paenibacillus* sp. RB01 (Anurutphan & Prousoontorn, 2015). The synthesis of fisetin glucosides and rhamnosides was previously reported by Parajuli *et al.* (2015) using nucleotide diphosphate (NDP)-sugar biosynthetic pathway of engineered *E.coli* (P. Parajuli, R. P. Pandey, N. T. H. Trang, A. K. Chaudhary, & J. K. Sohng, 2015). However, to the best our knowledge, the synthesis of fisetin glycoside by CGTase has not yet been reported.

The objectives of this research were:

I) To study the ability of CGTase from *Paenibacillus* sp. RB01 in transglycosylation reaction from glycosyl donor to fisetin.

II) To determine the optimum conditions for the fisetin glycoside production.

III) To purify and elucidate the structure of fisetin glycoside products.

CHAPTER II
MATERIALS AND METHODS

2.1 Equipments

Autoclave	: MLS-3020, Sanyo electric Co., Ltd., Japan
Autopipette	: Pipetman, Gilson, France
Balance	: AB204-S, Mettler Toledo, Switzerland
Balance	: PB303-S, Mettler Toledo, Switzerland
Centrifuge, refrigerated	: Avanti™ J-301, Beckman Instrument Inc., USA
CentriVap Concentrator	: CentriVap Concentrator, Labconco Corporation, USA
Electrophoresis unit	: Mini-PROTEAN® 3 Electrophoresis, Bio-Rad, USA
Filter paper	: No.1, Whatman®, England
Freeze-dryer	: Labconco corporation, USA
High Performance Liquid Chromatography	: Shimadzu, Japan
Incubator	: Gallenkamp, England
Incubator shaker	: New Brunswick Innova 4000, USA
Laminar flow	: SCV-4A1, Esco, USA

Magnetic stirrer and heater	: Thermolyne Corporation, USA
Mass spectrometry	: MicrOTOF, Bruker Daltonics Inc., USA
Membrane filter	: Nylon, 0.45 µm, National Scientific Company, USA
Oven	: Contherm, New Zealand
pH meter	: Mettler Toledo, Switzerland
Power supply	: PowerPac BasicTM , Bio-Rad, USA
Rotary evaporator	: Büchi, Switzerland
Separating funnel	: Schott duran, Germany
Sonicator bath	: Wiseclan®, India
Spectrophotometer	: G10S UV-Vis Spectrophotometer, Thermo Scientific™,USA
Tandem mass spectrometry	: Waters, Germany
Thin Layer Chromatography plates	: Silica gel 60 F_{245}, Merck, Germany
Ultrafiltration membrane (30,000 MWCO)	: Merck, Germany
UV box	:Chemical express co., LTD., Thailand
Vortex	: GS60E, VORTEX-GENIE®, Scientific Industries Inc., USA
Water bath	: Memmart , Germany

2.2 Chemicals

Absolute ethanol	: Merck, Germany
Acetone	: Carlo Erba Reagents, France
Acetonitrile	: RCI Labscan, USA
Acrylamide	: Merck, Germany
Agar	: Scharlau, Spain
Alpha-glucosidase	: Sigma, USA
Ammonium persulfate	: Pharmacia fine chemicals, Sweden
Aquacide II	: Calbiochem, USA and Sweden
Ascorbic acid	: Fluka, Switzerland
β-cyclodextrin	: Sigma, USA
Beef extract	: Biomark Laboratories, India
Bovine serum albumin	: Sigma, USA
Bromophenol blue	: Merck, Germany
1-Butanol	: Carlo Erba Reagents, France
Calcium chloride	: Scharlau, Spain
Chloroform	: Merck, Germany
Commercial grade corn starch	: Unilever, Thailand
Coomassie blue G-250, R-250	: Sigma, USA

Dichloromethane	: Merck, Germany
Dimethyl sulfoxide (DMSO)	: Sigma, USA
2,2-Diphenyl-1-picrylhydrazy	: Sigma, USA
di-Potassium hydrogen orthophosphate	: Univar, Australia
Ethyl acetate	: Carlo Erba Reagents, France
Ethylenediamine tetraacetic acid (ETDA)	: Univar, Australia
Fisetin	: Sigma, USA
Formic acid	: Merck, Germany
Glacial acetic acid	: Mallinckrodt Chemicals, Thailand
Glucoamylase	: Sigma, USA
Glucose	: Univar, Australia
Glycerol	: Merck, Germany
Glycine	: Sigma, USA
Hexane	: Carlo Erba Reagents, France
Hydrochloric acid	: Carlo Erba Reagents, France
Iodine	: Merck, Germany
Magnesium sulfate	: Scharlau, Spain
Maltose	: Laboratorios CONDA, Spain

Maltotriose	: Fluka, Switzerland
Maltoheptaoste	: Hayashibara Biochemical Laboratories Inc., Japan
Methanol	: Merck, Germany
Orcinol	: Sigma, USA
85% Orthophosphoric acid	: Merck, Germany
Peptone	: Scharlau, Spain
Phenolphthalein	: M&B Laboratory Chemicals, England
Potassium dihydrogen phosphate	: Univar, Australia
Potassium iodine	: Merck, Germany
2-Propanol	: Carlo Erba Reagents, France
Sodium acetate	: Univar, Australia
Sodium carbonate	: Univar, Australia
Sodium chloride	: Carlo Erba Reagents, France
Sodium dodecyl sulfate	: Sigma, USA
Sodium hydroxide	: Univar, Australia
Soluble starch	: Scharlau, Spain
Standard molecular weight marker protein	: GE Healthcare, England
Sulfuric acid, concentrated	: J.T.Baker, Thailand

TEMED (N,N,N‘,N‘-tetramethylene-ethylenediamine)	: Fluka, Switzerland
Trifluoroacetic acid	: Sigma, USA
Yeast extract	: Scharlau, Spain

2.3 Bacteria strain

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

2.4 Media preparation

2.4.1 Medium I

Medium I broth was prepared by using 0.5% (w/v) beef extract, 1.0% (w/v) peptone, 0.2% (w/v) NaCl, 0.2% (w/v) yeast extract and 1.0% (w/v) soluble starch. The solution was adjusted to pH 7.2 using 2 N NaOH. For solid medium, 1.5% (w/v) agar was added. The liquid and solid medium were sterilized at 121°C for 15 minutes using an autoclave.

2.4.2 Horikoshi’s medium

Horikoshi’s medium was used for CGTase expression of *Paenibacillus* sp. RB01. The medium was prepared by the method that previously has been reported by (Horikoshi, 1971) with slight modification. The medium ingredients, namely 1.0% (w/v) soluble starch, 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4$ and 0.5% (w/v) peptone, were dissolved in DI water. After that, the solution was sterilized at 121°C for 15 minutes by an autoclave. Sodium carbonate solution

which was sterilized separately was then added to reach the final concentration of 0.75% (w/v). The pH of derived Horikoshi's medium was 10.1 – 10.2.

2.5 Bacteria cultivation and CGTase expression

2.5.1 Starter inoculum

Paenibacillus sp. RB01 was streaked on solid medium I and incubated at 37°C for 18 hours. One colony was inoculated into liquid medium I. The starter flask was shaken at 37°C, 250 rpm in orbital shaker until OD_{660} reached 0.3-0.5.

2.5.2 Enzyme production

Starter inoculum of 1.0% (v/v) was transferred into Horikoshi's medium and cultivated at 37°C, 250 rpm in shaking incubator. After incubation for 72 hours, the cells were separated by centrifugation at 5,000 rpm at 4°C for 15 minutes. Supernatant, called crude enzyme, was collected and kept at 4°C until further used.

2.6 Partial purification of CGTase

The starch adsorption method (Kato & Horikoshi, 1984) was conducted for CGTase purification with some modifications. Commercial grade corn starch, which was prior dried at 121°C for 30 minutes, was slowly sprinkled into crude CGTase solution to reach a final concentration of 5% (w/v). The starch suspension was stirred at 4°C overnight. The Starch cake was harvested by centrifugation at 8,000 rpm, at 4°C for 30 minutes. The obtained starch cake was then washed with cool TB1 buffer (10 mM Tris-HCl pH 8.5 containing 10 mM $CaCl_2$) until the solution was clear. Then, CGTase was eluted from starch cake by stirring it in TB2 buffer (0.2 M maltose in TB1 buffer) at 4°C overnight. The ratio of TB2 buffer per crude enzyme used was 62.5 ml/1 L. The eluent was collected by centrifugation at 8,000 rpm at 4°C for 30 minutes. The

supernatant, called partial purified enzyme, was dialyzed against 50 mM phosphate buffer (pH 6.0) at 4°C to remove remaining maltose. The partial purified enzyme was concentrated by ultrafiltration membrane (30,000 molecular weight cut-off of the membrane was).

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

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

The separating gel was 7.5% (w/v) acrylamide while the stacking gel used here was 5.0% (w/v) acrylamide. The protein samples were mixed with sample buffer before loaded into the wells. The running buffer was Tris-glycine buffer pH 8.0. The electrophoresis was performed at constant current of 25 mA per slab, at room temperature on Mini-Gel electrophoresis unit from cathode towards anode. As the dye front reached the bottom of the gel, the gel was cut into 2 parts for protein staining and activity staining.

2.7.2 Coomassie blue staining

A half gel from native-gel was exposed to protein staining by Coomassie blue staining solution which consisted of 0.1% (w/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid. It was incubated at room temperature for 1 hour. After that the gel was destained with destaining solution (10% (v/v) methanol and 10% (v/v) acetic acid) until the gel background was clear.

2.7.3 Dextrinizing activity staining

The other half of the native gel was soaked with 2.0% (w/v) soluble starch in 200 mM phosphate buffer (pH 6.0) at 40°C for 10 minutes. It was rinsed several times with distilled water to remove the remaining substrate on the gel. Iodine staining

reagent (0.2% (w/v) I_2 in 2.0% (w/v) KI) was then added for color development at room temperature. The clear zone on the dark background of the gel was starch degrading activity of the protein.

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

The denaturing gel electrophoresis was composed of 0.1% (w/v) SDS in the separating gel (7.5% (w/v) acrylamide) and the stacking gel (5.0% (w/v) acrylamide). The electrode buffer was Tris-glycine buffer pH 8.0, containing 0.1% (w/v) SDS. The samples were treated with sample buffer and boiled for 5 minutes prior to gel application. The electrophoresis was performed at constant current of 25 mA per slab, at room temperature on Mini-Gel electrophoresis unit from cathode towards anode. After that, the gel was stained with Coomassie blue staining solution for visualization of separated protein. The molecular weight marker protein includes carbonic anhydrase (30 kDa), ovalbumin (45 kDa), albumin (66 kDa) and phosphorylase b (97 kDa).

2.8 Enzyme activity assay

2.8.1 Dextrinizing activity

The dextrinizing activity (also called starch hydrolytic activity) was assayed by using iodine as indicator. The method was modified from (Fuwa, 1954) in which 50 µL of enzyme sample was incubated with 150 µL of substrate solution (0.2% (w/v) soluble starch in 0.2 M phosphate buffer pH 6.0) at 40ºC for 10 minutes. Then, the reaction was terminated by adding 2 mL of 0.2 M HCl, followed by addition of 250 µL iodine reagent (0.02% (w/v) I_2 in 0.2% (w/v) KI). The solution was adjusted to a final volume of 5 mL with distilled water. Finally, the absorbance at 600 nm was read by a spectrophotometer. For control reaction, HCl solution was added prior to the enzyme

sample. One unit of enzyme was defined as the amount of enzyme which reduced 10% of the blue color of starch-iodine complex per minute under the described conditions.

2.8.2 Cyclization activity

The method measures the β-cyclodextrin synthesized from CGTase by using phenolphthalein (indicator). In addition, the calibration curve of β-cyclodextrin concentration was used to calculate the synthesized β-cyclodextrin concentration. First, 250 µL of enzyme sample was incubated with 1.25 mL of 6% (w/v) soluble starch at 70 ºC for 30 minutes. Next, the sample reaction was boiled for 10 minutes to stop reaction. Then, 500 µL of diluted sample reaction was mixed with 2 mL of phenolphthalein solution and left at room temperature for 15 minutes. The absorbance was then measured at 550 nm by a spectrophotometer. One unit of enzyme was defined as the amount of β-cyclodextrin per minute under the above condition.

2.9 Protein determination

The protein concentration of enzyme sample was measured by (Bradford, 1976). Bovine serum albumin (BSA) was used as protein standard. One milliliter of Bradford's reagent was added into 0.1 mL protein sample or standard BSA solution. The solution was mixed by vortex and then kept in dark for 10 minutes. After that, the reaction mixture was measured at 595 nm using spectrophotometer. Bradford's reagent was prepared by dissolving 100 mg Coomassie blue G-250 in 50 mL of absolute ethanol and 100 mL of 85% H_3PO_4, and then adjusted to final volume of 1 L distilled water. The reagent was filtered with whatman@ No.1 filter paper before used.

2.10 Effect of reaction condition on fisetin glycoside synthesis

To obtain the highest yield of fisetin glycosides, the effect of reaction condition (including DMSO, fisetin, β-CD, enzyme concentration and incubation time) on fisetin glycosides synthesis had to be explored. The amount of fisetin and glycoside products was monitored using TLC and HPLC. The product yield and fisetin conversion were used as parameters to optimize the reaction condition of fisetin glycosides synthesis. Fisetin conversion (%) was calculated as follow:

$$\text{Fisetin conversion (\%)} = [(A_0 - A_t)/ A_0] \times 100$$

Where A_0 is the peak area of fisetin at reaction time = 0 minute and A_t is the peak area of fisetin after 24-hour incubation.

2.10.1 Effect of co-organic solvent on CGTase stability

Acetone and DMSO were selected as co-organic solvent because they can dissolve fisitin and they do not act as acceptors in this study. CGTase of 900 U/ml was incubated in solution containing various concentration of organic solvent (0 - 50%(v/v)) and 50 mM phosphate buffer (pH 6.0) at 40ºC for 24 hours, After that, the remaining dextrinizing activity of each enzyme samples was measured as the method described above.

2.10.2 Effect of DMSO concentration on the synthesis of fisetin glycosides

To investigate a suitable DMSO concentration used, fisetin glycosides were synthesized by incubating 1% (w/v) fisetin, 1% (w/v) soluble starch and 1000 U/ml of CGTase in 50 mM phosphate buffer (pH 6.0) containing 10-50% (v/v) DMSO at 40ºC in static condition. After 24-hour incubation, the reaction was terminated by boiling for 10 minutes and the glycoside products were analyzed by TLC and HPLC.

2.10.3 Donor specificity

In order to determine an appropriate glycosyl donor used for fisetin glycoside synthesis, three candidate glycosyl donors, namely soluble starch, β-cyclodextrin and maltoheptaose, were considered. The reaction was conducted by incubating 1% (w/v) fisetin, 1000 U/ml of CGTase and 1% (w/v) glycosyl donor in 50 mM phosphate buffer, pH 6.0 containing appropriate amount of DMSO. After incubation for 24 hours, the reaction was boiled for 10 minutes to stop reaction and the products were analyzed by TLC.

2.10.4 Effect of fisetin concentration on glycoside products

Thousand units per milliliter of CGTase and 1% (w/v) of appropriate donor were incubated with fisetin to reach final concentration of 0.05 - 1% (w/v) in 50 mM phosphate buffer (pH 6.0) containing appropriate concentration of DMSO. After incubation at 40ºC for 24 hours, the reaction was boiled for 10 minutes. Then, the obtained glycoside products were analyzed by TLC and HPLC.

2.10.5 Effect of glycosyl donor concentration on glycoside products

To study the effect of donor concentration, 0.05 – 1% (w/v) of glycosyl donor was incubated with 1000 U/ml CGTase and appropriate fisetin concentration. The reactions were conducted in 50 mM phosphate buffer (pH 6.0) containing appropriate concentration of DMSO at 40ºC for 24 hours. After termination of reaction by 10 minutes boiling, the derived glycosides products were analyzed by TLC and HPLC.

2.10.6 Effect of CGTase concentration on glycoside products

In order to determine the optimum CGTase concentration for fisetin glycoside synthesis, CGTase at final concentration of 0 - 1200 U/ml were incubated with

appropriate glycosyl donor and fisetin concentration in 50 mM phosphate buffer (pH 6.0) containing DMSO at 40ºC for 24 hours. Then, the glycoside products were analyzed by TLC and HPLC.

2.10.7 Effect of incubation time on glycoside products

To find the optimum incubation time, fisetin glycosides was synthesized using appropriate concentration of fisetin, glycosyl donor and CGTase as identified above. The reactions were incubated in 50 mM phosphate buffer (pH 6.0) containing appropriate concentration of DMSO at 40ºC and the reaction mixture was taken at time interval from 0-24 hours for product analysis by TLC and HPLC.

2.11 Quantitative and semi-quantitative analysis of fisetin glycoside products

2.11.1 Thin Layer Chromatography (TLC)

The transglycosylation products were preliminary analyzed by using Thin Layer Chromatography (TLC). The reaction mixture was loaded on silica gel 60 F_{254} aluminium sheets (10 cm in height). The glycoside products were separated by TLC using 1-butanol: 2-propanol: water (10: 5: 1) or ethyl acetate: methanol: water: toluene 10: 1.5: 1.3: 0.2 (v: v: v: v) as mobile phase. The product spots were detected by using UV lamp (360 nm) or by spraying a solution (27 ml of ethanol, 10 ml of conc. H_2SO_4, 8 ml of water and 0.1 g of orcinol) prior to heating at 120ºC for 10 minutes. Semi-quantitation of fisetin glycosides can be performed by measuring the spot intensities using Quantity One@ software (Bio-Rad).

2.11.2 High Performance Liquid Chromatography (HPLC)

Determination of fisetin glycosides were carried out using High Performance Liquid Chromatography (HPLC) equipped with photodiode array detector (PDA)

(Shimadzu, Japan). Fisetin glycoside products were separated by C18 column using the gradient between SolA: Acetonitrile and SolB: 0.1% (v/v) of trifluroacetic acid or formic acid as a mobile phase at a flow rate of 1mL/min for 25 minutes. The gradient program was described as follow: 20% SolA (0-4 min), 40% SolA (4-8 min), 100% SolA (8-14 min), 50% SolA (14-17 min) and 20% SolA (17-25 min). Fisetin and their glycosides were detected at 350 nm. External standard of fisetin was used for quantitation approach.

2.11.3 Determination of fisetin glycoside concentration using acidic hydrolysis and HPLC

The molar concentration of fisetin glycosides can be determined from the molar concentration of fisetin released. The acidic hydrolysis method was conducted according to (Hertog, Hollman, & Venema, 1992) with some modifications. The fisetin glycoside samples were hydrolyzed using 1.2 M HCl in 50% (v/v) methanol at 100 ºC for 10 minutes. After that, the reactions were neutralized by addition of 2 M Tris base solution in 50% (v/v) methanol. The hydrolytic products were then analyzed by HPLC using fisetin as external standard. For the control sample, acid and base were mixed prior to the addition of the samples.

2.12 Purification of fisetin glycosides

2.12.1 Purification of fisetin glycosides by preparative TLC

The transglycosylation reaction was performed under optimum condition and the products were purified using preparative TLC, silica gel 60 coated on glass plate (20 cm in height, 1 mm thickness). The samples were loaded on TLC plate and separated using solvent system containing ethyl acetate: methanol: water: toluene in the ratio of 100:15:13:2 (v: v: v: v). After that, TLC plate was dried by hair dryer. The

locations of glycoside products were then detected by UV lamp. Each expected product band was then scraped and extracted from silica 3 times with ethyl acetate using sonicator bath for 5 minutes. The solvent was then separated by filtration using whatman@ No.1 filter paper. After that, the solution was dried by rotary evaporator at pressure 240 bar, 40ºC. Finally, the dried products were dissolved in 40% (v/v) DMSO prior to HPLC analysis.

2.12.2 Purification of fisetin glycosides by organic solvent extraction

After the transglycosylation reaction was performed, the reaction mixture was diluted with distilled water until DMSO final concentration reached 2% (v/v). After that, the diluted sample was extracted with 2 volume of selected organic solvents, namely ethyl acetate, dichloromethane, 1-butanol, chloroform, butyl acetate, toluene and hexane by vigorous mixing using vortex. Aqueous and organic phases were separated by centrifugation at 5,000 g at 25ºC for 10 minutes. Each fraction was then dried by CentriVap Concentrator at 40ºC, and their composition was analyzed by HPLC.

2.12.3 Purification of fisetin glycosides by HPLC

The transglycosylation reactions that had been extracted by a suitable organic solvent were further purified by HPLC equipped with PDA. Each glycoside product was separated by C18 column using the gradient of acetonitrile and 0.1 % (v/v) formic acid solution. The expected product peaks were collected using fraction collector and the purified products were dried by freeze-dryer or CentriVap Concentrator at 40ºC.

2.13 Structural characterization of fisetin glycosides

2.13.1 Evaluation of the transglycosylation products using enzymatic analysis of glucoamylase and α-glucosidase

In order to be preliminary condition that the obtained glycoside products were fisetin glycosides produced by CGTase, The obtained transglycosylation products were investigated with glucoamylase and α-glucosidase. Glucoamylase from *Aspergillus niger* is inverting exo-action enzyme which plays a role to hydrolyze the glucose moieties from the non-reducing end of the glycoside product. On the other hands, α-glucosidase from *Saccharomyces cerevisiae* hydrolyzes the alpha glucose from non-reducing terminal and alpha linkage between glucose and receptor. For the first enzymatic reaction, the reaction mixture (containing fisetin glycosides) was incubated with final activity of 60 U/mL of glucoamylase in 50 mM acetate buffer (pH 6.0) at 40ºC for 4 hours. Then, the reaction was terminated by boiling for 10 minutes and dried by CentriVap Concentrator. For the second reaction, sixty unit per milliliter of α-glucosidase in final concentration was incubated with the reaction mixture in 50 mM acetate buffer (pH 6.0) at 40ºC for 16 hours. After that, the reaction was boiled for 5 minutes and dried by CentriVap Concentrator. For the reaction that uses both enzymes, the reaction mixture was firstly treated with 60 U/ml of glucoamylase in 50 mM acetate buffer (pH 6.0) at 40ºC for 4 hours followed by 60 U/mL of treatment with α-glucosidase in 50 mM acetate buffer (pH 6.0) at 40ºC for 16 hours. The obtained hydrolysis products were then dried by CentriVap Concentrator as described previously. The derived dried reaction mixtures were then dissolved in 40% (v/v) DMSO prior to TLC and HPLC analysis.

2.13.2 Mass spectrometry (MS)

In order to confirm the obtained products as fisetin glycosides, the molecular mass of crude glycosides was preliminary determined by MS. Electrospray ionization-time of flight mass spectrometry (ESI-MS) profile was performed by a microOTOF at Department of chemistry. Faculty of science, Mahidol University, Thailand. The compounds were ionized by electrospray ionization on positive-ion mode.

2.13.3 Tandem mass spectrometry (MS/MS)

The tentative structures of each fisetin glycoside was analyzed by MS/MS (TSQ Endura^{TM} triple Quadrupole Mass Spectrometer, ThermoFisher SCIENTIFIC). The compounds were ionized by electrospray ionization on negative-ion mode. Mass spectrum were used to determine the fisetin glycoside structure by analyzing the fragmentation pattern.

2.14 Chemical and biological properties of synthetic fisetin glycosides

2.14.1 Solubility of derived fisetin glycoside in water

To determine the solubility of fisetin glycosides, the evaporated dried samples were dissolved in exact volume of deionized water (50 µL). After that, the molar concentration of fisetin glycosides were determined by acid hydrolysis and HPLC as described above (section 2.11.3) using fisetin as external standard. For solubility of fisetin in water, the excess amount of fisetin were dissolved in water by vortex. Then, insoluble fisetin was removed by centrification at 12,000 g for 10 minutes, and filtered by 0.22 um syringe filter. Due to the fact that the concentration of water soluble fisetin is very low, the high sensitivity florescent spectroscopy was applied. The fluorescent signal of soluble fisetin was determined by microplate reader in fluorescent mode, using

excitation and emission wavelength of 370 nm and 470 nm, respectively. Various concentrations of fisetin solution were used as external standards.

2.14.2 Antioxidant activity

The antioxidant activities were determined using DPPH assay. DPPH was dissolved in methanol to reach final concentration of 0.015 mg/ml. one hundred microlitres of DPPH solution were added into 96 well plate. After that, the different concentration of samples solution, namely fisetin, purified glycoside product and ascorbic acid, were added and then mixed with former DPPH solution to reach the final volume of 250 µl. The plate was kept in the dark for 30 minutes before subjected to the absorbance measurement 520 nm using microplate reader. Blank for the reactions was the solvent that dissolved the samples (methanol or water). The radical scavenging activities (RSA) of each sample concentrations were calculated according to the formula as follow:

$$\% \text{ RSA} = [(A_B - A_A)/A_B] \times 100$$

Where % RSA = percentage inhibition,

A_B and A_A are the absorbance values of the blank sample and of the sample, respectively.

After that, the graph of % RSA versus sample concentrations were plotted. Linear regression analysis was carried out in order to calculate the effective concentration of sample required to scavenge DPPH radical by 50% (ED_{50}).

CHAPTER III
RESULTS

3.1 Expression and partial purification of CGTase

CGTase was successfully expressed by *Paenibacillus* sp. RB01 using Horokoshi's medium at 40ºC for 72 hours. Since CGTase is extracellular enzyme, it is easy to harvest by centrifugation, and crude CGTase in the supernatant can be obtained. The obtained crude CGTase displayed the dextrinizing activity approximately of 8223.92 U/ml culture. After that, CGTase was purified by starch adsorption technique as described in Section 2.6. Purification table of CGTase is shown in Table 4. The results showed that the yield of CGTase was 31% after purified by starch adsorption. Specific activity of purified CGTase was 5033 U/mg which was higher than that of the crude CGTase (146.21 U/mg) approximately 34 folds.

The purity of CGTase was then checked by polyacrylamide gel electrophoresis (PAGE) as described in Section 2.7. Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) (Figure 9) was performed to check the activity of the enzyme. It was found that the Coomassie straining bands of both crude purified CGTase were corresponded to those of dextrinizing activity straining, indicating that CGTase was successfully purified. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also performed to determine the molecular weight of derived CGTase. As shown in Figure 10, one thick band was observed and its molecular mass was found to be approximately 66 kDa which was consistent with the molecular mass of CGTase that was previously reported (Anurutphan & Prousoontorn, 2015). Although

other bands were also observed, the purity of the obtained CGTase was sufficed for the synthesis of fisetin glycosides.

Moreover, CGTase activity was confirmed by phenolphthalein method. Since CGTase has cyclization activity, it can synthesize β-cyclodextrin from starch. When the concentration of β-cyclodextrin increased, it can form inclusion complex with phenolphthalein, resulting in the reduction of pink color of solution. It was found that the purified protein was really CGTase because it showed cyclization activity of 27 U/ml.

Table 4 Purification table of CGTase using starch adsorption

Purification step	Total activity* (U)	Total protein (mg)	Specific activity* (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	80,327.2	549.4	146.21	1	100
Starch adsorption	24,671.76	4.90	5033.00	34	31

* Dextrinizing activity

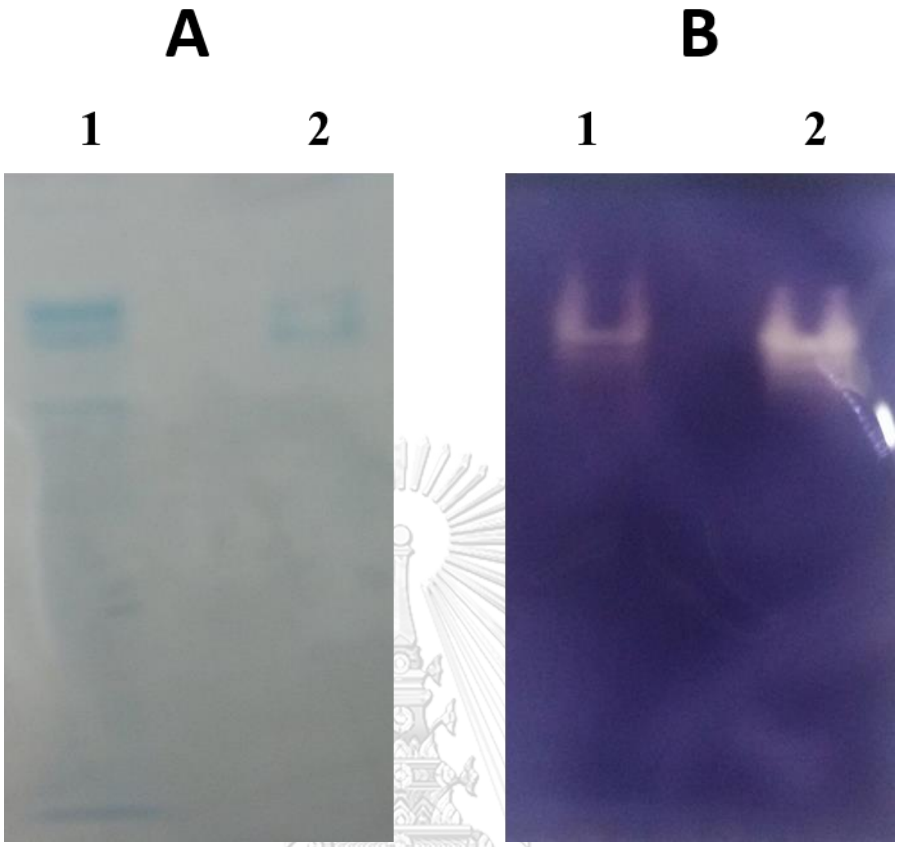


Figure 9 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) of *Paenibacillus* sp. RB01.

(A) Coomassie blue staining

Lane 1: Crude enzyme (2.5 µg)

Lane 2: Partial purified enzyme (0.06 µg)

(B) Dextrinizing activity staining

Lane 1: Crude enzyme (0.5 U)

Lane 2: Partial purified enzyme (0.5 U)

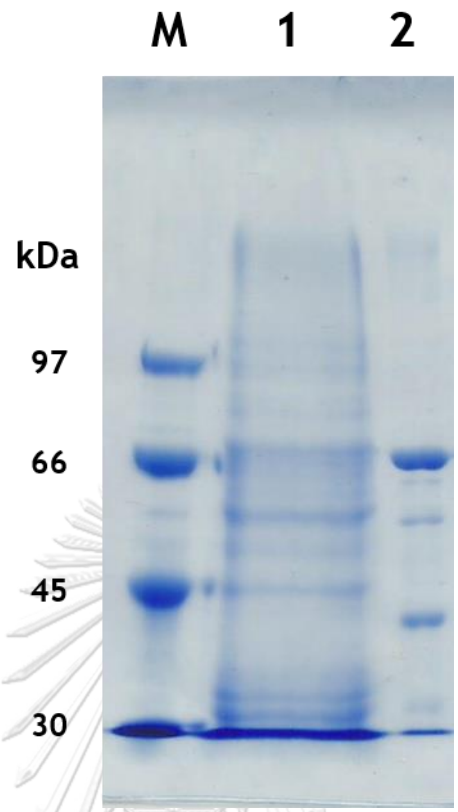


Figure 10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *Paenibacillus* sp. RB01.

M: Low molecular weight protein markers

Phosphorylase b (97 kDa)

Albumin (66 kDa)

Ovabumin (45 kDa)

Carbonic anhydrase (30 kDa)

Lane 1: Crude enzyme (25 µg)

Lane 2: Partial purified enzyme (1.0 µg)

3.2 Effect of co-organic solvent on CGTase stability

To increase the solubility of substrate fisetin, the reactions were performed in the presence of co-organic solvent. DMSO and acetone were candidates since they lack hydroxyl groups, and cannot be used as glycosyl acceptors. However, there are some limitations for the use of co-organic solvent in enzymatic reaction because enzyme is easily denatured and resulted in the loss of its activity. Thereby, in this study, the stability of CGTase in different concentration of co-organic solvent had to be investigated. The remaining activity of CGTase after incubation in 50 mM phosphate buffer, pH 6.0 containing different concentration of organic solvent at 40°C for 24 hours were analyzed and compared. As shown in Figure 11 and 12, the remaining activity of CGTase decreased with the increase concentration of both organic solvents. The enzyme activity rapidly decreased with the increase of acetone concentration (Figure 11), and completely inactivated when the acetone concentration was above 40% (v/v). On the contrary, CGTase which was incubated in DMSO solutions showed higher remaining activity (Figure 12). It retained >80% of initial activity after incubated in 10 – 30% (v/v) DMSO, and showed >40% of starting activity after incubated in 40% (v/v) DMSO. Therefore, it can be concluded that DMSO provided higher stability for CGTase than acetone, and DMSO at concentration of 40% (v/v) was selected as a co-organic solvent in further studies.

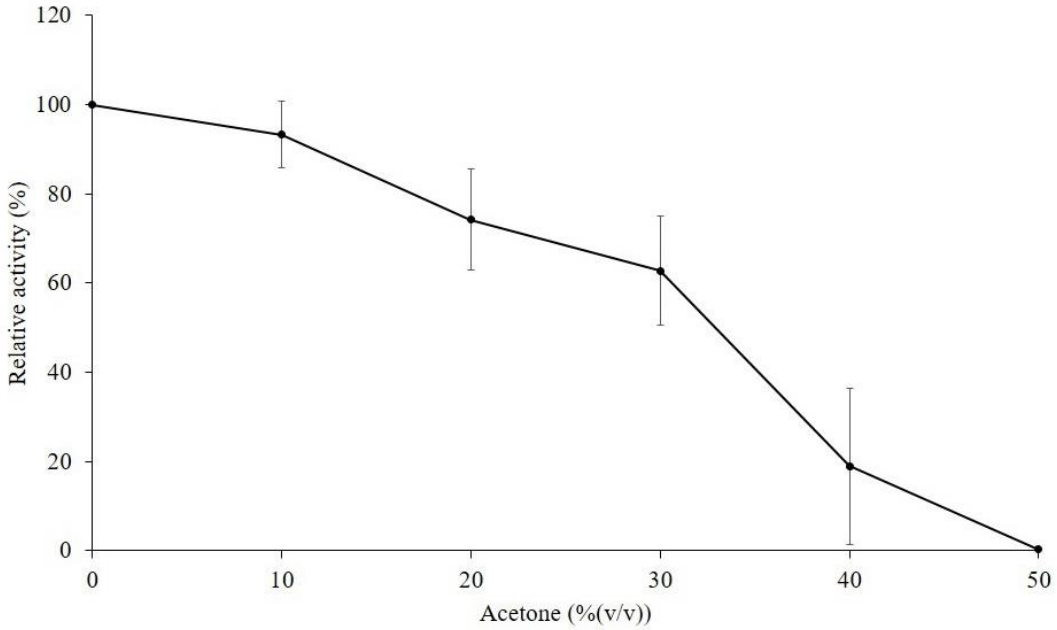


Figure 11 Relative dextrinizing activity of CGTase in various acetone concentrations. All experiments were performed in triplicates. Bars display mean ± S.D.

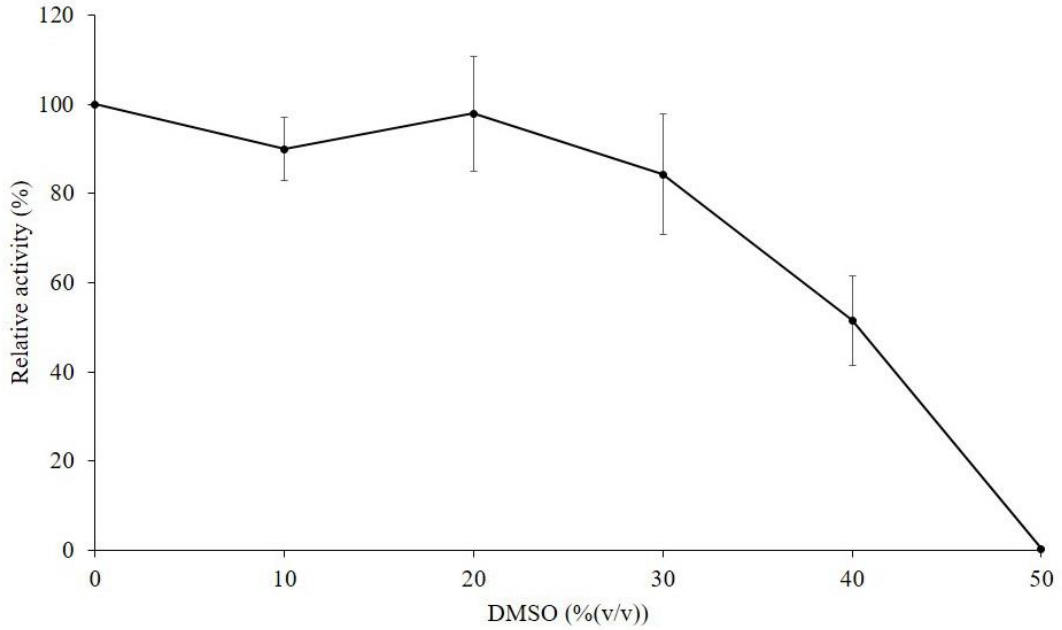


Figure 12 Relative dextrinizing activity of CGTase in various DMSO concentrations. All experiments were performed in triplicates. Bars display mean ± S.D.

3.3 Effect of DMSO concentration on the synthesis of fisetin glycosides

Since DMSO had lower effect on CGTase stability, it was selected as a co-organic solvent for the synthesis of fisetin glycosides. In this study, fisetin glycosides were synthesized by CGTase under different concentration of DMSO (10 – 50% (v/v)) in 50 mM phosphate buffer, pH 6.0. Various control reactions were conducted in order to verify the presence of fisetin glycoside signals. The first control experiment was carried out where fisitin was not added to the reaction. For the second control experiment, the enzyme was omitted. In the last control experiment, the reaction proceeded at o minute. All reactions were then analyzed by TLC and HPLC (Figure 13-15). Since TLC used was silica gel TLC, the spots of the expected products should be between the spots of fisetin and standard saccharides. Due to the fact that fisetin has lower polarity than that of saccharides, fisetin will appear on the top of the TLC plate. On the other hand, saccharides will appear at the bottom of the plate. Thus, the spots of fisetin glycosides which consist of both fisetin and sugar moieties should appear between the spots of fisetin and sugar molecules. TLC analysis revealed that CGTase was able to synthesize fisetin glycosides since the reaction mixture (Figure 13, lane d) showed the extra bands at R_f of 0.53, 0.49, 0.33 and 0.30 while no extra bands were seen in other control reactions (Figure 13). These bands appeared in both orcinol/H_2SO_4 spray and under UV lamp, indicating that the products contain both glycosyl moieties and fisetin. The existence of flavonoid can be detected under UV lamp because of their aromatic structure whereas sugar moieties can interact with orcinol to form the dark spot on TLC. The intensity of product bands increased with the increase of DMSO and seemed to be optimum at 40% (v/v). However, the intensity of expected product bands dramatically decreased when the DMSO concentration was 50% (v/v). The result

coincided with previous study in which the CGTase lost almost all activity in 50% (v/v) DMSO (Figure 12). Then, HPLC analysis was performed in order to confirm TLC results. HPLC chromatogram showed at least 5 fisetin glycosides, appeared at retention time of 10.4, 8.2, 7.8, 6.4 and 5.4 minutes, were synthesized by CGTase (Figure 14). Similarly, the peak area of glycoside products increased with the increased of DMSO concentration and was optimum at 40% (v/v) (Figure 15). Therefore, DMSO at final concentration of 40% (v/v) in 50 mM phosphate buffer, pH 6.0 was selected as a solvent for fisetin glycoside synthesis by CGTase.

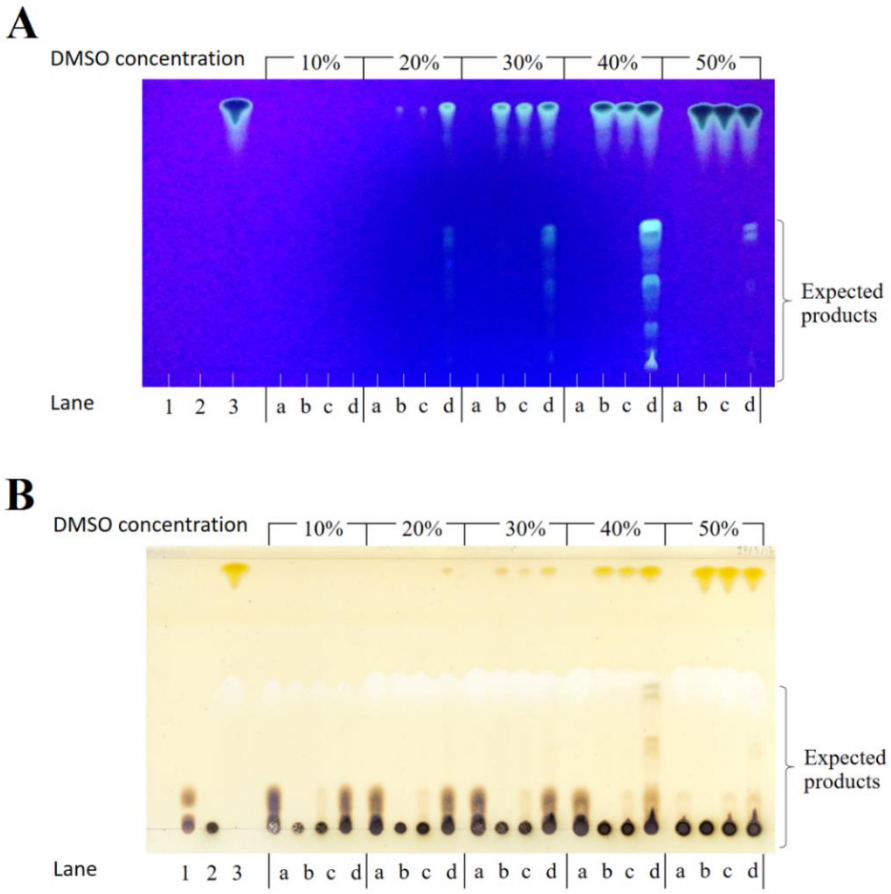


Figure 13 TLC analysis of the transglycosylation reaction from cyclodextrin and fisetin by CGTase at various DMSO concentrations (10-50%). The spots of glycoside products were detected by UV lamp (360 nm) (A) and sprayed with ethanol, conc. H_2SO_4, water and orcinol (B). Mobile phase was ethyl acetate: methanol: water: toluene at 10: 1.5: 1.3: 0.2 (v: v: v: v).

Lane 1: Standard sugar G1-G3

Lane 2: β-cyclodextrin

Lane 3: Standard fisetin

Lane a: The reaction without fisetin

Lane b: The reaction without enzyme

Lane c: The reaction at 0-minute incubation

Lane d: The reaction at 24-hour incubation

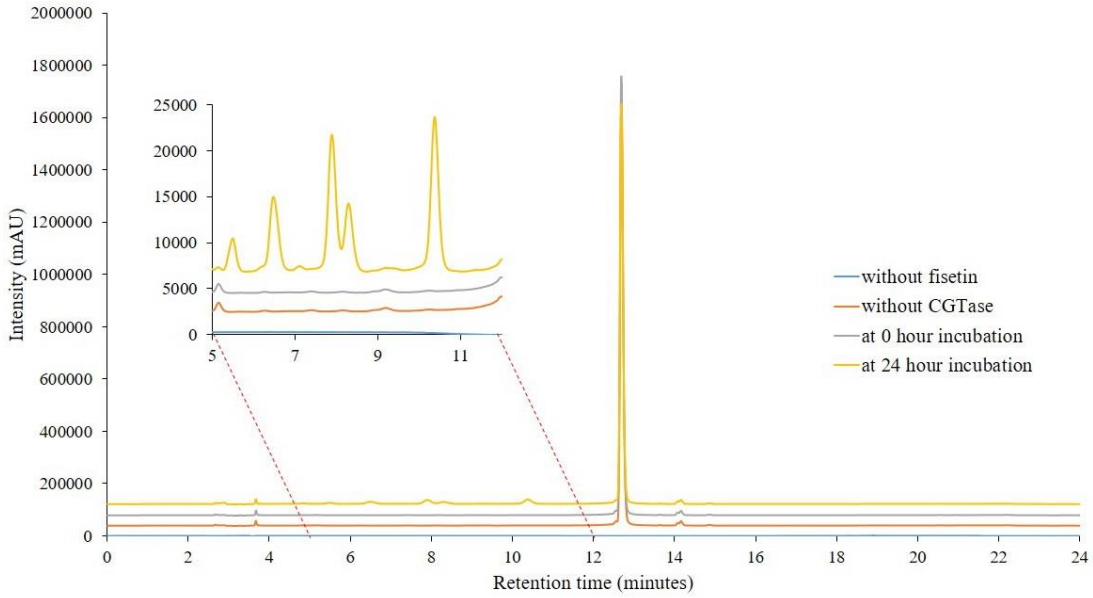


Figure 14 HPLC chromatogram of the transglycosylated products of CGTase with fisetin and β-cyclodextrin.

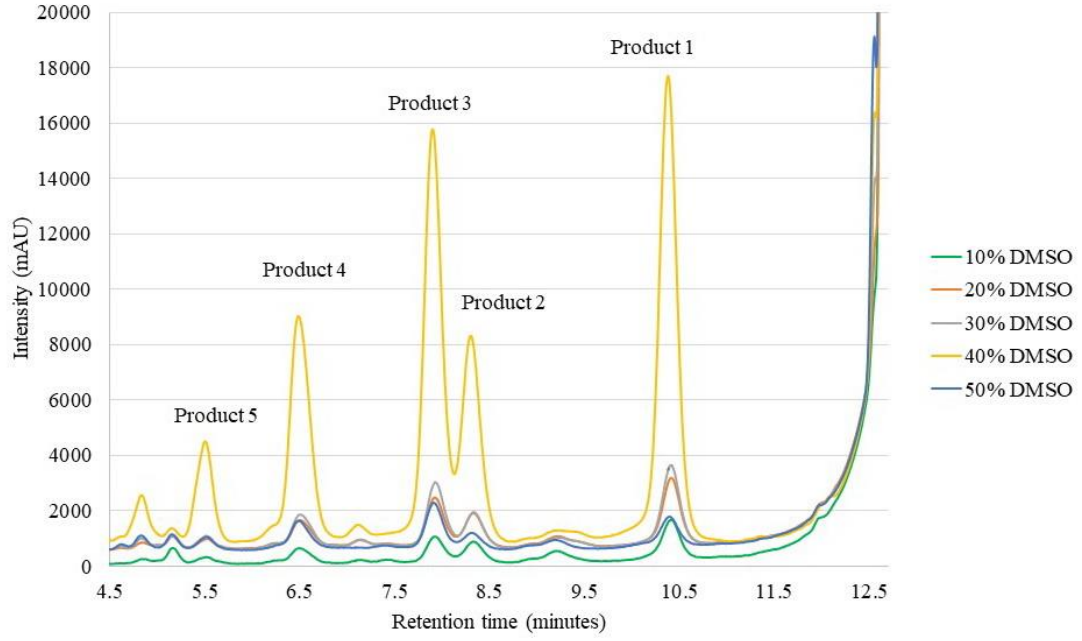


Figure 15 HPLC chromatogram of reaction products of CGTase using different concentration of DMSO.

3.4 Donor specificity

Since CGTase can use various carbohydrate molecules as a glycosyl donor, it is interesting to explore which glycosyl donor is most suitable for fisetin glycoside synthesis. Soluble starch, β-cyclodextrin (β-CD) and maltoheptaose (G7) were used as glycosyl donors for the synthesis of fisetin glycosides. The reactions were analyzed by TLC (Figure 16). It was found that all three glycosyl donors can be used as glycosyl donor for fisetin glycoside production since the product bands were detected. However, the intensity of fisetin glycosides synthesized by soluble starch was seen to be highest while the intensity of products derived from β-CD and G7 were lower (Figure 16). Semi-quantitative analysis of TLC showed that the synthesis of fisetin glycosides using β-CD as a glucosyl source provided the total yield of fisetin glycosides approximately 82% of the products derived from soluble starch, whereas G7 provided only 68% (Figure 17). However, due to the limitation of equipment (polymer form of soluble starch is possible to be precipitated in HPLC), β-CD was then selected as a glycosyl donor in further studies.

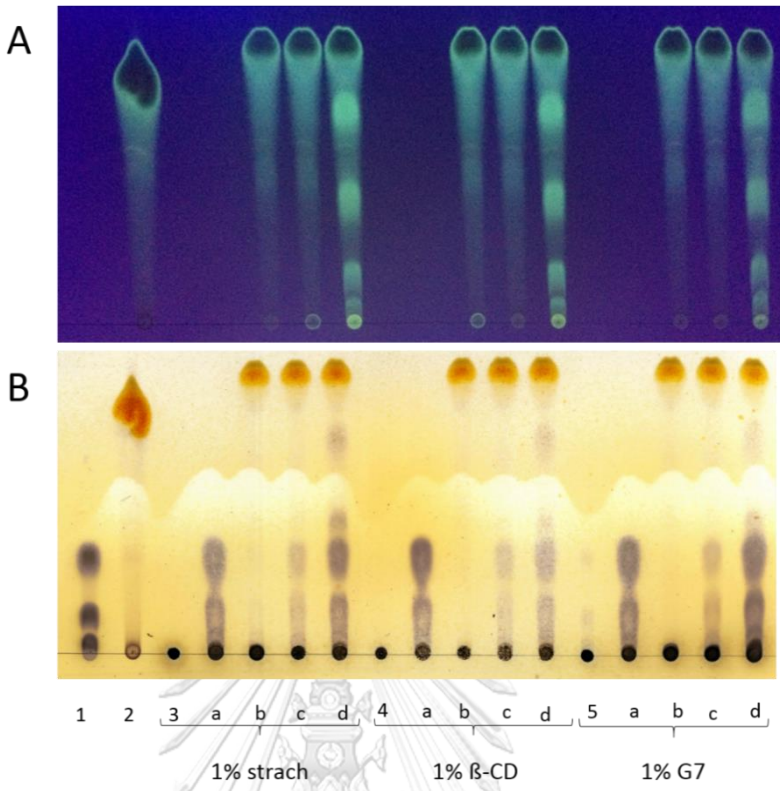


Figure 16 TLC analysis of the transglycosylation reaction from various donors for the synthesis of fisetin glycosides. The spots of glycoside products were detected by UV lamp (360 nm) (A) and sprayed with ethanol, conc. H_2SO_4, water and orcinol (B). Mobile phase was n-butanol: 2-propanol: water at 10: 5: 1 (v: v: v).

Lane 1: Standard sugar G1-G3

Lane 2: Standard fisetin

Lane 3: Soluble starch

Lane 4: β-cyclodextrin

Lane 5: Maltoheptaose

Lane a: The reaction without fisetin

Lane b: The reaction without enzyme

Lane c: The reaction at 0-minute incubation

Lane d: The reaction at 24-hour incubation

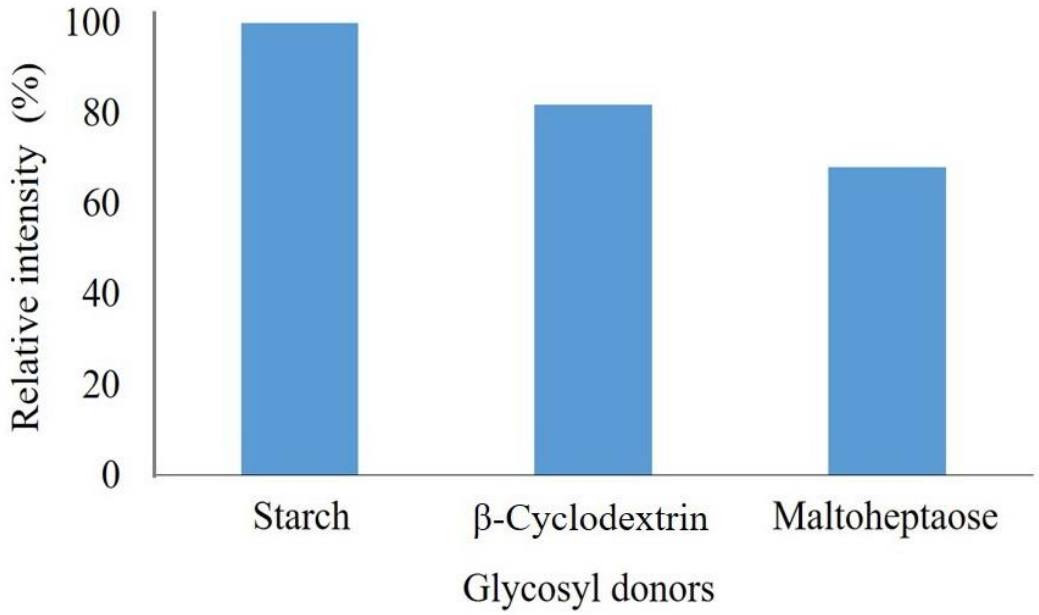


Figure 17 Relative intensity of transglycosylation products using various glycosyl donors.

3.5 Preliminary structural characterization of glycoside products

3.5.1 Structural characterization of glycoside products using glucoamylase and α-glucosidase

In this study, the structure of the products was roughly elucidated by enzymatic analysis. Enzymatic analysis has been widely used to characterize the structure of glycoside derivatives. Glucoamylase was used to hydrolyze the α-(1,4) glycosidic bond between glucose and glucose moieties (Chiba, 1997; Sauer et al., 2000) while α-glucosidase was used to hydrolyze the bonding between glucose and an acceptor (Chiba, 1997). Various control reactions were carried out, the reaction without fisetin (Figure 18, lane 4), reaction without enzyme (Figure 18, lane 5) and reaction at 0-minute incubation (Figure 18, lane 6) and the results were compared to the reaction mixture at 24-hour incubation (Figure 18, lane 7) in order to be sure that the observed products were fisetin glycosides. As can be seen in Figure 18 lane 7, four expected product bands can be detected by orcinol spray. It was found that the hydrolysis reaction of glucoamylase (Figure 18, lane 8) showed lower intensity of P3 and P4 bands (R_f of 0.33 and 0.30, respectively) while the intensity of glucose band was higher, indicating that P3 and P4 was hydrolyzed by glucoamylase. On the contrary, the hydrolysis products by α-glucosidase (Figure 18 lane 9) exhibited the lower intensity of P1 and P2 (R_f of 0.53 and 0.49, respectively), whereas the intensity of P3 and P4 was still constant. This reflected the hydrolysis activity of α-glucosidase towards P1 and P2. However, when the TLC plate was visualized under UV lamp, the P1 and P2 can still be detected. This was because the detection has higher sensitivity. When both enzymes were used for the hydrolysis, it was found that the intensity of all products dramatically decreased. Some bands can be detected under UV lamp because of the higher sensitivity of the

detection method (Pandey, Parajuli, Chu, Kim, & Sohng, 2016). From these results, it can be concluded that P1 and P2 were fisetin monoglucoside, while P3 and P4 contain at least 2 glucosyl units. In order to obtain the clearer results, HPLC analysis was applied. HPLC chromatogram showed at least 5 product bands of fisetin glycosides. The results were corresponded to TLC analysis (Figure 19) in which the peaks of products 2, 4 and 5 disappeared after the reaction mixture was treated with glucoamylase, but the products 1 and 3 can still be seen. The hydrolysis products of α-glucosidase showed the reduction of product 1 and 3, and hydrolysis products using both enzymes showed the reduction of all peak areas. From this results, we can concluded that the product 1 and 3 is fisetin which covalently linked to single glucose via α-glycosidic linkage (fisetin monoglucoside). Product 2, 4 and 5 were fisetin glycosides that contain at least 2 glucose units.

3.5.2 Mass spectroscopy (MS)

In order to conform the existence of fisetin glycoside species, the reaction mixture was then analyzed using mass spectroscopy (Figure 20). It showed an $[M+Na]^+$ ion at m/z of 471 and 633 which corresponded to the molecular weight of fisetin mono- and diglucoside, respectively. The results confirmed the existence of fisetin glycosides, at least 2 species, in the reaction mixture.

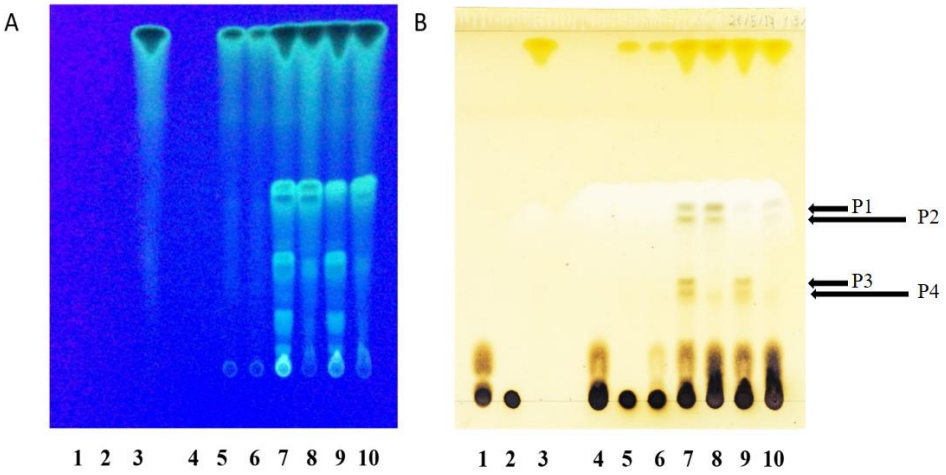


Figure 18 TLC analysis of transglycosylated products treated with glucoamylase and α-glucosidase. The spots of glycoside products were detected by UV lamp (360 nm) (A) and sprayed with ethanol, conc. H_2SO_4, water and orcinol (B). Mobile phase was ethyl acetate: methanol: water: toluene at 10: 1.5: 1.3: 0.2 (v: v: v: v).

Lane 1: Standard sugar G1-G3

Lane 2: Standard fisetin

Lane 3: β-cyclodextrin

Lane 4: The reaction without fisetin

Lane 5: The reaction without enzyme

Lane 6: The reaction at 0-minute incubation

Lane 7: The reaction at 24-hour incubation

Lane 8: The reaction at 24-hour incubation with glucoamylase

Lane 9: The reaction at 24-hour incubation with α-glucosidase

Lane 10: The reaction at 24-hour incubation with glucoamylase and α-glucosidase

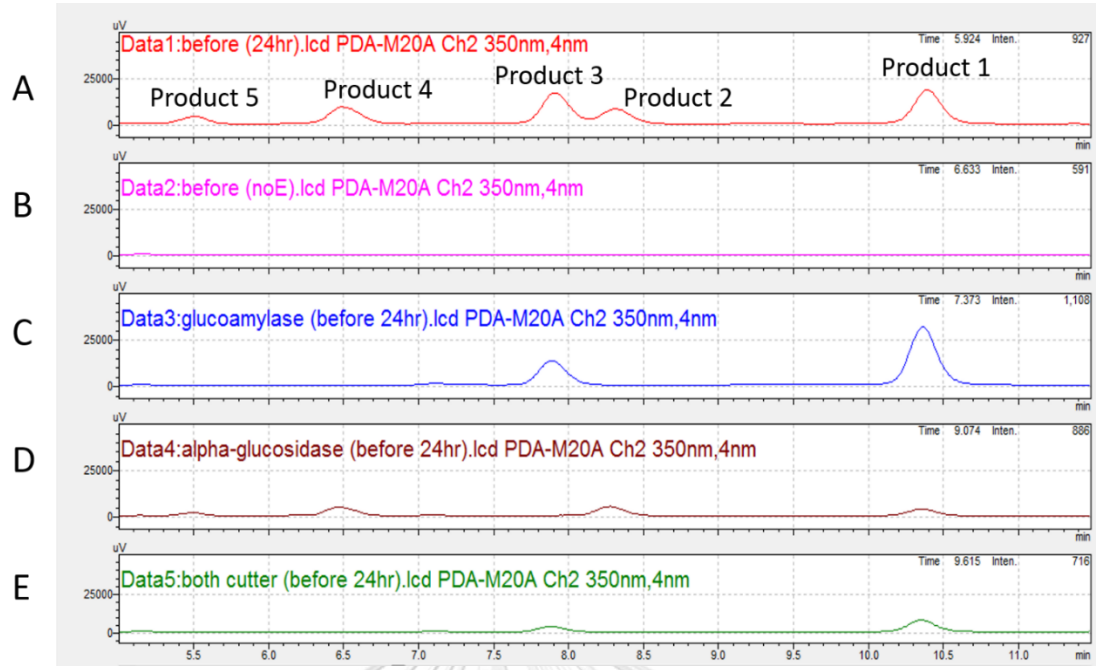


Figure 19 HPLC chromatogram of transglycosylated products of CGTase treated with glucoamylase and α-glucosidase.

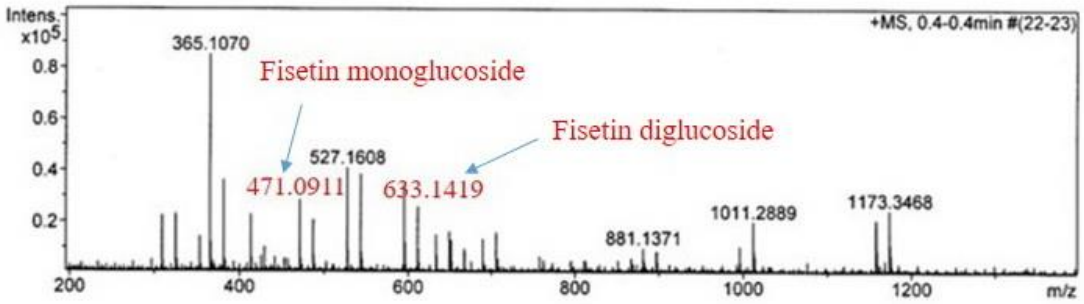
A: The reaction at 24-hour incubation

B: The reaction without enzyme

C: The reaction at 24-hour incubation with glucoamylase

D: The reaction at 24-hour incubation with α-glucosidase

E: The reaction at 24-hour incubation with glucoamylase and α-glucosidase



#	m/z	I	I %	S/N	FWHM	Res.
1	309.0361	22565	26.5	30.0	0.0640	4831
2	325.0113	23213	27.3	31.2	0.0690	4712
3	353.2055	14417	17.0	19.4	0.0758	4661
4	365.1070	84995	100.0	117.9	0.0786	4645
5	366.1114	12433	14.6	16.7	0.0769	4764
6	381.0811	36259	42.7	50.1	0.0778	4901
7	413.2684	22866	26.9	31.5	0.0907	4555
8	429.2440	10497	12.4	14.1	0.0959	4477
9	471.0911	28563	33.6	40.1	0.0902	5221
10	487.0681	20835	24.5	29.1	0.1012	4811
11	527.1608	40946	48.2	58.6	0.1089	4840
12	543.1357	38604	45.4	55.4	0.1153	4712
13	595.0877	34265	40.3	49.5	0.1207	4929
14	596.0914	12086	14.2	16.8	0.1218	4894
15	611.0627	25912	30.5	37.3	0.1307	4675
16	612.0681	9905	11.7	13.7	0.1305	4691
17	633.1419	15098	17.8	21.4	0.1260	5023
18	649.1192	16410	19.3	23.4	0.1352	4801
19	651.1500	13008	15.3	18.4	0.1453	4482
20	689.2142	13473	15.9	19.2	0.1440	4786
21	705.1892	16095	18.9	23.2	0.1380	5109
22	881.1371	10019	11.8	13.6	0.1680	5244
23	995.3139	10658	12.5	14.5	0.2007	4959
24	1011.2889	20308	23.9	28.6	0.2021	5003
25	1157.3678	21079	24.8	30.4	0.2279	5078
26	1158.3759	10474	12.3	14.6	0.2232	5189
27	1173.3468	24791	29.2	36.0	0.2326	5044
28	1174.3444	11480	13.5	16.1	0.2476	4743
29	1443.4144	17017	20.0	25.4	0.2618	5513
30	1459.3917	10224	12.0	14.8	0.2903	5027

Figure 20 ESI/MS[1] analysis profile in positive mode ionization of the transglycosylated products.

3.6 Optimization of fisetin glycoside production

To obtain the highest yield of fisetin glycosides, various parameters such as fisetin, β-CD and enzyme concentration, and incubation time were further optimized. The reaction was performed in phosphate buffer (pH 6.0) containing 40% (v/v) DMSO at 40ºC. The remaining fisetin and fisetin glycosides synthesized were then analyzed by HPLC as described in section 2.11.2. The fisetin conversion, which was used as a parameter to define the optimum condition, can be calculated as follow:

$$\text{Fisetin conversion (\%)} = (A_0 - A_t)/ A_0 \times 10$$

Where A_0 is the peak area of fisetin at reaction time = 0 min and A_t is the peak area of fisetin after 24-hour incubation.

The amount of transglycosylated products was compared directly by using peak area.

3.6.1 Optimization of fisetin concentration

Effect of fisetin concentration on fisetin glycoside synthesis was investigated by using 0.05-1.0% (w/v) of fisetin. HPLC chromatogram showed that when fisetin concentration increased, the amount of transglycosylated products also increased (Figure 21). However, fisetin conversion was optimum at 0.25% (w/v) fisetin, and decreased when too high concentration of fisetin was used (Figure 25). It indicates that the increase of transglyosylated products cannot compensate the increase of fisetin concentration. Thus, fisetin concentration at 0.25% (w/v) was chosen to be optimum concentration.

3.6.2 Optimization of appropriate donor concentration

Since β-cyclodextrin (β-CD) was selected as glycosyl donor for the synthesis of fisetin glycosides, concentration of β-CD that provided the highest yield of fisetin

glycoside was studied. Since β-CD can hardly be dissolved in 40% (v/v) DMSO at final concentration higher than 1% (w/v), β-CD at final concentrations of 0.05-1.0% (w/v) were consequently used as substrate. The results showed that the amount of fisetin glycosides increased with β-CD concentration, and was maximum at 1% (w/v) β-CD (Figure 22). Similarly, percent conversion of fisetin increased with the increase of β-CD concentration (Figure 25).

So β-CD concentration at 1.0% (w/v) was used for further experiments.

3.6.3 Optimization of CGTase concentration

In order to determine the optimum CGTase concentration for fisetin glycoside synthesis, CGTase at final concentration of 0 - 1200 U/ml were incubated with 0.25% (w/v) of fisetin concentration and 1.0% (w/v) of β-CD concentration at 40ºC for 24 hours. The result showed that transglycosylated products increased with the increase of CGTase concentration. After that, the amount of expected products were slightly decreased when higher CGTase concentration was used (Figure 23). For fisetin conversion result, it showed the highest percent conversion of fisetin at CGTase concentration of 200 U/ml (Figure 25).

3.6.4 Optimization of incubation time

The incubation time for synthesis of fisetin glycoside was studied from 0 to 48 hours. The transglycosylation reaction was conducted by incubating 0.25% (w/v) of fisetin and 1.0% (w/v) of β-CD with 200 U/mL of CGTase at 40ºC. The result showed that transglycosylated products increased when the incubation time increased, and reached plateau at 24 hours of incubation (Figure 24). For percent fisetin conversion, the optimum fisetin conversion was found to be at 24 hours. When the incubation time

was longer than 24 hours the fisetin conversion was reduced (Figure 25). So, for further study, 24 hours of incubation was chosen for the synthesis of fisetin glycosides.

3.6.5 Comparison of the yield of fisetin glycosides before and after optimization

To confirm the increase of fisetin glycosides after optimization, the fisetin conversion under optimized condition (0.25% (w/v) of fisetin, 1%(w/v) of β-CD, 200 U/ml of CGTase at 24- hour incubation) was then compared to that of the non-optimized condition (1% (w/v) of fisetin, 1% (w/v) of β-CD, 1000 U/ml of CGTase at 24-hour incubation). The result showed that the fisetin conversion under optimum condition (17.1% ± 4.21%) significantly increased when compared to that of non-optimized condition (10.1% ± 1.15%) (Figure 26).

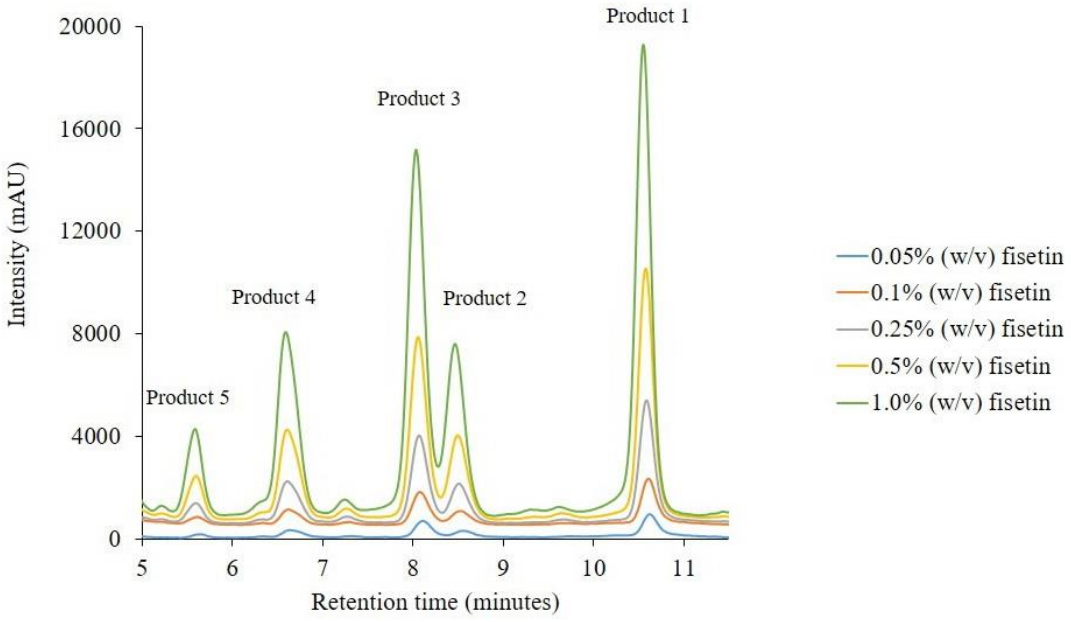


Figure 21 HPLC chromatogram of reaction products with CGTase using various fisetin concentrations.

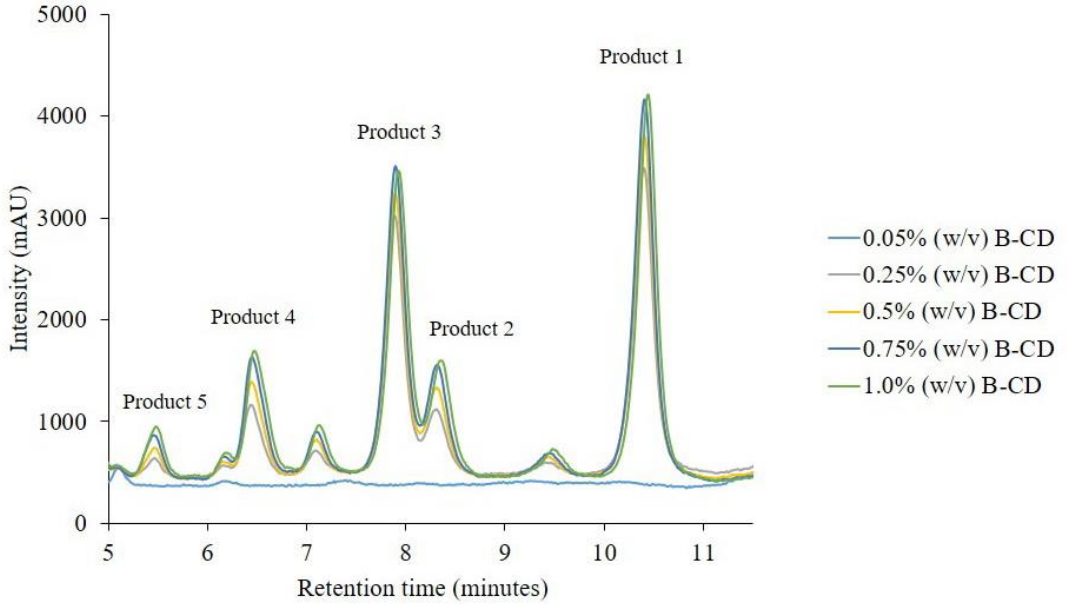


Figure 22 HPLC chromatogram of reaction products with CGTase using various β-CD concentrations.

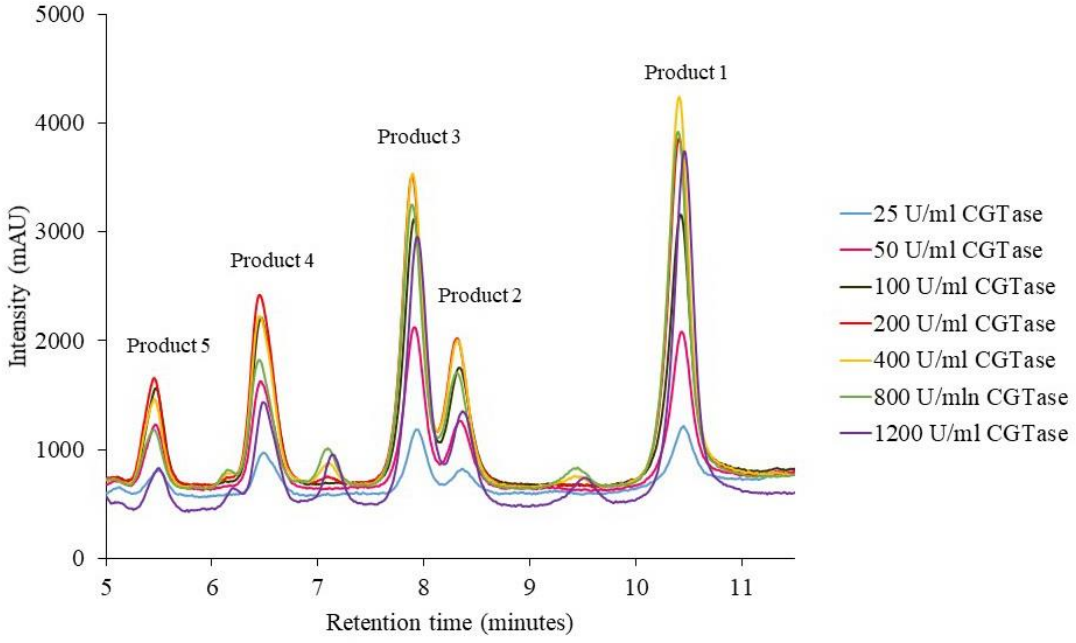


Figure 23 HPLC chromatogram of reaction products with CGTase using various CGTase concentrations.

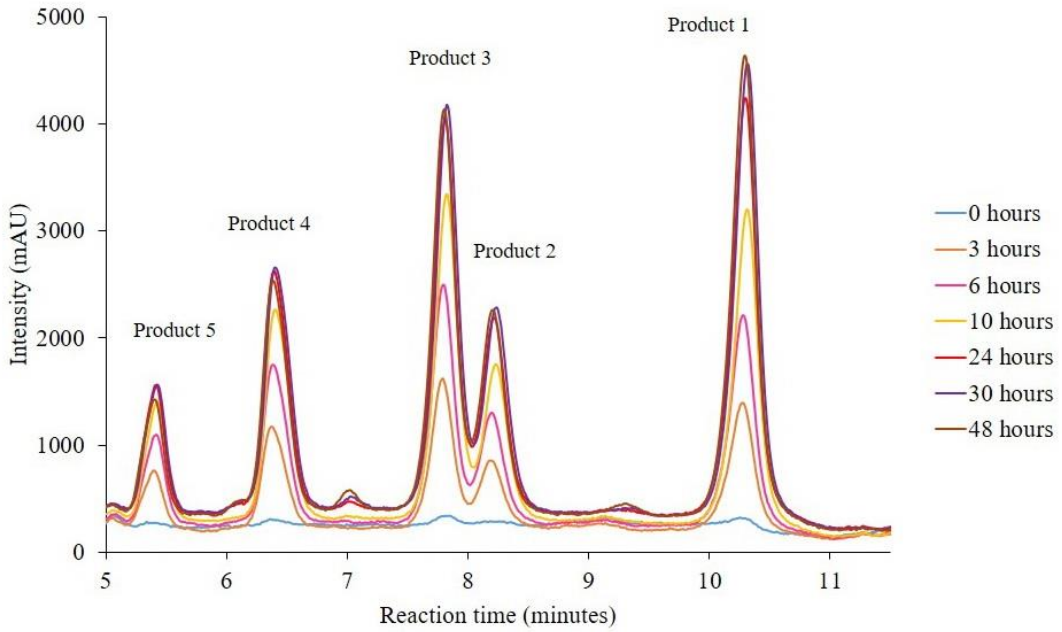


Figure 24 HPLC chromatogram of reaction products with CGTase incubation at various times.

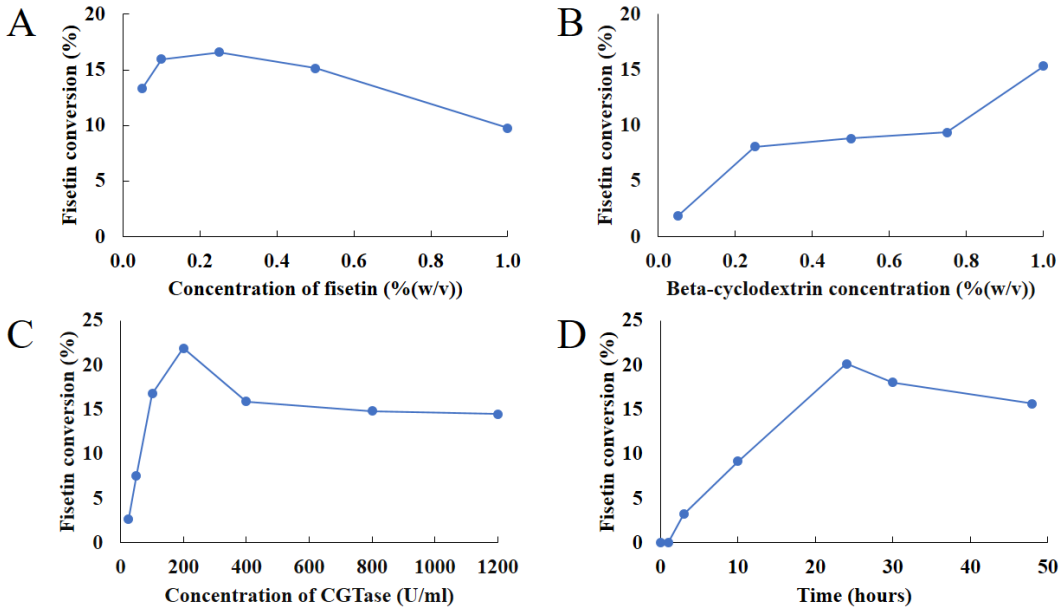


Figure 25 Fisetin conversion (%) under optimized condition for fisetin glycoside production by CGTase.

A: Fisetin conversion of fisetin concentration

B: Fisetin conversion of β-CD concentration

C: Fisetin conversion of CGTase concentration

D: Fisetin conversion of incubation time

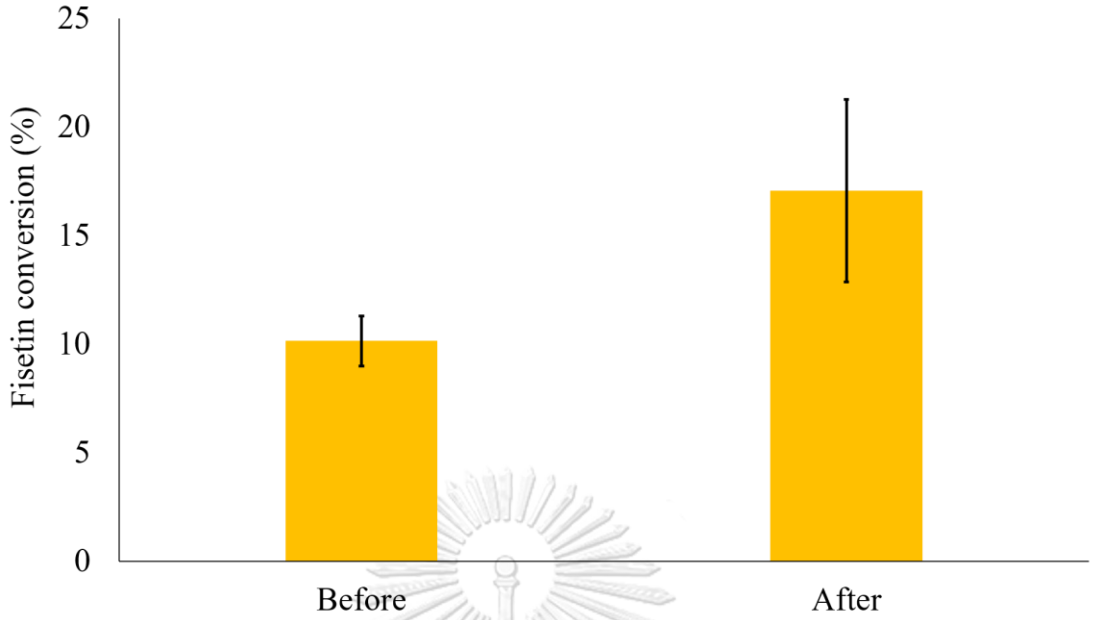


Figure 26 Fisetin conversion before and after optimization. Data points are the average of triplicate experiments and error bars represent standard deviation.

3.7 Purification of fisetin glycosides

3.7.1 Purification of fisetin glycosides using preparative TLC

Fisetin glycosides were synthesized under optimum condition as described above. The obtained reaction mixture was loaded onto TLC plate, and was separated by solvent system as described in section 2.12.1. The bands of each fisetin glycoside was monitored under UV lamp (Band I, II and III, Figure 27). Each band was scraped and pooled. The glycoside products were then extracted by co-solvent as described in section 2.12.2. The purity of each band was analyzed by HPLC. As shown in Figure 28, the products extracted from band I showed 2 peaks at retention time of 12.6 and 10.1 minutes in which one of them correlated to the retention time of fisetin (Rt of 12.6 minutes) while the other band did not correlate with any peaks of the glycoside products. For the band II extract, three products (fisetin (Rt of 12.6 minutes), product 1 (Rt of 10.4 minutes) and product 3 (Rt of 7.8 minutes)) were detected. No products were detected from the band III extract. Although some products can be extracted but the concentration was extremely low when compared to that of the crude product. Thus, the preparative TLC might not be suitable for fisetin glycoside purification.

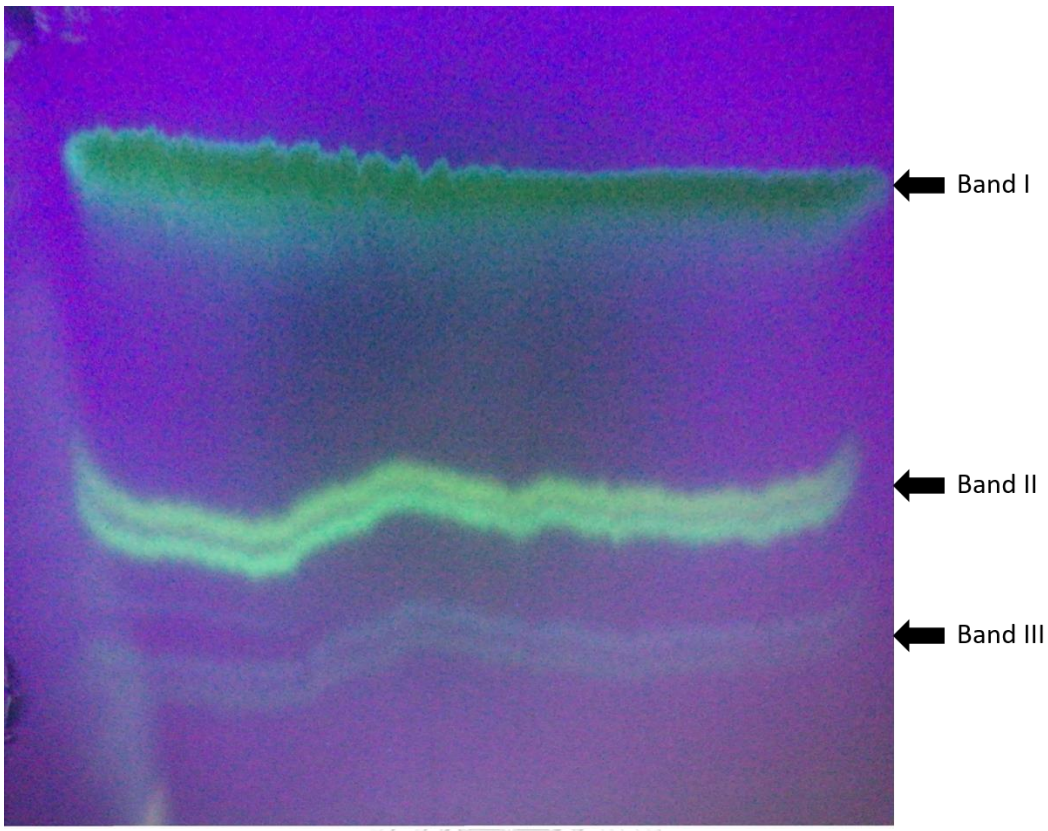


Figure 27 Purification of fisetin glycosides using preparative TLC. The band of glycoside products were detected by UV lamp (360 nm). Mobile phase was ethyl acetate: methanol: water: toluene at 10: 1.5: 1.3: 0.2 (v: v: v: v).

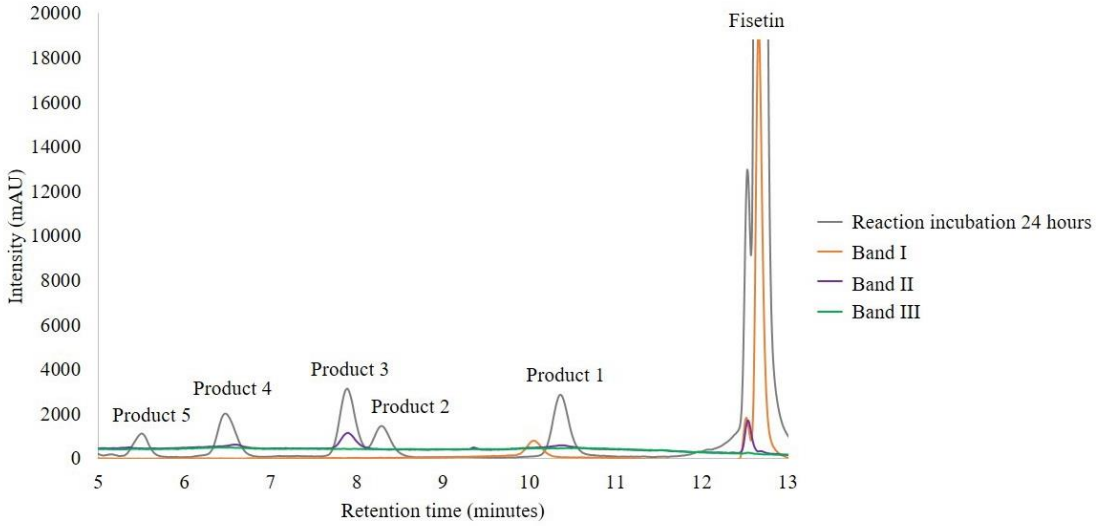


Figure 28 HPLC analysis of fisetin glycoside products extracted from preparative TLC.

3.7.2 Extraction of fisetin by organic solvents

The Fisetin glycoside products were further purified by solvent extraction. Due to the fact that fisetin can hardly dissolve in water, some of organic solvents were selected to extract an excess fisetin in the reaction mixture. In this study, 1-butanol, butyl acetate, chloroform, dichloromethane, ethyl acetate, hexane and toluene were used. Prior to organic solvent extraction, the reaction mixture containing 40% (v/v) DMSO had to be diluted with distilled water to get DMSO concentration of 2% (v/v). After that, two volumes of selected organic solvents were added to reaction mixture solution, and mixed vigorously by vortex. Aqueous and organic phases were separated by centrifugation at 5,000 g at 25ºC for 10 minutes. Each fraction was then dried by CentriVap Concentrator at 40ºC and analyzed by HPLC. For 1-butanol extraction (Figure 29), it was found that all species of glycoside products including the remaining fisetin were extracted in organic phase, resulting in no products in aqueous phase. On the contrary, chloroform, dichloromethane, hexane and toluene extraction can extract only slight amount of fisetin from the reaction mixture. Thus, the large amount of fisetin still remained in aqueous solution (Figure 30-33). Ethyl acetate and butyl acetate seemed to be suitable for this work since large amount of fisetin was extracted and only little fisetin was found in aqueous solution (Figure 34-35). Comparison between these two solvents, butyl acetate might be more suitable since no fisetin was found in aqueous phase after extraction, indicating that butyl acetate can completely remove fisetin from the reaction mixture. Accordingly, butyl acetate was selected as an organic solvent for fisetin extraction prior to the purification of fisetin glycosides.

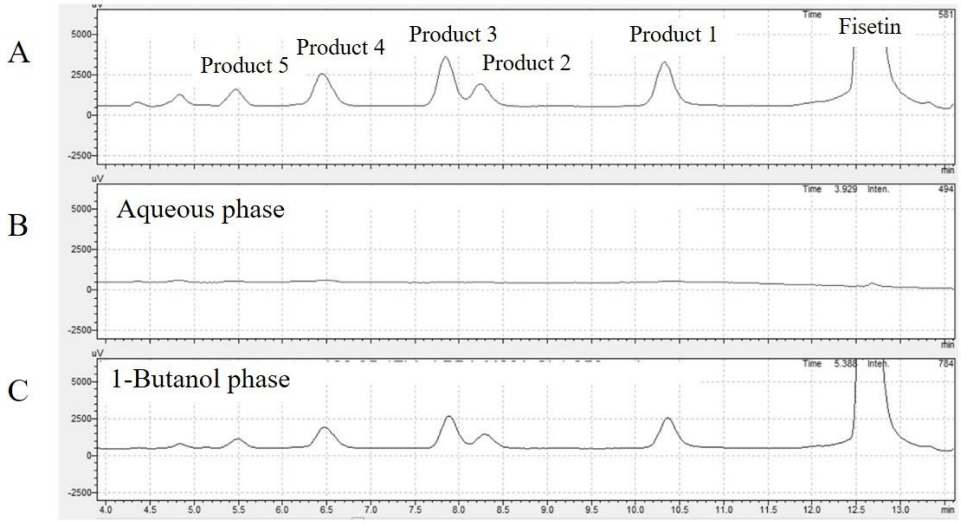


Figure 29 HPLC chromatogram of reaction products using CGTase extracted with 1-butanol.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

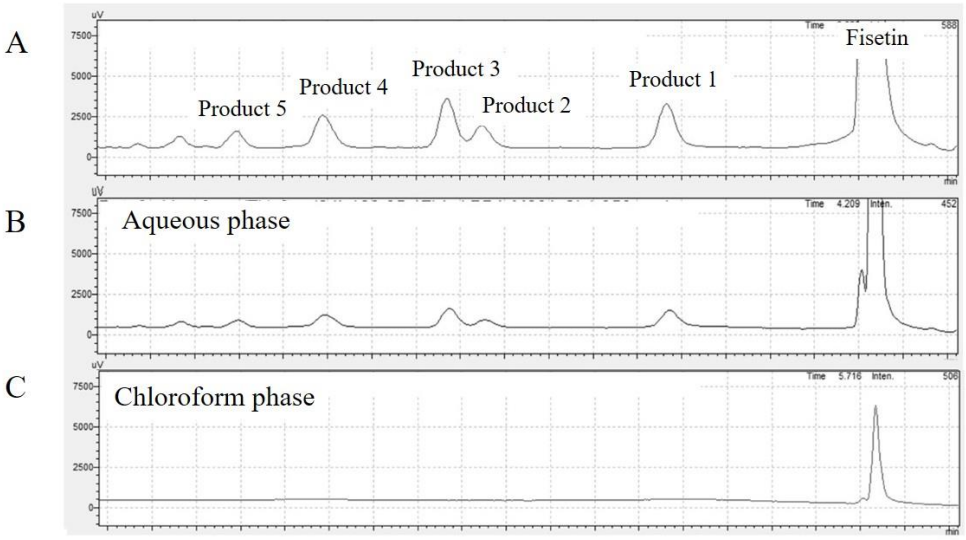


Figure 30 HPLC chromatogram of reaction products using CGTase extracted with chloroform.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

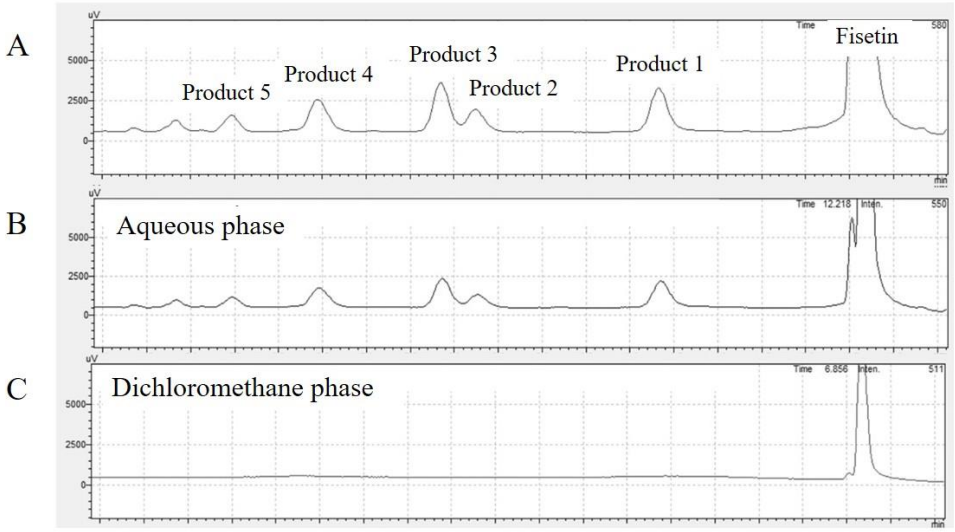


Figure 31 HPLC chromatogram of reaction products using CGTase extracted with dichloromethane.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

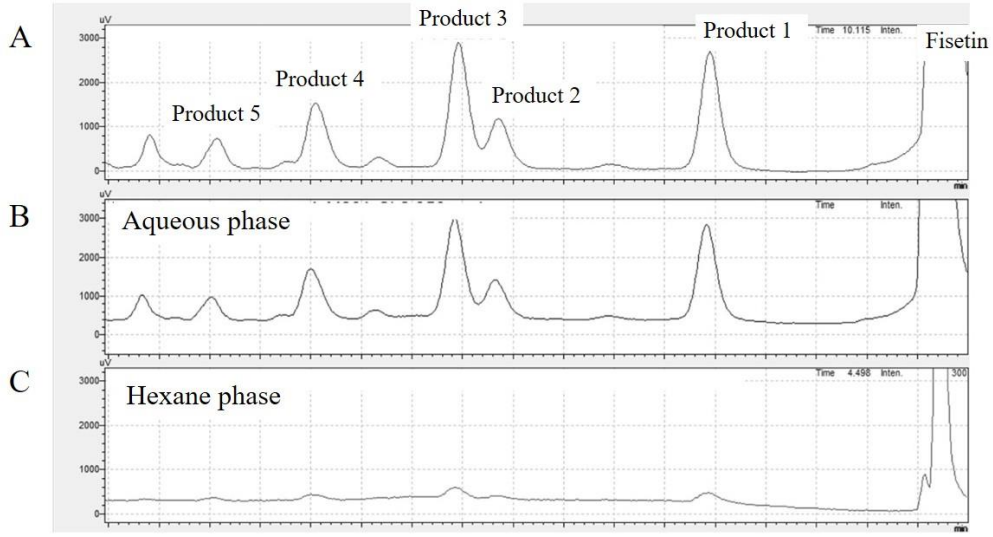


Figure 32 HPLC chromatogram of reaction products using CGTase extracted with hexane.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

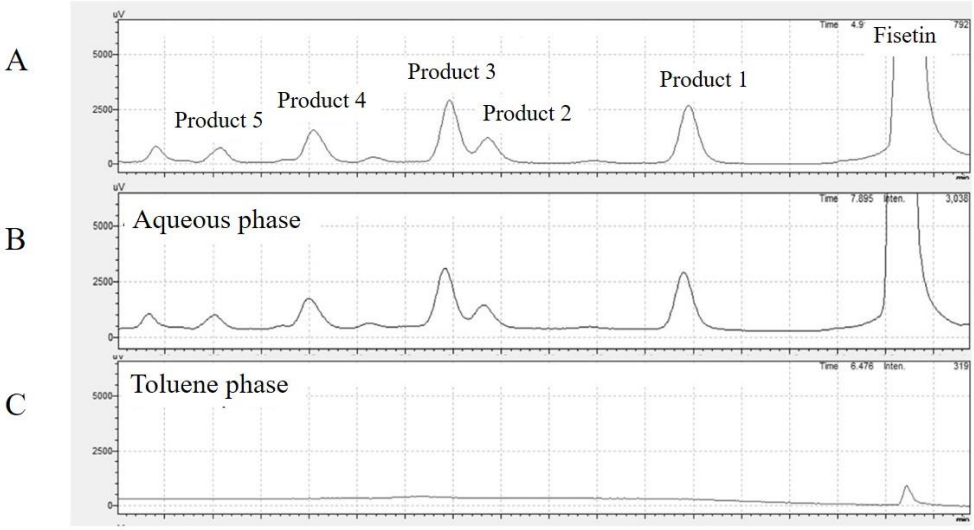


Figure 33 HPLC chromatogram of reaction products using CGTase extracted with toluene.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

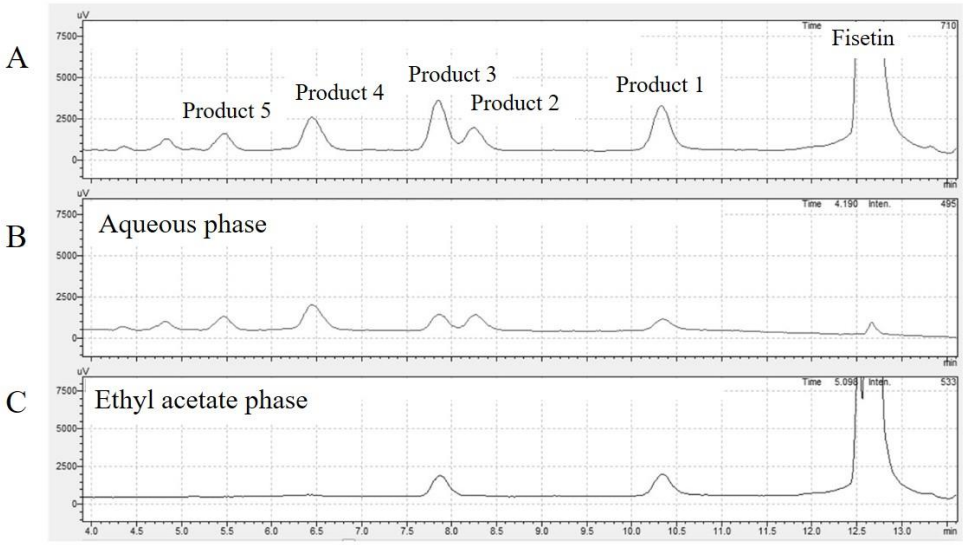


Figure 34 HPLC chromatogram of reaction products using CGTase extracted with ethyl acetate.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

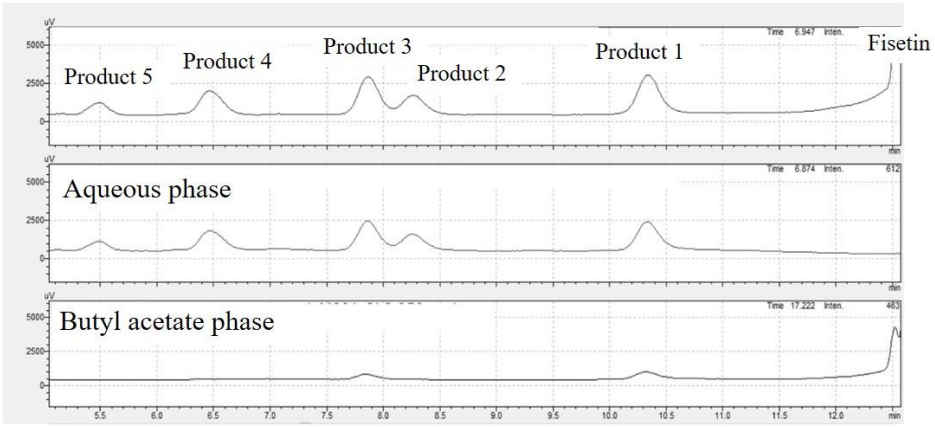


Figure 35 HPLC chromatogram of reaction products using CGTase extracted with butyl acetate.

A: The reaction at 24-hour incubation

B: The reaction in aqueous phase

C: The reaction in organic phase

3.7.3 Purification of fisetin glycosides using HPLC

After the crude product of fisetin glycosides was extracted by butyl acetate, the derived glycoside products was further purified by analytical HPLC. The glycoside products were separated by C18 column using the gradient of acetonitrile as elution solvent as described in section 2.12.3. Due to analytical HPLC was employed, several injections were applied. All fractions were pooled and dried by evaporator. Then, the purity of each fraction was checked by HPLC. The results showed that only product 1, 3 and 5 (retention time of 16.5, 14.7 and 13.4 minutes) can be purified by HPLC since they showed only single peak. In contrast, product 2 and 4 (retention time of 15.3 and 13.9 minutes) cannot be purified (Figure 36). So only product 1, 3 and 5 were selected for further biological activity assay and solubility determination.

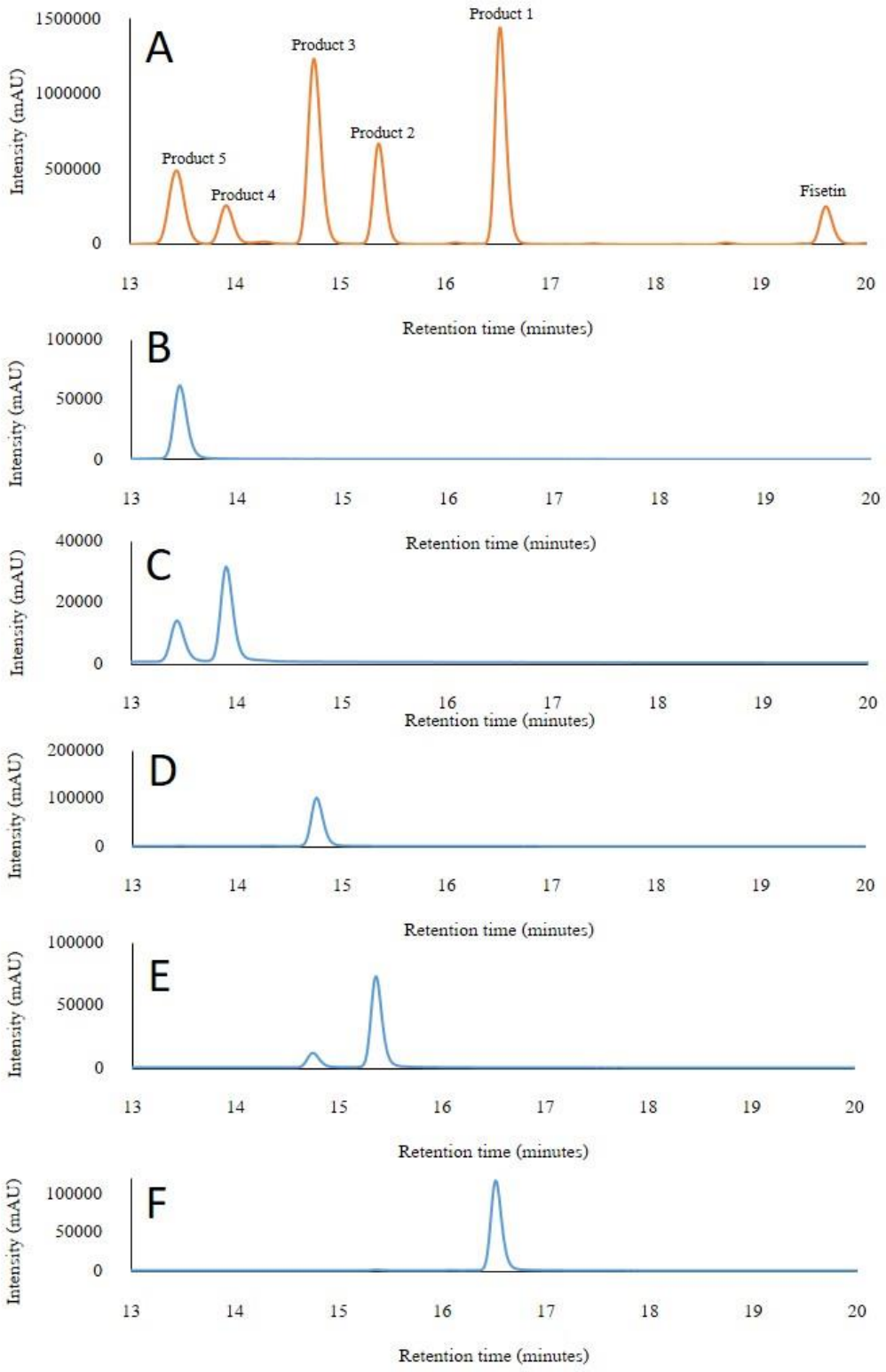


Figure 36 HPLC chromatogram of fisetin glycosides after purified by HPLC. (A) The extracted product with butyl acetate, (B-F) the purified fraction 1-5.

3.8 Structural characterization of fisetin glycoside products by MS

After fisetin glycosides were synthesized and purified, structures of derived glycosides were then investigated. Since the very low amount of fisetin glycosides can be produced, the low sensitive structural determination techniques, such as NMR, could not be applied. So, in this study, the high sensitive technique, LC-MS/MS, were used to determine the possible structure of obtained fisetin glycosides by the reaction of CGTase (Filip & Magda, 2004; Tsimogiannis, Samiotaki, Panayotou, & Oreopoulou, 2007). Although fraction C and E cannot be purified by HPLC, it was negligible since these fractions will be further purified by LC system of LC-MS/MS. First, the mass of each purified fraction (fraction 1 – 5, from HPLC chromatogram Figure 36) was subjected to MS1 analysis (Figure 38-42). It was found that product 1 and 3 were fisetin monoglucoside (m/z 447) while product 2 and 5 were fisetin diglucoside (m/z 609) and product 4 was fisetin triglucoside (m/z 771). Form these results, we can see that the order of purified fractions of fisetin glycosides by HPLC did not correlate with the amount of glucosyl units that were attached to fisetin. Fisetin glycosides with the same mass, i.e. product 1 and 3 were eluted from the column at different Rt. This suggested that they may have different polarity which was dependent on the glycosylated position. The mass of purified products was summarized in Table 5. After that, the fragmentation patterns of secondary mass spectrometer were used to predict the position of glucose attached to fisetin glycosides. In this study, only fraction 3 and 5 (containing product 1 and 3, respectively) were investigated since they are less complicated among all products. To interpret the fragments of derived glycosides, many of fragmentation patterns of various flavonoids and flavonoid glycosides were studied and compared, and finally the possible fragmentation patterns of fisetin were predicted (Figure 37).

The results showed that the glycoside group possibly linked to fisetin at ring A and C since some characteristic peaks can be detected (Figure 43- 44). As shown in Figure 43, fragmentation pattern of product 1 (retention time of 16.5 minutes) had m/z corresponded to the mass of Structure c (Figure 37A) + glucoside (m/z of 326.99), Structure d (Figure 37A) + glucoside (m/z of 255.00) and fisetin (m/z of 285.00). For the product 3, it showed the mass of Structure e (Figure 37A) + glucoside (m/z of 281.00), Structure h (Figure 37B) + glucoside (m/z of 298.99) and fisetin (m/z of 285.00). Moreover, MS2 spectrum also confirmed that all of derived glycoside products were fisetin glycosides as the peak at m/z of 285.00 was found which corresponded to the mass of fisetin.

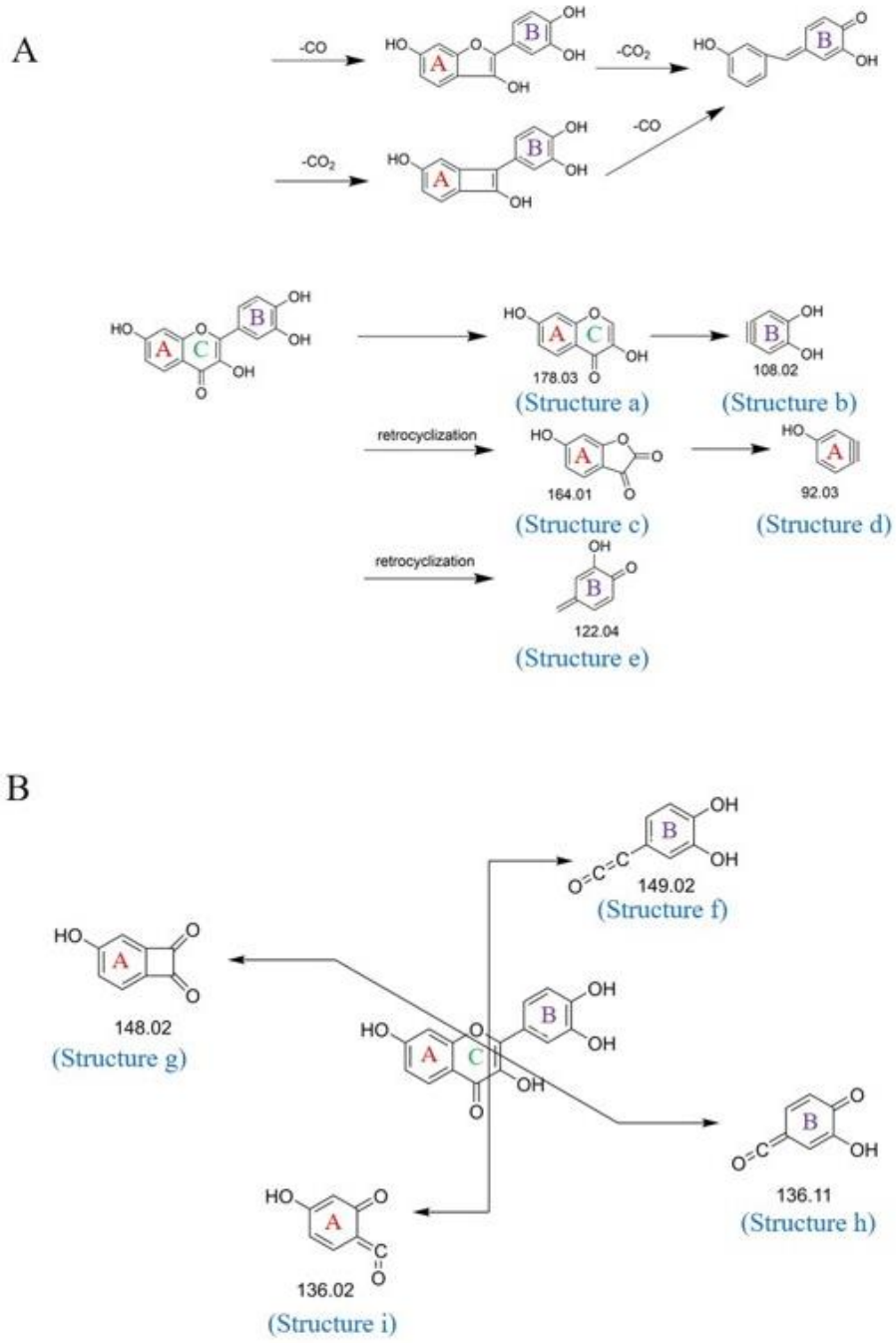


Figure 37 Prediction of fragmentation patterns of fisetin derived from mass spectroscopy. (A) the fragment modified from quercetin (Weber, Shen, Yang, Prajda, & Li, 1999), and (B) Formal notation used for the retrocyclization fragmentation of the [M − H]− ions of flavonoids (Troalen, Phillips, Peggie, Barran, & Hulme, 2014).

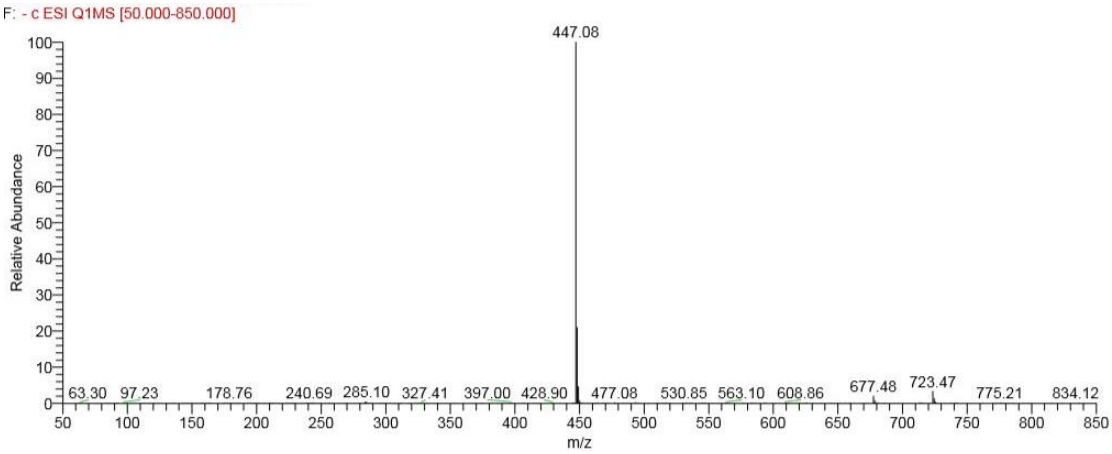


Figure 38 ESI/MS[1] analysis profile in negative mode ionization of the product 1 (retention time of 16.5 minutes).

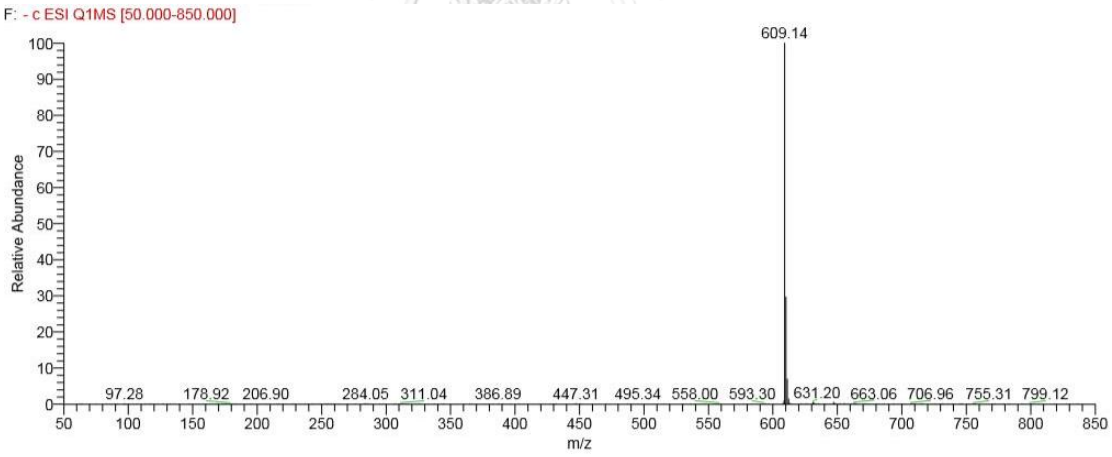


Figure 39 ESI/MS[1] analysis profile in negative mode ionization of the product 2 (retention time of 15.3 minutes).

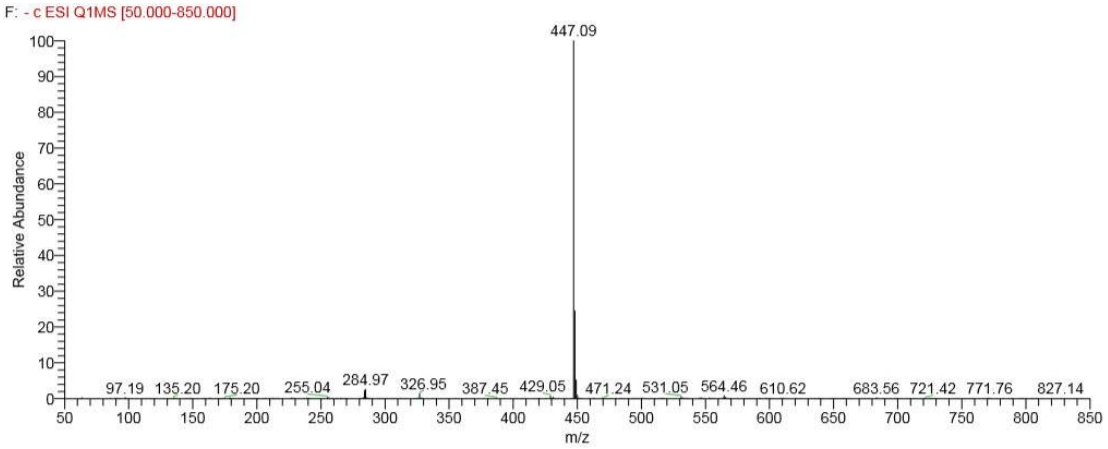


Figure 40 ESI/MS[1] analysis profile in negative mode ionization of the product 3 (retention time of 14.7 minutes).

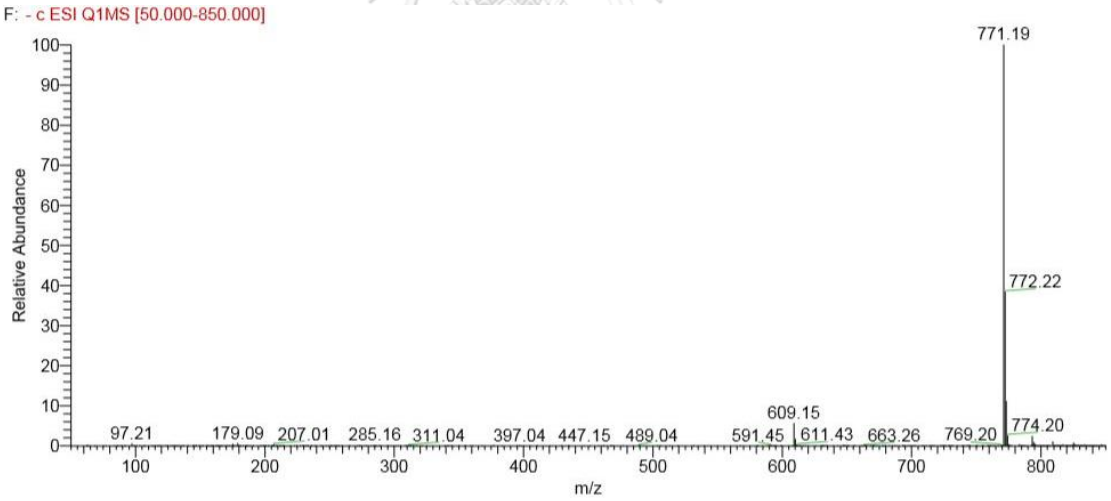


Figure 41 ESI/MS[1] analysis profile in negative mode ionization of the product 4 (retention time of 13.9 minutes).

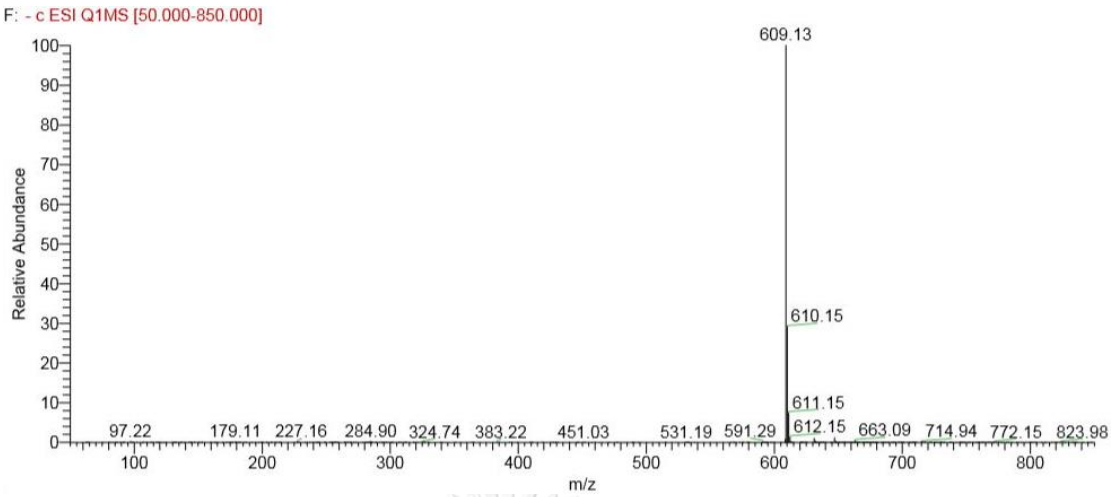


Figure 42 ESI/MS[1] analysis profile in negative mode ionization of the product 5 (retention time of 13.4 minutes).

Table 5 m/z ratio of purified products by ESI/MS[1] analysis profile in negative mode ionization.

Sample	m/z
Fraction 5 (containing the peak at 16.5 min)	447.08
Fraction 4 (containing the peak at 15.3 and 14.7 min)	609.14
Fraction 3 (containing the peak at 14.7 min)	447.09
Fraction 2 (containing the peak at 13.9 and 13.4 min)	771.19
Fraction 1 (containing the peak at 13.4 min)	609.13

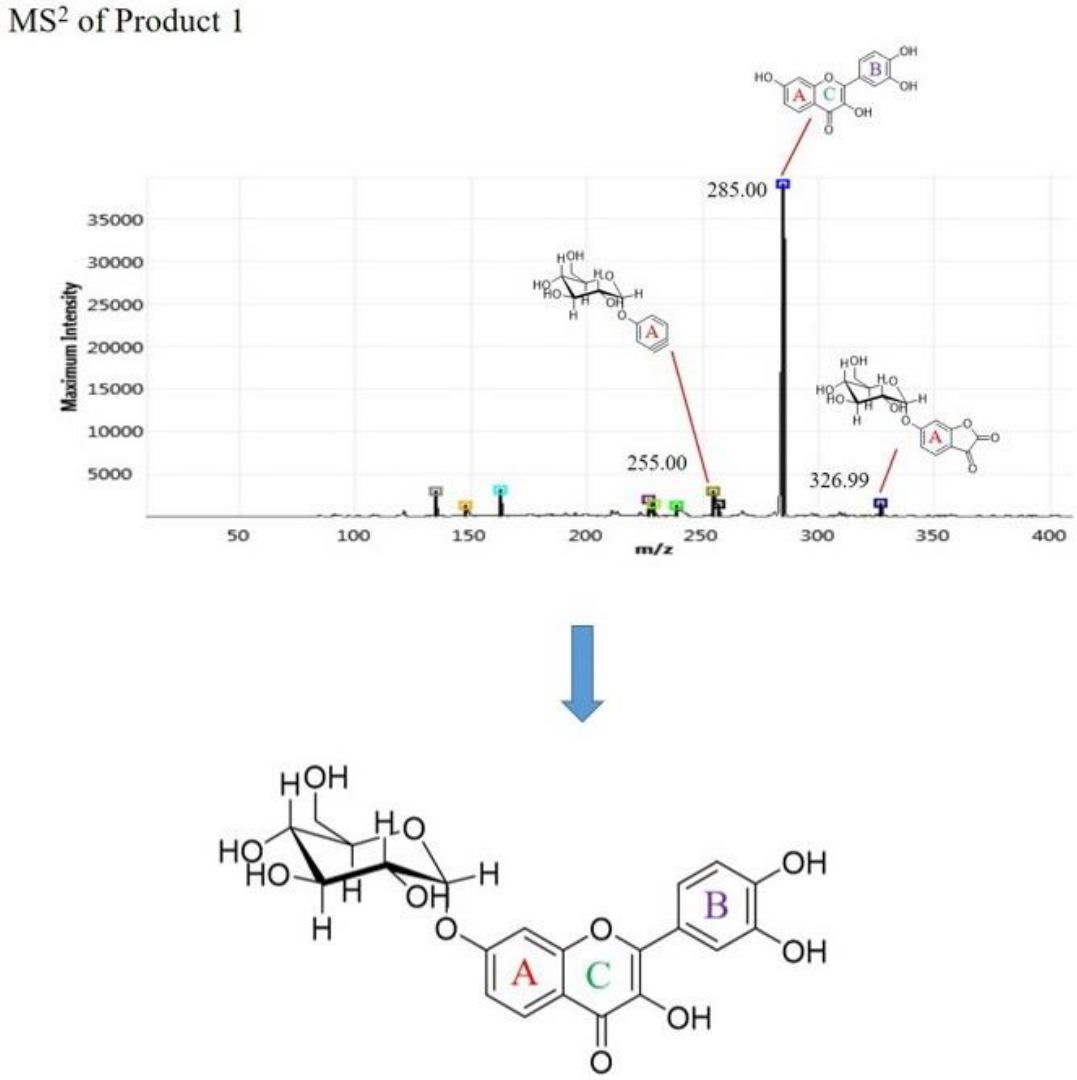


Figure 43 ESI/MS^2 analysis profile in negative mode ionization of the product 1 (retention time of 16.5 minutes).

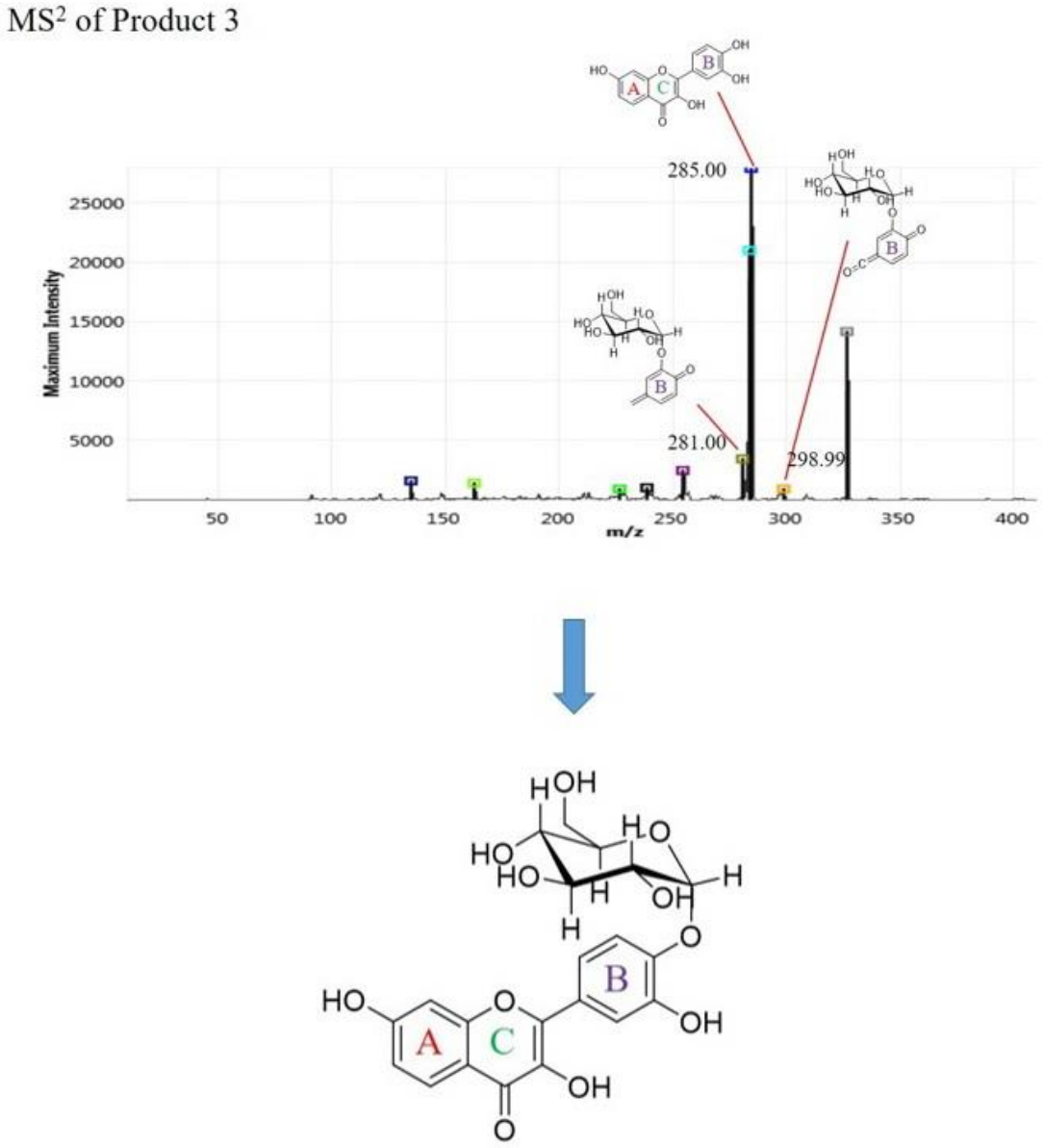


Figure 44 ESI/MS^2 analysis profile in negative mode ionization of the product 3 (retention time of 14.7 minutes).

3.8.1 Water solubility of fisetin glycoside products

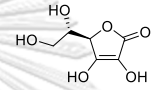
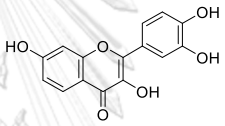
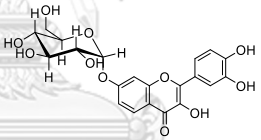
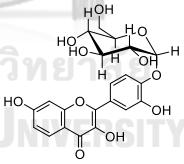
The aim of this study was to increase the solubility of fisetin by transglycosylation. So the solubility of obtained fisetin glycosides have to be investigated. Due to the low amount of fisetin glycosides yield, the amount of glycoside products cannot be weighed directly. Thus, the molar concentration of each fisetin glycoside (purified fraction; product 1, 3 and 5) had to be determined from the molar concentration of fisetin after hydrolysis in acid solution using HPLC (Section 2.11.3). After the molar concentration of each species was known, the solution of purified products was then dried by Centrivap. Due to the low fisetin glycoside production yield, all dried products were re-dissolved in 50 µl of ultra-pure water, and the molar concentration of each glycoside was then checked by the same method as described above. The result showed that at least 0.827, 0.408 and 0.231 mM of product 1, 3 and 5 was able to dissolve in water while the original fisetin was able to dissolve at concentration as low as 0.459 µM. It can clearly be seen that the product 1, 3 and 5 had water solubility at least 1800, 888 and 503 times higher than that of fisetin. Hence, the transglycosylation reaction to fisetin using CGTase can dramatically increase the water solubility of fisetin. Nevertheless, due to limited amount of each fisetin glycoside product, the absolute value of fisetin glycoside solubility could not be resolved.

3.8.2 Antioxidant activity

After fisetin glycosides were synthesized and purified, the anti-oxidant activity of each glycoside was then analyzed and compared to that of the original fisetin. In this study, the antioxidant activity of each product species was determined by DPPH assay as described in Method section 2.14.2. The results showed that the concentration of fisetin, product1, 3 and 5 required for scavenging radical of DPPH by 50% (ED_{50}) was

2.72, 2.28, 2.52 and 1.97 µM, respectively. This indicated that the antioxidant activity of fisetin glycosides were comparable to that of the parent molecule even if they were transglycosylated by CGTase (Table 6). Moreover, it was found that ED_{50} of fisetin and all fisetin glycosides were lower than that of L-ascorbic acid, indicating that the derived fisetin glycosides had higher antioxidant activity than vitamin C.

Table 6 Antioxidant activity of L-ascorbic acid, fisetin and fisetin glycosides.

Compound	Structure	ED_{50} (µM)
Ascorbic acid		5.59
Fisetin		2.72
Product 1 (fisetin monoglucoside)		2.28
Product 3 (fisetin monoglucoside)		2.52
Product 5 (fisetin diglucoside)	-	1.97

CHAPTER IV
DISCUSSION

Transglycosylation has widely been used to improve the chemical properties such as water solubility and/or stability of some organic compounds while still retaining their biological activity. Enzymatic transglycosylation has usually been employed since it is more specific than chemical reaction and provides products with high purity. Numerous enzymes have been reported for the production of glycoside derivatives. For example, dextransucrase from *Leuconostoc mesenteroids* was used as a catalyst for the synthesis of kaempferol-3-O-β-D-nigeroside (Kim et al., 2012), amylosucrase from *Deinococcus geothermalis* was used for the synthesis of catechin-3'-O-β-D-glucoside (H.-K. Cho et al., 2011), and α-amylase was used for alkyl glucose production (Larsson, Svensson, & Adlercreutz, 2005).

Cyclodextrin glycosyltransferase (CGTase) is one of the enzymes that has extensively been exploited for the synthesis of many glycoside products, for example, ascorbyl glycosides (Gudiminchi, Towns, Varalwar, & Nidetzky, 2016), epicatechin glycosides (Aramsangtienchai, Chavasiri, Ito, & Pongsawasdi, 2011b), piceid glycosides , neohesperidin glycosides and naringin glycosides (Mathew, Hedström, & Adlercreutz, 2012) (Figure 45). These glycoside derivatives showed higher water solubility than their parent molecules and they still possessed the antioxidant activity. However, to the best of our knowledge, there are no reports on enzymatic synthesis of fisetin glycosides by CGTase.

Fisetin (3, 3', 4', 7-tetrahydroxyflavone) is one of the polyphenols in the flavonoid group which belongs to flavonol subgroup. It has very interesting

spectroscopic and pharmacological properties and has widely been used in many applications. Fisetin can be found in many plants including fruits and vegetables. Thus, in this study, it is of great interest to synthesize fisetin glycosides by using CGTase in order to increase its solubility in water and still retain the antioxidant activity. This will undoubtedly be useful in pharmaceutical industries.

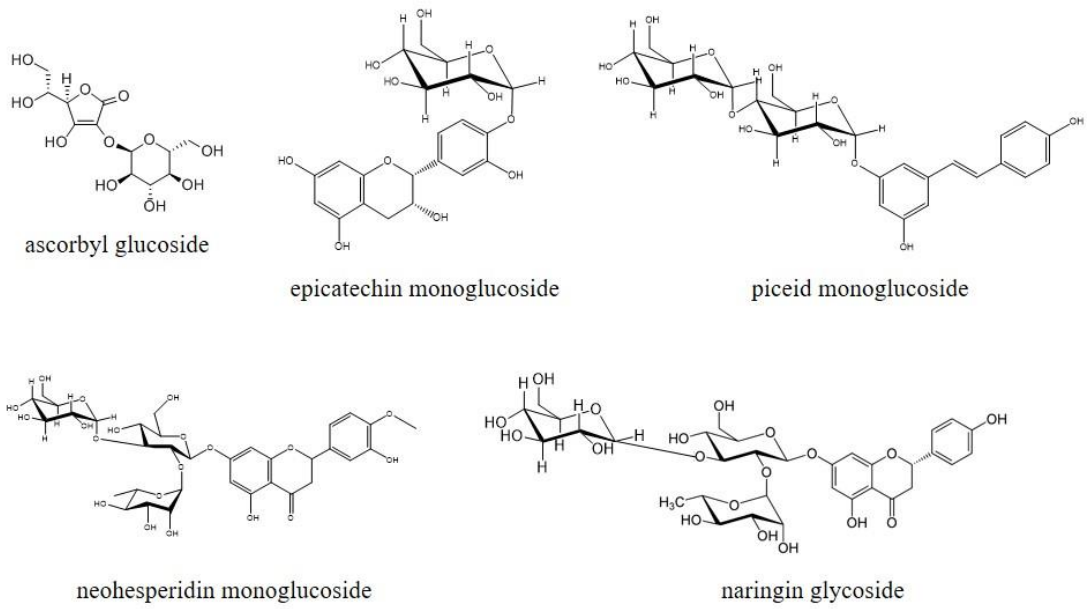


Figure 45 Glycosides products synthesized by CGTase.

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

CGTase from *Paenibacillus* sp.RB01 were partially purified by starch adsorption. This technique is powerful since only a few enzyme can specifically adsorb to starch, resulting in single step separation of CGTase from crude enzyme. Although CGTase can tightly bind to starch, it can be merely eluted from starch particles by using maltose solution due to higher affinity for CGTase binding. Moreover, maltose can

easily be removed by dialysis. The yield of purified CGTase was 31 % with 34 purification fold and specific activity of 5033.00 U/mg protein. In comparison with previous studies, Chotipanang stated that the yield of CGTase after purifiy by starch adsorption was 38% with 46% fold purity (Chotipanang, Bhunthumnavin, & Prousoontorn, 2011), while Anurutphan reported that the obtained CGTase had 32.7% yield and 95 purification fold (Anurutphan & Prousoontorn, 2015). The different of these values suggested that the purity of derived enzyme was largely dependent on the quality of person performing purification process. Furthermore, it was found that not only CGTase but also other proteins that contain starch binding domain can bind to starch granules. Therefore, it is possible that CGTase derived from this technique were not absolutety purified enzyme but might contain other enzyme species. SDS-PAGE showed that at least 3 protein species were co-purified by starch adsorption. Nevertheless, it was found that the major protein band on SDS-PAGE showed the estimated molecular weight of 66 kDa, which corresponded to the molecular weight of CGTase previously reported by Chotipanang *et al.* (2011) and Anurutphan *et al.* (2015). This result suggested that CGTase was a major protein that was purified by starch adsorption and the purity of enzyme was enough to be used for glycoside synthesis. Moreover, native-PAGE results also showed that the purified protein possibly be the CGTase since it showed hydrolysis activity on the native-PAGE. The existence of CGTase was further comfirmed by cyclization activity assay using phenolphthalein methods.

4.2 Effect of organic solvent on CGTase stability

Due to the fact that fisetin had low solubility in water, co-organic solvent system were then used in order to increase the transglycosylation efficiency. Moreover,

scientific researches indicated that co-organic solvent might increase transglycosylation reaction over hydrolysis reaction. In this study, DMSO and acetone were selected as candidate organic solvent since they lack of hydroxyl group and cannot be used as glycosyl acceptors. The result showed that DMSO showed lower effect on CGTase stability than that of acetone since enzyme was still active even if the high concentration of DMSO was used. The result implied that the stability of CGTase was largely dependent on the type and concentration of organic solvent used. This finding was corresponded to Anurutphan *et al*. (2015) in which CGTase was still active even if the concentration of DMSO was increased up to 50% (v/v). Moreover, Park *et al.* (1998) stated that CGTase was stabilized in the presence of low concentration of DMSO while it could be inactive in high DMSO concentration (Park, Oh, Choe, Park, & Lee, 1998). Thus, DMSO was selected as co-organic solvent for this study. Although the high concentration of organic solvent promotes the solubility of fisetin, the ability of CGTase to synthesize fisetin glycosides might be decreased. Due to the fact that inactivation of enzyme in organic solvent can be resulted from conformational change and active center blockage (Klibanov, 1997), the effect of DMSO concentration on fisetin glycoside synthesis had to be determined.

4.3 Effect of DMSO concentrations on the fisetin glycoside synthesis

Although the higher concentration of organic solvent increased the solubility of fisetin, it also resulted in the reduction of enzyme activity. Thus, the effect of DMSO concentration on the yield of fisetin glycoside had to be further investigated. After incubation for 24 hours, the amount of fisetin glycoside products was determined by TLC. It was found that the amount of fisetin glycosides produced by CGTase increased when DMSO concentration increased and reached optimum at 40% (v/v) DMSO. This

results suggested that the increase of fisetin glycoside yield was because the substrate fisetin was more soluble and easily reacted with glucosyl-enzyme intermediate. Moreover, the higher concentration of organic solvent reduced the amount of water in the reaction mixture and thus, hydrolysis activity was decreased while the transglycocsyltation activity was increased (Oikawa, Tsukagawa, Chino, & Soda, 2001). The increase of transglycosylation activity by using co-organic solvent has previously been reported for the synthesis of resveratrol glucosides by CGTase (Anurutphan & Prousoontorn, 2015). The results showed that the amount of fisetin glycosides was dramatically decreased when DMSO concentration used was as high as 50% (v/v) which was corresponded to the previous finding in which the activity of CGTase was completely lost at 50% (v/v) DMSO.

4.4 Optimization of fisetin glycoside production

In order to obtain the highest yield of fisetin glycosides, the reaction condition had to be optimized. Previous studies revealed that the yield of glycoside products was dependent on the reaction condition, such as pH, temperature, substrate concentration, enzyme concentration and incubation time (Anurutphan & Prousoontorn, 2015). Chotipanang *et al.* stated that optimum pH and temperature for transglycosylation reaction of CGTase was 6.0 and 40°C, respectively. Hence, in this study, we explored only the effects of fisetin concentration, donor concentration, CGTase concentration and incubation time on the transglycosylation reaction. First, the effect of fisetin concentration on fisetin glycoside synthesis was examined. It was found that the increase of fisetin concentration increased the synthesis of fisetin glycosides. This result coincided with previous study by Anurutphan *et al.* (2015) in which the amount of resveratrol glylcosides synthesized by CGTase increased with increase of resveratrol

concentration. The increase of fisetin glycoside products might be caused by the increase of diffusion rate of fisetin into the active site of CGTase. However, the percent conversion of glycoside products decreased when fisetin concentration was higher than 0.25% (w/v). This indicated that the increasing rate of fisetin glycoside production was lower than the rate of utilization of the substrate. So, for the commercial propose, fisetin concentration at 0.25% (w/v) was selected for further studies.

Similarly, the effect of donor concentration was then studied. Moreno *et al.* (2010) and Mótyán *et al.* (2011) reported that initial substrate concentration influenced transglycosylation reaction (Moreno et al., 2010; Mótyán et al., 2011). In this study, β-CD was selected as an appropriate glycosyl donor. Due to the fact that the CGTase activity was largely dependent on initial substrate concentration used, it was clear that the amount of fisetin glycosides produced by CGTase increased with the increase of β-CD. The optimum concentration of β-CD for fisetin glycoside synthesis was found to be 1%. Higher concentration of β-CD cannot be used due to limitation of β-CD solubility.

Then, the effect of enzyme concentration was determined. The results showed that the percentage of fisetin conversion increased according by to the enzyme concentration and seemed to be optimum at 200 U/ml. However, the fisetin conversion decreased when enzyme concentration was too high. This results was similar to that of Ye *et al*. (2011). They reported that the addition of excess enzyme might cause the formation of enzyme aggregate (Ye et al., 2013).

Finally, the effect of reaction time was studied. The results showed that the amount of glycoside products increased with the increase of incubation time and the

amount of products saturated at 24 hours. Hence, the optimal condition obtained was to incubate 0.25% (w/v) fisetin and 1% (w/v) β-CD with 200 U/ml of CGTase in 50 mM phosphate buffer (pH 6.0) containing 40% (v/v) DMSO at 40°C for 24 hours. Comparison between fisetin conversion of before and after optimization, we found that percent conversion under optimized condition was 70% which was higher than that of an un-optimized condition. It can clearly be seen that the reaction condition affected the yield of fisetin glycosides.

4.5 Purification of fisetin glycosides

Prior to the structure analysis of obtained fisetin glycosides, the glycoside products were subjected to purification. We expected that the different fisetin glycoside products possibly had different chemical properties (polarity and molecular weight), thus purification techniques including solvent extraction and chromatography were used.

4.5.1 Purification by preparative TLC

Preparative TLC is a common technique that has been used to purify various chemical compounds and natural products. Moreover, it has also been widely used for flavonoid glycoside purification. For example, resveratrol glycosides synthesized by CGTase (Anurutphan & Prousoontorn, 2015) and fisetin glycosides synthesized by engineered *E.coli* (Pandey et al., 2016). In this work, the TLC separating system was performed according to Pandey *et al.* (2016). The reaction mixture was loaded onto silica gel based TLC plate and was separated by mixture of solvents as described in section 2.12.1. Due to the fact that the surface of the silica gel is very polar and because of the hydroxyl groups, they can form hydrogen bonds, van der waals and dipole-dipole interactions with the proper compounds. As a result, the high polar products will be

adsorbed on the silica gel more strongly than the non-polar substrate (fisetin). Each separating band was scraped and pooled, and glycoside products were extracted by ethyl acetate (Pandey et al., 2016). From these results, we found that the concentration of glycoside products eluted from silica gel was extremely low. Moreover, some peaks that were not correlated to glycoside products were detected, indicating that purification of fisetin glycosides by preparative TLC was not effective. The reduction of fisetin glycoside yield after purification by TLC might be resulted from the interaction between glycosyl moieties and silica was too strong.

4.5.2 Purification by solvent extraction and HPLC

The aim of this work was to increase water solubility of fisetin by transglycosylation reaction. The derived fisetin glycosides had to have higher polarity than that of substrate fisetin. So, we hypothesized that substrate fisetin could be separated from reaction mixture using low-polar organic solvent extraction. In this work, various organic solvents with different polarity were exploited. The polarity index is shown in Table 7. We found that the large amount of fisetin was still retained when the reaction mixture was extracted by low polar solvent (such as dichloromethane, hexane and toluene). In contrast, almost all fisetin can be removed form aqueous phase when high polar solvents were applied. This finding might result from the fact that fisetin contain many of hydroxyl groups, and it might prefer to interact with polar molecules rather than non-polar ones. From this result, it can be concluded that butyl acetate was the most suitable organic solvent for fisetin glycoside extraction since the low amount of fisetin was detected in aqueous phase, and no peaks of glycoside products were detected in an organic phase.

Table 7 Polarity index of some organic solvents.

(https://people.chem.umass.edu/xray/solvent.html)

Solvent	**Polarity index**
Hexane	0
Toluene	2.4
Dichloromethane	3.1
1-Butanol	3.9
n-Butyl acetate	4.0
Chloroform	4.1
Ethyl acetate	4.4
Water	10.2

After that, the glycoside extract was further purified by HPLC. Since almost all fisetin was removed, the high concentration of fisetin glycoside mixture can be injected into HPLC equipped with fraction collector. Each product species was separated by solvent system as described in Section 2.12.3. From this experiment, we found that only 3 purified glycoside products can be purified by HPLC while the other two products showed some impurity. However, the purity of derived glycosides was enough for further structural analysis since they will be separated by liquid chromatography again prior to MS/MS analysis (LC-MS/MS).

4.6 Structural analysis of obtained fisetin glycosides

In order to be sure that the derived glycoside products were fisetin glycosides, many structural analysis techniques were performed. For the first analysis, after the transglycosylation reaction was carried out the reaction mixture was analyzed directly by MS. It revealed an $[M+Na]^+$ ion at m/z of 471 and 633 which correlated to the molecular weight of fisetin mono- and diglucosides with sodium ion, respectively. To confirm the type of bonding between glycosyl and fisetin and between glucose units, enzymatic analysis was performed. Schematic diagram of enzymatic analysis of fisetin glycosides by glucoamylase and α-glucosidase is shown in Figure 46. The hydrolysis patterns of fisetin glycosides by α-glucosidase visualized by TLC and HPLC indicated that glucose moieties linked to fisetin via alpha-glycosidic linkage. Moreover, it suggested that at least 2 species of fisetin monoglucosides were formed. The hydrolysis patterns of di- or triglucosides by glucoamylase indicated that the bonding between glucose units was α-1,4 glycosidic linkage.

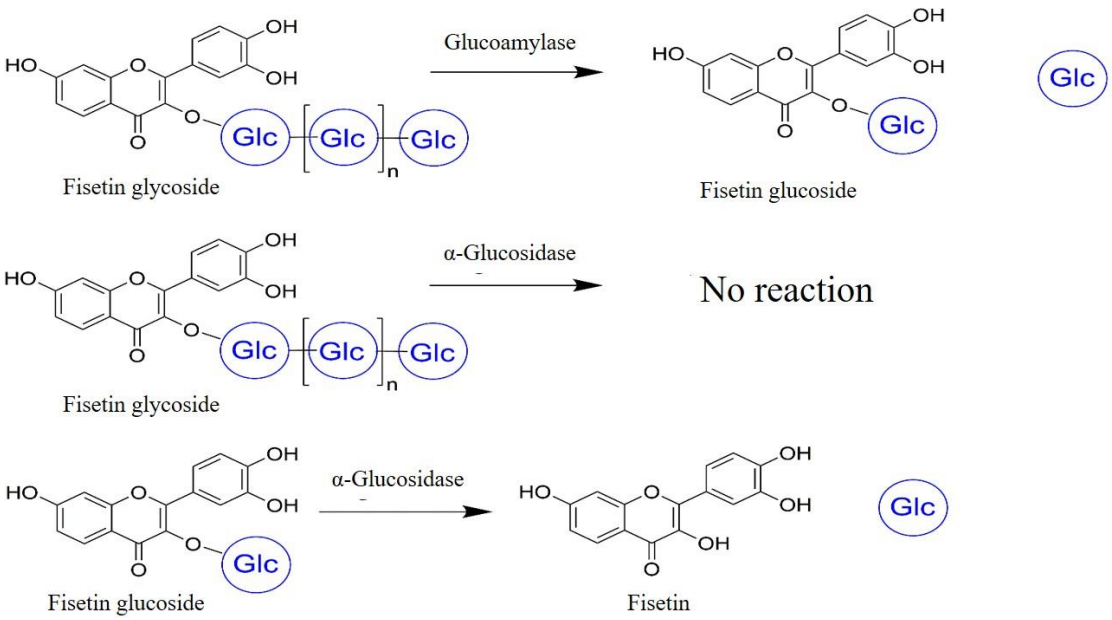


Figure 46 Schematic diagram of enzymatic analysis of fisetin glycosides by glucoamylase and α-glucosidase.

The position of glucose attached to fisetin was further determined by LC-MS/MS. The technique has widely been used to determine the possible structure of flavonoid glycosides (Viktoria & András, 2010). In mass spectrometry, the molecules that passed though the ionization chamber of a mass spectrometer will be dissociated into the fragment of energetically unstable molecular ions. These fragment ions are the evidence pattern that will used to determine the structure of the interested compounds. In this study, the transglycosylated products were first purified by solvent extraction and HPLC. The structure of fisetin monoglucosides were then investigated since their structures were less complicated among all glycoside products. The results showed that the glucose might be linked to fisetin at *O*-7 and *O*-4'/*O*-5' of 3-hydroxyflavone backbone of fisetin. However, it was difficult to identify the exact location of glucosyl unit by MS/MS since the fragmentation pattern of carbohydrate compounds was quite

complicated and very difficult to interpret (Figure 47). Moreover, the structure of fisetin 4'-*O*-glucoside and fisetin 5'-*O*-glucoside could not be distinguished by LC-MS/MS since they provided the same m/z of daughter fragment ion (Figure 48). In comparison to previous studies, Parajuli *et al.* (2015) and Pandey *et al.* (2016) reported that fisetin glycosides synthesized from engineered *E. coli* were both fisetin 3-*O*-glucoside and fisetin 3-*O*-rhamnoside (Figure 49). It implied that the molecular structure of fisetin glycosides is largely dependent on the enzyme used.

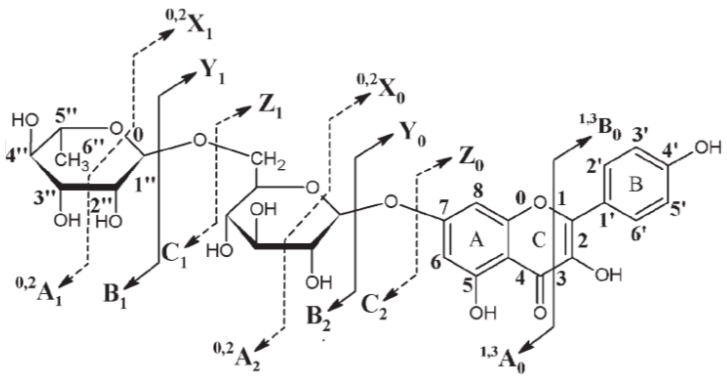


Figure 47 Fragment nomenclature commonly applied for O-glycosides (Viktoria & András, 2010).

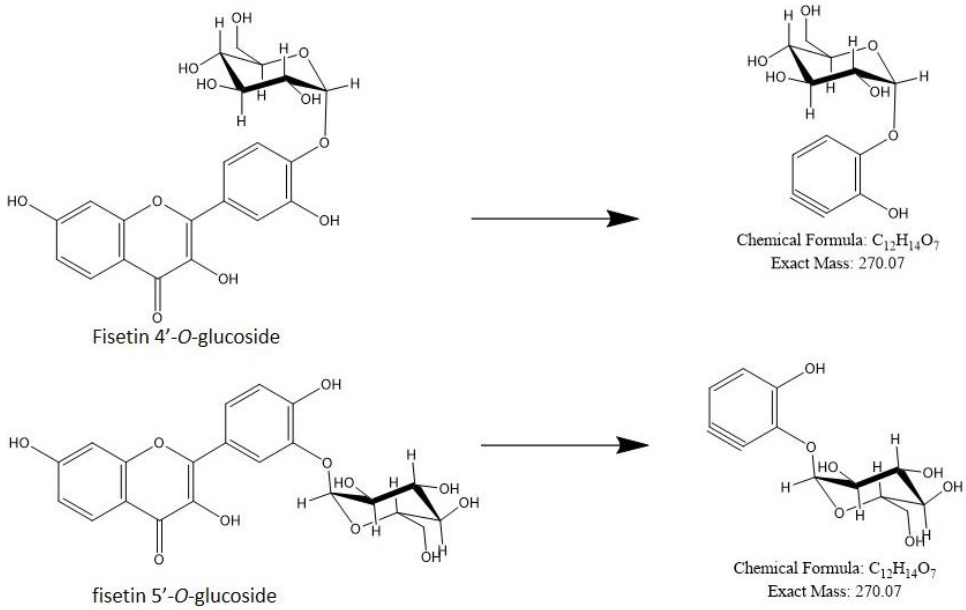


Figure 48 The possibility of fragmentation of fisetin 4'-O-glucoside and fisetin 5'-O-glucoside.

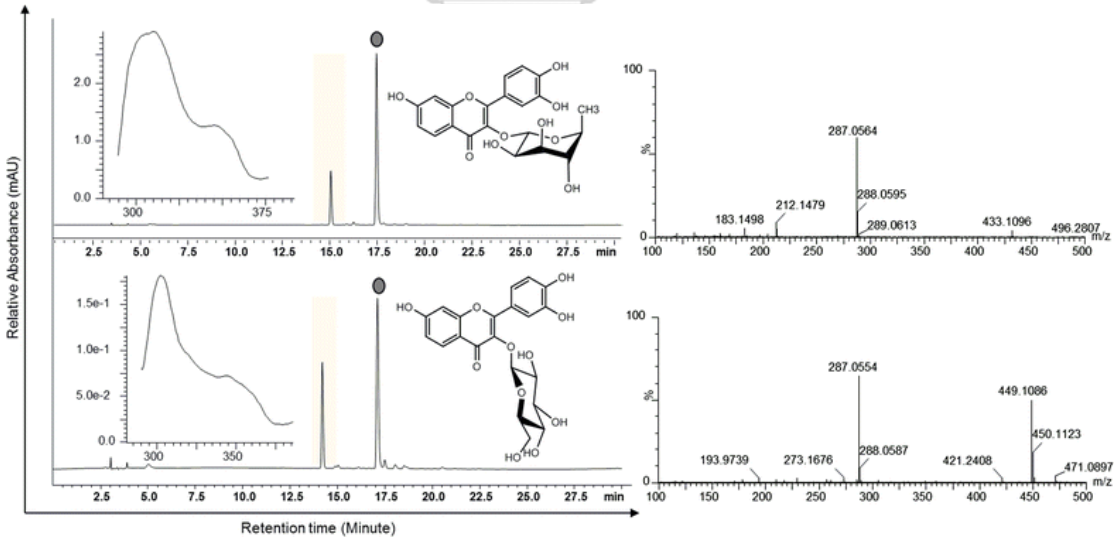


Figure 49 Fisetin glucoside and fisetin rhamnoside synthesized by engineered *E. coli* (P. Parajuli, R. Pandey, N. Trang, A. K. Chaudhary, & J. K. Sohng, 2015).

4.7 Water solubility of synthetic fisetin glycosides

The aim of this work was to increase the solubility of fisetin by transglycosylation. So the solubility of derived fisetin glycosides had to be measured. The purified fisetin glycosides were dissolved in exact known volume of water, and then the amount of soluble fisetin glycoside was measured by HPLC. However, the solubility of derived fisetin glycosides could not directly be determined since very low amount of glycoside products was produced, and thus the solution could not be saturated by the compounds. The molar concentration of each fisetin glycoside was determined from the molar concentration of fisetin after hydrolysis using HPLC. To determine the molar concentration of each product, the solution was then dried and re-dissolved in ultra-pure water prior to the analysis by HPLC. The result indicated that water solubility of fisetin glycosides was at least 500 times higher than the parent fisetin. The increase in water solubility of an organic compound after transglycosylation might be resulted from interaction between hydroxyl group of glycoside and water. There are several reports on the transglycosylation reaction to improve the bioavailability of some interesting compounds. For example, water solubility of hesperidin and naringin glycosides synthesized by CGTase were 300 and 1000 times higher than their substrates (Kometani et al., 1996). Epicatechin-3'-*O*-α-D-glucopyranoside had 44 times higher water solubility than unglucosylated epicatechin (Aramsangtienchai et al., 2011a). Resveratrol 3,5-*β*-D-diglucoside synthesized by UGT71A15 (a uridine 5'-diphosphate α-D-glucose-dependent glucosyltransferase from apple) was more water-soluble than unglucosylated molecule about 1,700 fold (Alexander, 2015).

4.8 Antioxidant activity

Scientific studies revealed that transformation of flavonoid to glycoside derivative might affect not only solubility, but also biological activity. For example, epicatechin glucosides synthesized by CGTase (Aramsangtienchai et al., 2011a) showed the lower antioxidant activity than that of epicatechin. Likewise, resveratrol-3-*β*-glucoside and resveratrol-3-*β*-maltoside showed lower antioxidant activity when compared to that of resveratrol (Sato et al., 2014). Moreover, Lepak *et al*. (2015) stated that "Although glycosylation be useful to enhance resveratrol solubility on resveratrol derivative to give the higher water solubility than unglucosylated molecule but unselective attachment of sugars could destroy the molecule's antioxidant activity" (Alexander et al., 2015). In comparison to this work, antioxidant activity of fisetin was not changed by transglycosylation reaction. All fisetin glycosides still expressed the similar antioxidant activity to the original fisetin. This result suggested that the existence of glycoside moieties did not involve in scavenging ability of fisetin.

CHAPTER V
CONCLUSIONS

1. CGTase from *Paenibacillus* sp. RB01 was partial purified by starch absorption technique. The yield and purification fold were found to be 31% and 34, respectively and specific activity of purified CGTase was 5033 U/mg protein (in terms of dextrinizing activity).

2. The concentration of DMSO in reaction mixture largely had an effect on CGTase stability. The dextrinizing activities of CGTase were dramatically decreased when DMSO concentration increased.

4. Potato soluble starch was found to be the best glycosyl donor. However, due to the limitation of solubility of starch in HPLC system, β-cyclodextrin with a slight lower production yield than that of potato soluble starch was then chosen as an appropriate glycosyl donor.

5. DMSO at 40% (v/v) gave the highest production yield of tranglycosylation reaction of fisetin glycosides by CGTase.

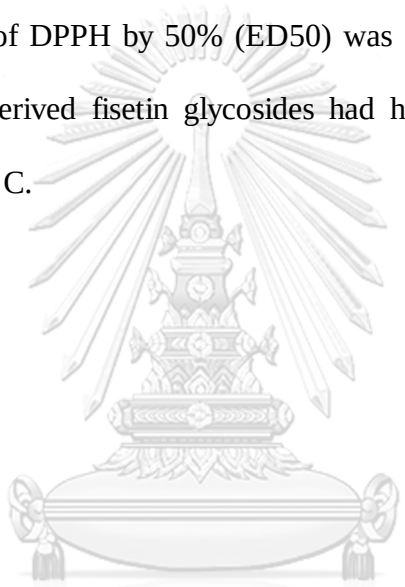
6. Under optimum condition (0.25% (w/v) of fisetin in 40% (v/v) DMSO, 1.0% (w/v) of β-cyclodextrin with 200 U/ml of CGTase at 40°C for 48 hours) for transglycosylation reaction, the fisetin conversion under optimum condition (17.1% ± 4.21%) significantly increased when compared to that of non-optimized condition (10.1% ± 1.15%).

7. The molecular mass of product 1 (Rt ~16.5 minutes) and 3 (Rt ~14.7 minutes) were corresponded to the mass of fisetin monoglucoside (m/z 447), while product 2 (Rt ~15.3

minutes) and 5 (Rt ~13.4 minutes) were fisetin diglucosides (m/z 609) and product 4 (Rt ~13.9 minutes) was fisetin triglucoside (m/z 771).

8. The water solubility of fisetin glycosides increased at least 500 times higher than that of the parent fisetin.

9. The concentration of fisetin monoglucoside (Rt ~16.5 minutes), fisetin monoglucoside (Rt ~14.7 minutes) and fisetin diglucoside (Rt ~13.4 minutes) required for scavenging radical of DPPH by 50% (ED50) was 2.72, 2.28, 2.52 and 2.28 µM, respectively. So, the derived fisetin glycosides had higher antioxidant activity than fisetin and also vitamin C.



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APPENDIX

Appendix A: Preparation of polyacrylamide gel electrophoresis

I) Stock reagents

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

Tris (hydroxymethyl)-aminomethane 24.2 g

Then, adjusted pH to 8.8 using 1 M HCl and adjusted volume to 100 ml with distilled water.

1.5 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

Then, adjusted pH to 8.8 using 1 M HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

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

0.5 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

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

10% (w/v) SDS

Sodium dodecyl sulfate 10 g

Then, adjusted volume to 100 ml with distilled water.

50% (v/v) Glycerol

Glycerol	50 mL

Then, adjusted volume to 100 ml with distilled water.

1% (w/v) Bromophenol blue

Bromophenol blue	0.1 g

Adjusted volume to 10 ml with distilled water. Next, solution was filtered to eliminate the aggregated dye.

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

Ammonium persulfate	0.5 g

Then, adjusted volume to 5 ml with distilled water.

- Solution B

2 M Tris-HCl pH 8.8	75 ml
10% (w/v) SDS	4 ml

Then, adjusted volume to 100 ml with distilled water.

- Solution C

1 M Tris-HCl pH 6.8	50 ml
10% (w/v) SDS	4 ml

Then, adjusted volume to 100 ml with distilled water.

II) Working solutions

Native-PAGE

- 7.5 % separating gel

Solution A	1.41 ml
1.5 M Tris-HCl pH 8.8	2.50 ml
Distilled water	3.49 ml
10% (w/v) $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

- 5.0 % stacking gel

Solution A	0.32 ml
0.5 M Tris-HCl pH 6.8	0.50 ml
Distilled water	1.70 ml
$(NH_4)_2S_2O_8$	25 µl
TEMED	3 µl

- Sample buffer

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

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

- Electrophoresis buffer

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g

Then, adjusted volume to 1 L with distilled water (pH should be approximately 8.3).

SDS-PAGE

- 7.5 % separating gel

Solution A	1.41 ml
Solution B	2.50 ml
Distilled water	3.49 ml
10 % (w/v) $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

- 5.0 % stacking gel

Solution A	0.32 ml
Solution C	0.50 ml
Distilled water	1.70 ml
10% (w/v) $(NH4)_2S_2O_8$	25 µl
TEMED	3 µl

- Sample buffer

1 M Tris-HCl pH 6.8	0.6 ml
50% (v/v) Glycerol	5.0 ml

10% (w/v) SDS	2.0 ml
2-Mercaptoethanol	0.5 ml
1% (w/v) Bromophenol blue	1.0 ml
Distilled water	0.9 ml

One part of sample buffer was added to four part of sample. The mixture was boiled for 5 minutes before loading to the gel.

- Electrophoresis buffer

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

Then, adjusted volume to 1 L with distilled water (pH should be approximately 8.3).

Appendix B: Preparation of buffer solution

200 mM phosphate buffer, pH 6.0

200 mM di-Potassium hydrogen phosphate (K_2HPO_4)	100 ml
200 mM Potassium di-hydrogen phosphate (KH_2PO_4)	50 ml

Used 200 mM KH_2PO_4 to adjust pH of 200 mM K_2HPO_4 to pH 6.0

50 mM phosphate buffer, pH 6.0

50 mM di-Potassium hydrogen phosphate (K_2HPO_4)	100 ml
50 mM Potassium di-hydrogen phosphate (KH_2PO_4)	50 ml

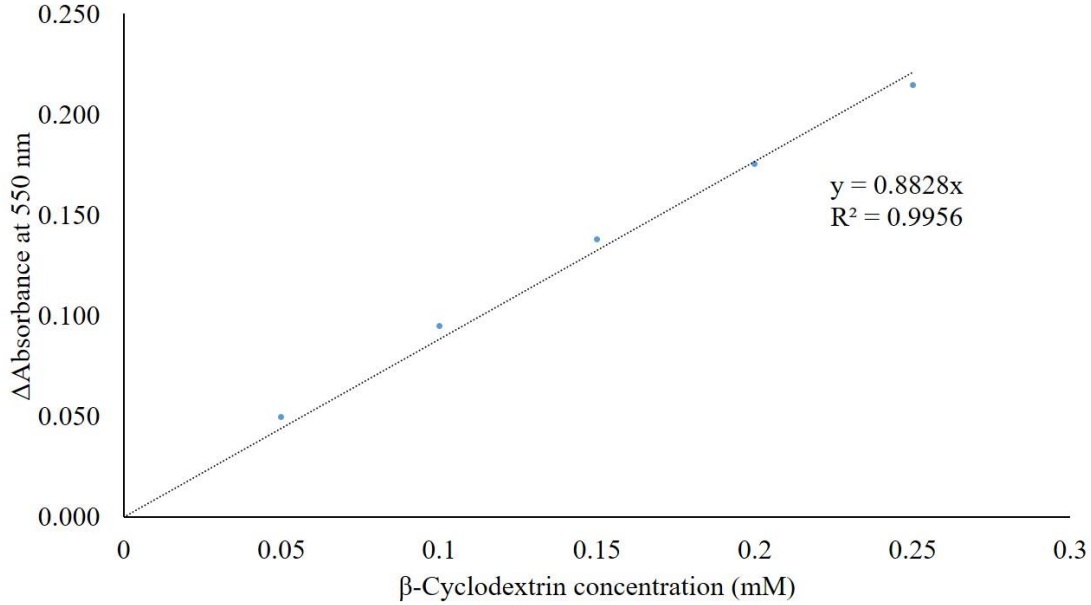
Used 50 mM KH_2PO_4 to adjust pH of 50 mM K_2HPO_4 to pH 6.0

50 mM Tris-glycine buffer, pH 8.0

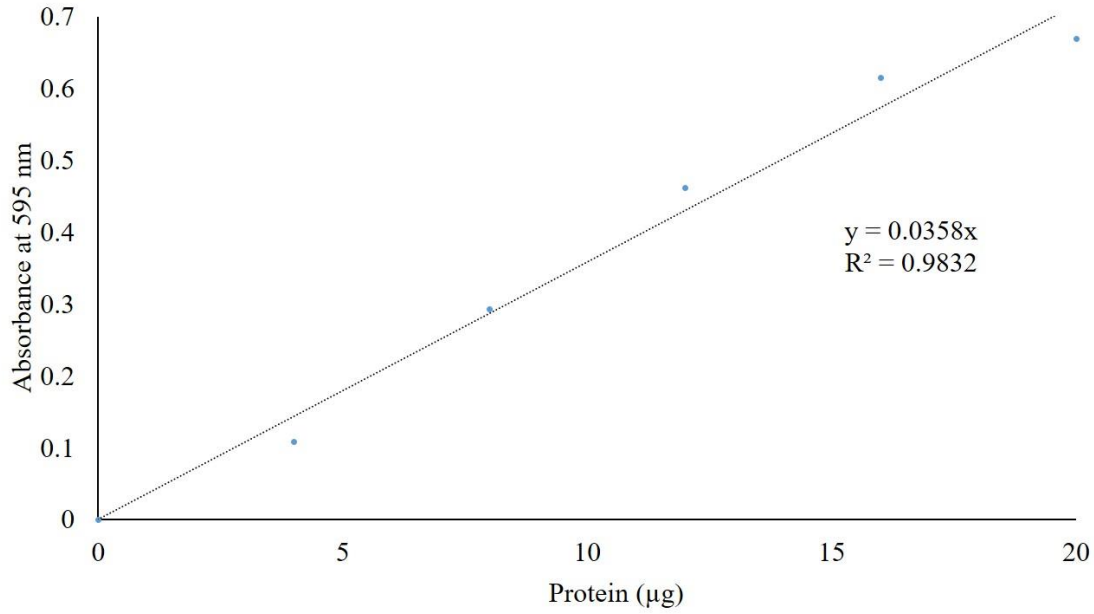
Tris (hydroxymethyl)-aminomethane	0.303 g
Glycine	0.188 g

Then, adjusted volume to 50 ml with distilled water and adjusted pH to 8.0 using 1 M NaOH.

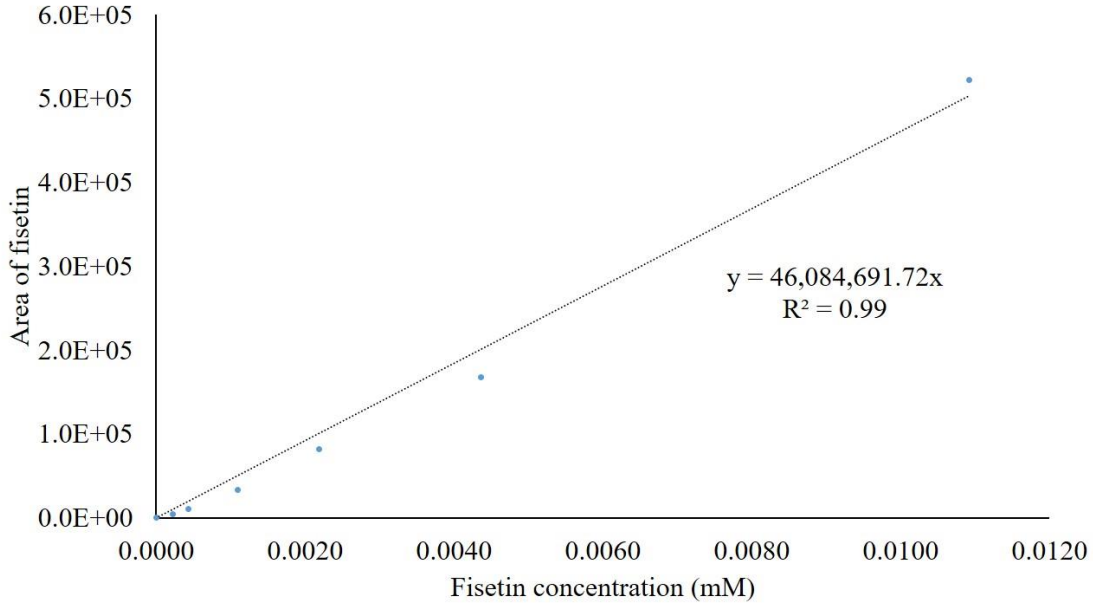
Appendix C: Standard curve for β-CD determination by Phenolphthalein method.



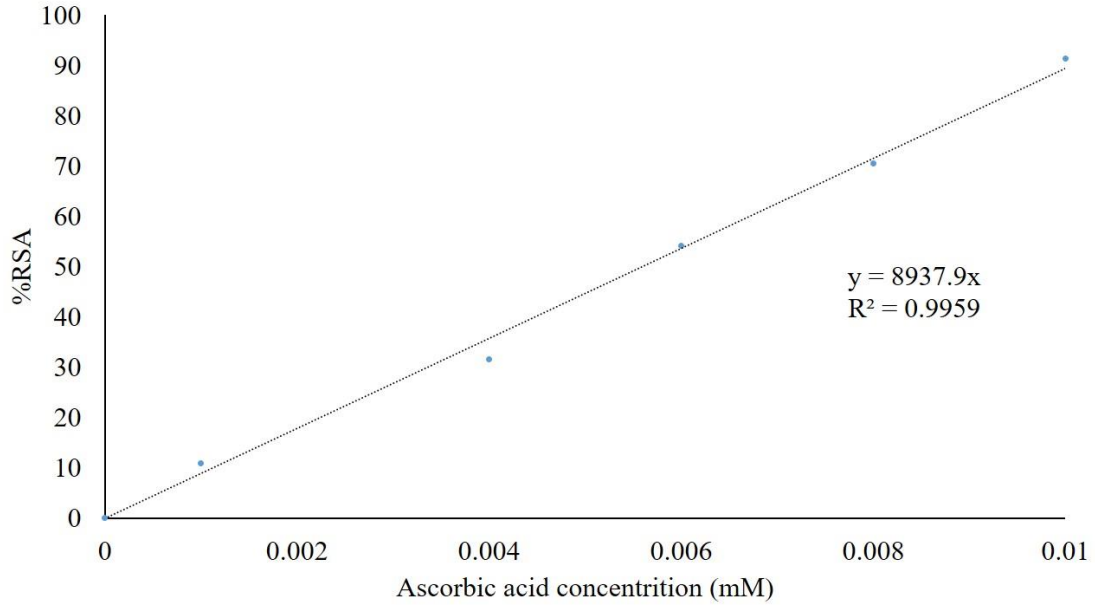
Appendix D: Standard curve for protein determination by Bradford's method.



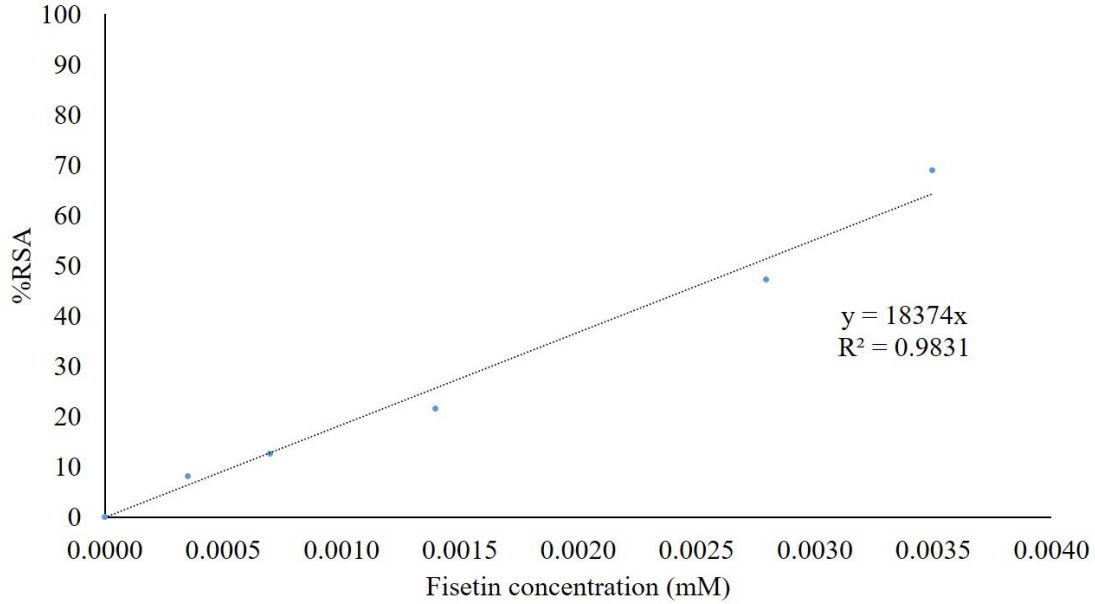
Appendix E: Standard curve for fisetin concentration determination by hydrolysis in acid solution.



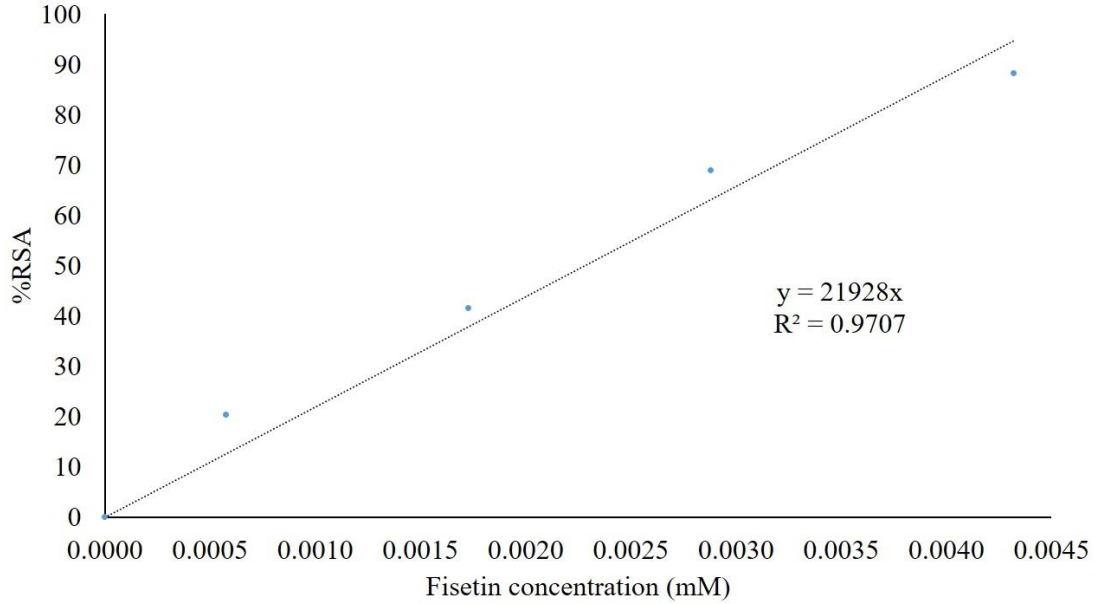
Appendix F: Standard curve for antioxidant determination of ascorbic acid by DPPH assay.



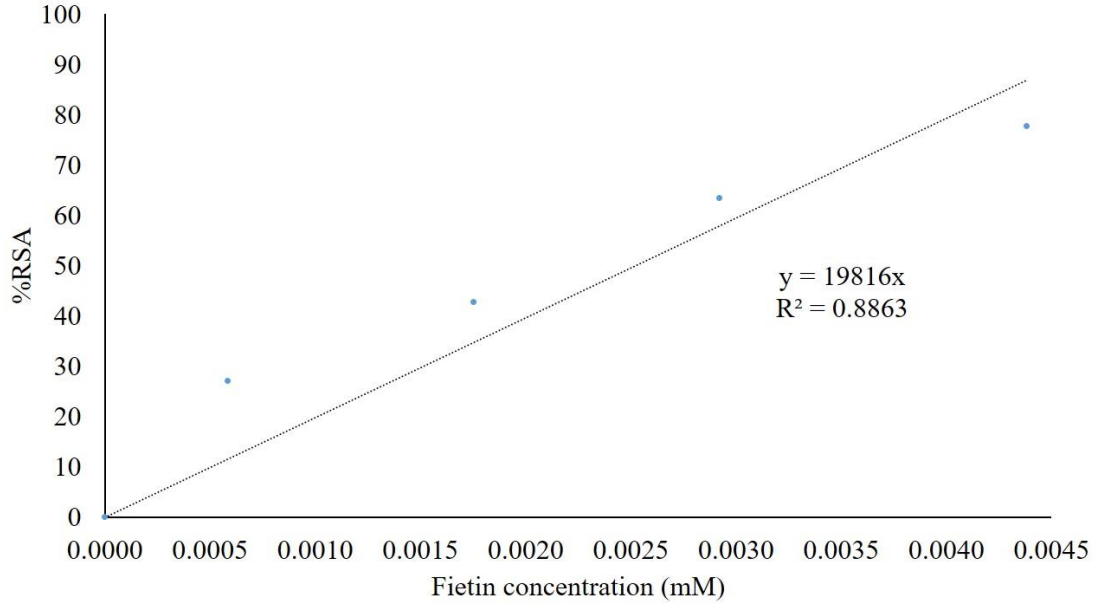
Appendix G: Standard curve for antioxidant determination of fisetin by DPPH assay.



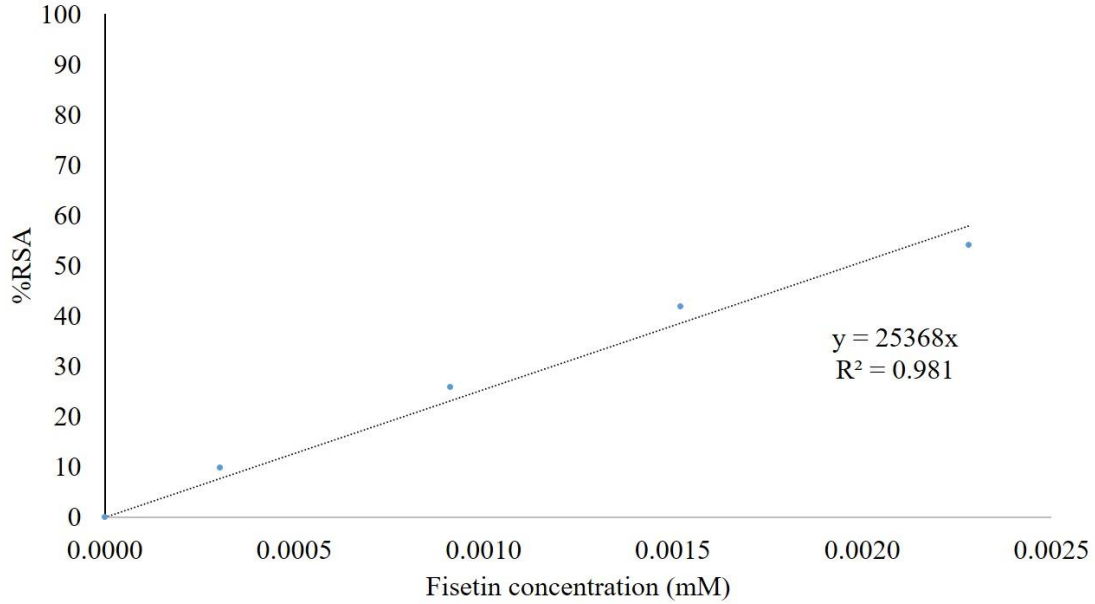
Appendix H: Standard curve for antioxidant determination of product 1 by DPPH assay.



Appendix I: Standard curve for antioxidant determination of product 3 by DPPH assay.



Appendix J: Standard curve for antioxidant determination of product 5 by DPPH assay.



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