การสังเคราะห์เรสเวอราทรอลไกลโคไซด์โดยไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรส
จาก *Paenibacillus* sp. RB01

นางสาวณัฐญา อนุรัฐพันธุ์

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

Miss Nattaya Anurutphan

A Thesis Submitted in Partial Fulfillment of the Requirements
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Field of Study	Biochemistry and Molecular Biology
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ณัฐญา อนุรัฐพันธุ์ : การสังเคราะห์เรสเวอราทรอลไกลโคไซด์โดยไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรสจาก *Paenibacillus* sp. RB01 (SYNTHESIS OF RESVERATROL GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. มัญชุมาส เพราะสุนทร, 118 หน้า.

งานวิจัยนี้ ได้ทำการสังเคราะห์เรสเวอราทรอลไกลโคไซด์ผ่านปฏิกิริยาทรานส์ไกลโคซิเลชันโดยใช้บีตาไซโคลเดกซ์ทรินเป็นตัวให้ไปยังตัวรับคือเรสเวอราทรอล ซึ่งเร่งปฏิกิริยาโดยเอนไซม์ไซโคลเดกซ์ทรินไกลโคซิลแทรนส์เฟอเรส (CGTase) จากแบคทีเรีย *Paenibacillus* sp.RB01 ทำการตรวจสอบผลของความเข้มข้น DMSO ต่อความเสถียรของเอนไซม์ CGTase พบว่า เมื่อความเข้มข้นของ DMSO เพิ่มขึ้น ความเสถียรของเอนไซม์ CGTase ลดลงอย่างเห็นได้ชัด โดยบีตาไซโคลเดกซ์ทรินถูกเลือกให้เป็นตัวให้หมู่ไกลโคซิลที่เหมาะสม และพบว่า DMSO ที่ความเข้มข้น 20% (v/v) เป็นความเข้มข้นที่ให้ผลิตภัณฑ์สูงสุด จากการตรวจสอบความสามารถในการสังเคราะห์เรสเวอราทรอลไกลโคไซด์ผ่านปฏิกิริยาทรานส์ไกลโคซิเลชัน พบว่า เมื่อทำการบ่ม เรสเวอราทรอล 1.0% (w/v) ใน DMSO 20% (v/v), บีตาไซโคลเดกซ์ทริน 1.0% (w/v) และเอนไซม์ CGTase 100 U/ml ที่ 40 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง พบผลิตภัณฑ์ที่เกิดขึ้น 2 ผลิตภัณฑ์โดยมีค่า R_f เท่ากับ 0.55 และ 0.77 จากการศึกษาสมบัติเบื้องต้นของผลิตภัณฑ์ โดยใช้กลูโคแอมิเลสและแอลฟากลูโคซิเดส พบว่า ผลิตภัณฑ์เป็นอนุพันธ์ของกลูโคไซด์ และโมเลกุลของกลูโคสเชื่อมต่อด้วยพันธะ α-1,4 กับเรสเวอราทรอล ภายใต้การผลิตในภาวะที่เหมาะสม พบปริมาณผลิตภัณฑ์เพิ่มขึ้น 3.71 เท่า ทำการแยกผลิตภัณฑ์หลักที่เกิดขึ้นโดยเทคนิค HPLC และทำการวิเคราะห์ขนาดและโครงสร้างของโมเลกุลโดยเทคนิค MS และ LC-MS/MS ตามลำดับ พบว่า ผลิตภัณฑ์หลัก (เรสเวอราทรอลไกลโคไซด์ I, Rt~13.6 นาที) มีขนาด 390 ดาลตัน ซึ่งตรงกับเรสเวอราทรอลมอนอกลูโคไซด์ที่เชื่อมต่อด้วยหมู่กลูโคสที่ตำแหน่ง 3-ไฮดรอกซิลของเรสเวอราทรอล จากการศึกษาสมบัติการละลายน้ำของเรสเวอราทรอลไกลโคไซด์ I (Rt~13.6 นาที) และ II (Rt~12.8 นาที) พบว่า มีค่าสูงกว่าค่าการละลายของเรสเวอราทรอล ประมาณ 72 และ 26 เท่า ตามลำดับ ขณะที่ค่าความสามารถในการเป็นสารต้านออกซิเดชันโดยวิธีการใช้อนุมูลอิสระ DPPH• ของผลิตภัณฑ์หลักทั้งสอง มีค่าต่ำกว่าค่าของเรสเวอราทรอลและวิตามินซี

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5571970223 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
KEYWORDS: CYCLODEXTRIN GLYCOSYLTRANSFERASE, RESVERATROL, TRANSGLYCOSYLATION

NATTAYA ANURUTPHAN: SYNTHESIS OF RESVERATROL GLYCOSIDES BY CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Paenibacillus* sp. RB01. ADVISOR: ASST. PROF. MANCHUMAS PROUSOONTORN, Ph.D., 118 pp.

In this work, resveratrol glycosides were synthesized by transglycosylation reaction from β-cyclodextrin (β-CD) donor to resveratrol catalyzed by cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp.RB01. The effect of DMSO on CGTase stability was determined. The result showed that the enzyme activities were dramatically decreased when DMSO concentration increased. β-cyclodextrin was chosen as an appropriate glycosyl donor and it was found that 20% (v/v) of DMSO gave the highest production yield. To investigate its synthesis ability, the reaction mixture containing 1.0% (w/v) of resveratrol in 20% (v/v) of DMSO and 1.0% (w/v) of β-cyclodextrin were incubated with 100 U/ml of CGTase at 40°C for 24 hours. Two spots of the expected products were observed at R_f of 0.55 and 0.77. The products were preliminary characterized by glucoamylase and α-glucosidase and it was concluded that the product was glycoside derivative and glucose molecules was attached to resveratrol with α-1,4-linkage. After optimization, the yield was increased by 3.71 times. The main product was collected by HPLC and its molecular mass and its possible structure were analyzed by MS and LC-MS/MS techniques, respectively. The molecular mass of the main product (resveratrol glycoside I, Rt~13.6 minutes) was found to be 390 Daltons which corresponded to resveratrol monoglucoside having one monoglucosyl group attached to the position of 3-hydroxyl group of resveratrol. The water solubility of resveratrol glycoside I (Rt ~13.6 minutes) and II (Rt ~12.8 minutes) was about 72 and 26 times higher than that of resveratrol, respectively. While the DPPH• scavenging effect (%) of both of resveratrol glycoside I and II were lower than those of resveratrol and vitamin C.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
Academic Year:	2015		

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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
R_f	Relative mobility
Rt	Relation time
SDS	Sodium dodecyl sulfate
TLC	Thin Layer Chromatography
U	Unit(s)
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I
INTRODUCTION

1.1 Dietary polyphenols

Dietary polyphenols have received considerable attention among nutritionists, food scientists and consumers due to their roles in human health. Research in recent years intensely supports a role of polyphenols in the prevention of development of cancers, degenerative diseases, cardiovascular diseases and neurodegenerative diseases (Pandey and Rizvi 2009). Polyphenols are strong antioxidants that complement and add to the functions of antioxidant vitamins and enzymes as a defense against oxidative stress caused by excess reactive oxygen species (ROS) (Tsao 2010). Several thousand different phenolic structures have been identified in plants and it can be devided into four main groups by the chemical structures, which are flavonoids, phenolic acids, stilbene and lignans (Figure 1).

Flavonoids are secondary metabolites of plants, found largely in the fruits, vegetables, cereals and beverages. It can be divided into two types: anthocyanins and anthoxantins. Anthoxantins can be further classified into 5 classes: flavonols, flavanones, flavanols, flavones and isoflavones and anthocyanins. Some of the most common flavonoids are quercetin, a flavonol found in tea, apple and tomatoes; hesperetin, a flavanone found in citrus fruits; catechin, a flavanol abundant in green tea; daidzein, the main isoflavone in soybeans and soy products. (D'Archivio *et al.* 2007)

Phenolic acids are found in a variety of plant-based foods especially in the seeds, the skins of fruits and the leaves of vegetables. There are many different

phenolic acids found in nature and they can be divided into two categories: benzoic acid derivatives such as gallic acid present in tea and grape seeds and cinnamic acid derivatives including caffeic acid, present in many fruits and vegetables. The cinnamic acids are more common than the benzoic acids.

In addition to the flavonoids and phenolic acids there are several non-flavonoid polyphenols found in foods and plants that are considered important to human health. One of these is resveratrol. Resveratrol has been the subject of intense interest due to its range of unique biological properties. These include antioxidant, anti-inflammatory, cardioprotective, neuroprotective, cancer preventive and therapeutic activities. Recent results provide interesting insights into the effect of this compound on the life span of yeast (Howitz *et al.* 2003) and mammals (Baur *et al.* 2006).

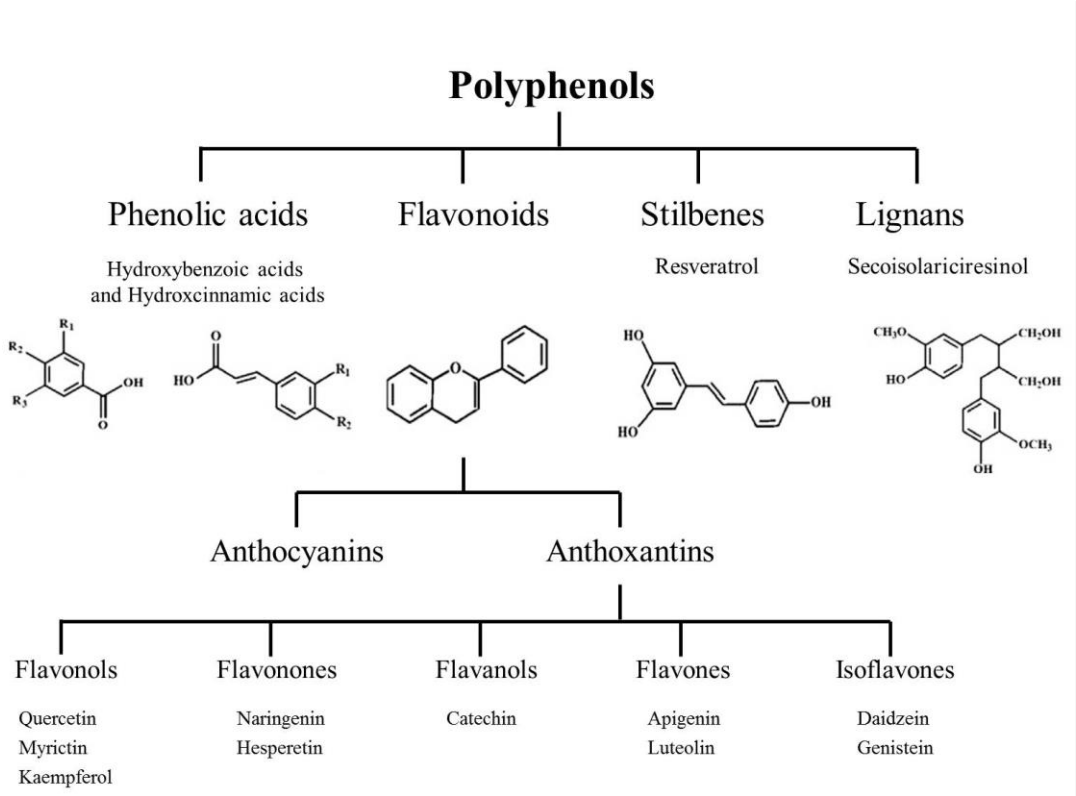


Figure 1 Classification and structure of the main polyphenols classes (adapted from (Câmara *et al.* 2013)).

1.2 Resveratrol

1.2.1 General information of resveratrol

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is one of polyphenols stilbene found in foods and plants; it can be produced in a wide variety of plants as phytoalexin as a part of a plant's defense system response to several harmful factors against disease, stress, injury, fungal infection and UV irradiation (Yan *et al.* 2010) and resveratrol is a compound that consists of 2 aromatic rings joined by a methylene bridge (Figure 2). The physical and chemical properties of *trans*-resveratrol is shown in table 1. The biosynthesis of resveratrol was catalyzed by stilbene synthase, consists in the repetitive decarboxylative condensation of a *p*-coumaroyl residue from *p*-coumaroyl-CoA with three C2-units from malonyl-CoA (Figure 3) (Signorelli and Ghidoni 2005).

1.2.2 Sources of resveratrol

Though the molecule of resveratrol was first isolated and characterized for its chemical structure from the roots of white hellebore (*Veratum grandiflorum O. Loes*) by Takaoka in 1940 and from the roots of *Polygonum cupsidatum* in 1963, recent studies have demonstrated that the most abundant natural sources of resveratrol are grapes. It can be found in the vines; roots, seeds and stalks but its highest concentration is in the peel of the grapes (Celotti *et al.* 1996), especially in red and purple grapes which are used to make red wines *(Vitis vinifera*, *labrusca*, and *muscadine)*. Resveratrol does not only present in grapes, but also found in a wide variety of plants such as eucalyptus, spruce, and lily and in other foods such as peanuts and some berries e.g. mulberries and blueberry (Table 2).

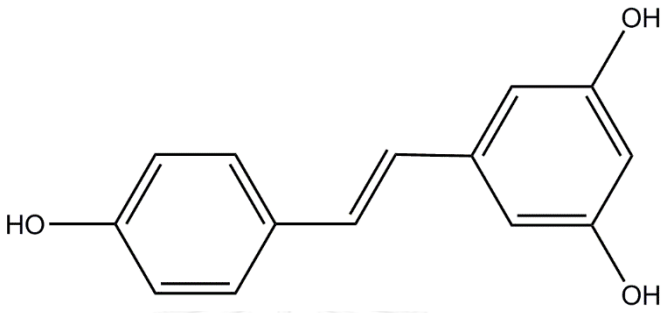


Figure 2 Structure of *trans*-resveratrol.

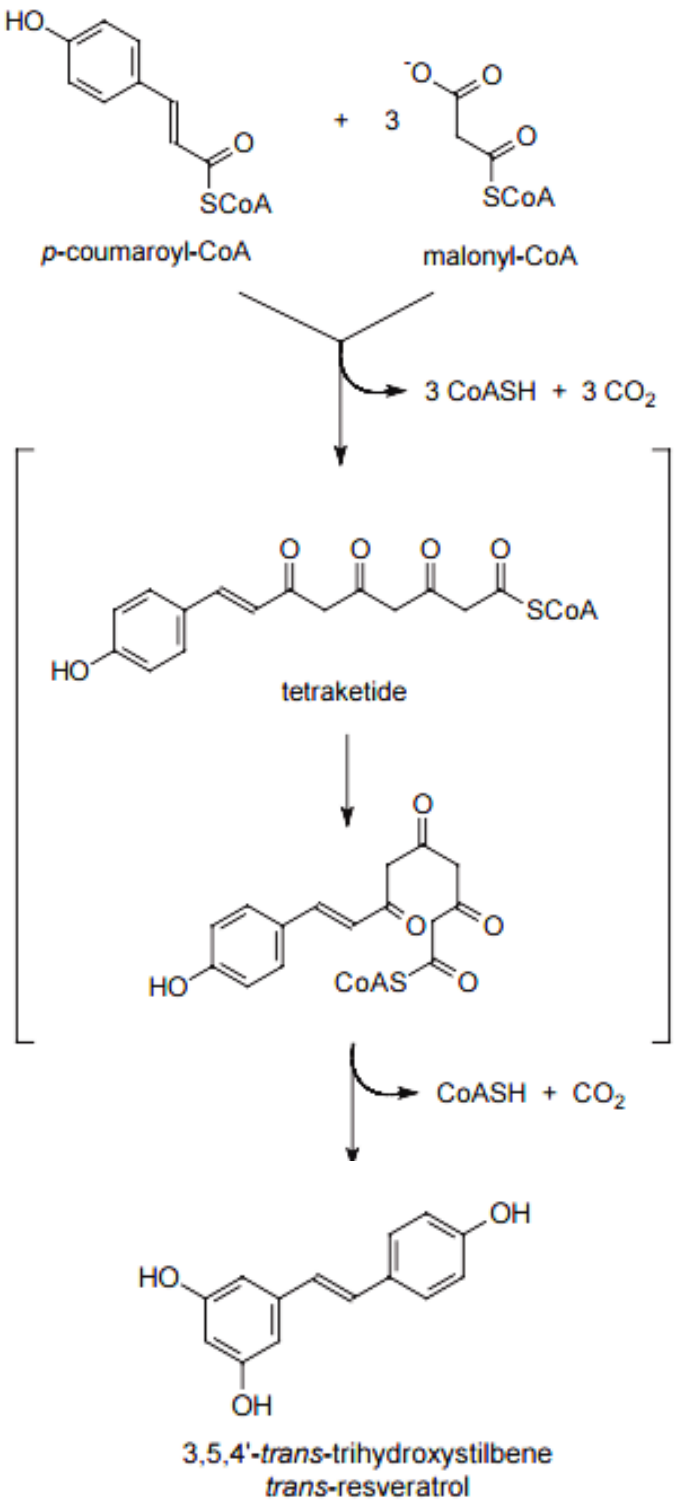


Figure 3 Biosynthetic pathway of resveratrol. (Signorelli and Ghidoni 2005)

Table 1 Physical and chemical properties of *trans*-resveratrol.

Properties	Characteristics
Name	3,4′,5-Trihydroxy-trans-stilbene 5-[(1E)-2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol
Formula	$C_{14}H_{12}O_3$
Molecular weight	228.24
Melting point	261 to 263 °C (502 to 505 °F; 534 to 536 K)
Solubility	50 mg/ml in acetone 0.03 g/L in water 16 g/L in DMSO 50 g/L in ethanol
Solubility (Color)	Clear
Solubility (Turbidity)	Faint Yellow
Toxicity	The 50% lethal dose (LD50) = 23.2 mM [in human LX-2 hepatic stellate cells] (Bechmann *et al*., 2009)

Table 2 Resveratrol content in foods from the database on polyphenol content in foods. (phenol-explorer.eu)

Sources	Content
Muscadine grape, red wine	3.02 mg/100 ml
Lingonberry	3.00 mg/100 g FW*
European cranberry	1.92 mg/100 g FW
Redcurrant	1.57 mg/100 g FW
Bilberry	0.67 mg/100 g FW
Strawberry	0.35 mg/100 g FW
Red wine	0.27 mg/100 ml
Fox grape, red wine	0.25 mg/100 ml
Black grape	0.15 mg/100 g FW
Rosé wine	0.12 mg/100 ml
Dehulled pistachio	0.11 mg/100 g FW
Peanut	0.08 mg/100 g FW
Dehulled peanut	0.07 mg/100 g FW
White wine	0.04 mg/100 ml
Chocolate, dark	0.04 mg/100 g FW
Peanut butter	0.04 mg/100 g FW
Roasted dehulled peanut	0.02 mg/100 g FW
Green grape	0.02 mg/100 g FW
Fox grape, white wine	0.01 mg/100 ml
Champagne	9.00e-03 mg/100 ml

* FW = fresh weight

1.2.3 Benefits of resveratrol

Resveratrol is one of the most studied polyphenol molecules and has gained a lot of attention on its reported in biological and pharmacological properties especially on disease-combating benefits. Many researches mostly done both *in vivo* and *in vitro* suggested that resveratrol might help protect the body against a number of diseases including:

1.2.3.1 Risk reduction for cardiovascular disease

Resveratrol can prevent the oxidation of LDL; a bad cholesterol, and resveratrol makes it more difficult for platelets to stick together and form the clots that can lead to a heart attack. Recent studies have demonstrated that resveratrol is able to reduce risk of cardiovascular disease. Resveratrol can pharmacologically precondition (PC) the heart through a nitric oxide (NO) dependent and adenosine receptors mediated mechanism (Das and Maulik 2006). In addition, it was demonstrated that the cardioprotective effects of resveratrol in hypoxic injury are mediated via the mechanisms of anti-apoptosis (Kim *et al.* 2007).

1.2.3.2 Antioxidant activity

Resveratrol was found to be an effective antioxidant in different in vitro assays including: total antioxidant activity, reducing power, DPPH•, ABTS•+, DMPD•+ and O2•− radical scavenging, hydrogen peroxide scavenging, and metal chelating activities. In summary, resveratrol is an effective antioxidant which can be used for minimizing or preventing lipid oxidation in pharmaceutical products, retarding the formation of toxic

oxidation products, maintaining nutritional quality, and prolonging the shelf life of pharmaceuticals (Gülçin 2010).

1.2.3.3 Anticancer activity

Various researches reporting the benefits of resveratrol in terms of anti-cancer properties in a variety of tumor cell lines were reported. The molecular mechanisms of resveratrol-induced apoptosis in colon cancer cells, with special attention to the role of the death receptor Fas in this pathway was study. Their results indicate that the ability of resveratrol to induce the redistribution of Fas receptor in membrane rafts may contribute to the molecule's ability to trigger apoptosis in colon cancer cells (Delmas *et al.* 2003).In addition, it was reported that resveratrol was shown to induce apoptosis in human breast cancer cells primarily through the caspase-3-dependent pathway in conclude that resveratrol be able to prevent the human breast cancer (Alkhalaf *et al.* 2008). And (Hsieh and Wu 2010) analysis on published data strengthen support that resveratrol displays novel roles in various cellular processes and help to establish an expanded molecular framework for cancer prevention by resveratrol *in vivo*.

1.2.3.4 Anti-inflammatory and neuroprotective

From Figure 4, the summary of the neuroprotective action of resveratrol was shown. Resveratrol also can attenuate the activation of immune cells and the subsequent synthesis and release of pro-inflammatory mediators through the inhibition of the transcriptional factors such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) (Zhang *et al.* 2010) and be able to mediate anti-inflammatory processes mainly inhibiting the

expression of cyclooxygenase-1 and 2 (COX-1 and 2) and hydroperoxidase functions. It also shows estrogenic properties towards different cell lines (Fremont 2000, King *et al.* 2006).

Resveratrol promotes the non-amyloidogenic cleavage of the amyloid precursor protein, enhances clearance of amyloid beta-peptides, and reduces neuronal damage by reducing GSK-3β activity and may promote Aβ clearance caused of alzheimer's disease that characterized by the presence of amyloid plaques in the brain, which comprise mainly β-amyloid peptide and α_1-antichymotrypsin (Vandenabeele and Fiers 1991)

Treatment with resveratrol can affect multiple signaling pathway effectors involved in cell survival, programmed cell death and synaptic plasticity; for cell survival, resveratrol is a potent activator of AMP-activated protein kinase (AMPK), a key regulator of cell survival in response to oxidative stress insults (Shibata *et al.* 2015); In programmed cell death, The apoptosis-inducing factor (AIF) is a mitochondrial oxido-reductase originally characterized as a mediator of caspase-independent programmed cell death (PCD) (Yu *et al.* 2010) and in the synaptic plasticity, resveratrol pretreatment effectively restore the synaptic plasticity in Chronic cerebral hypoperfusion (CCH) rats both functional and structural (Li *et al.* 2016). Direct and/or indirect activation of the deacetylase sirtuins by resveratrol has also been suggested (Bastianetto *et al.* 2015).

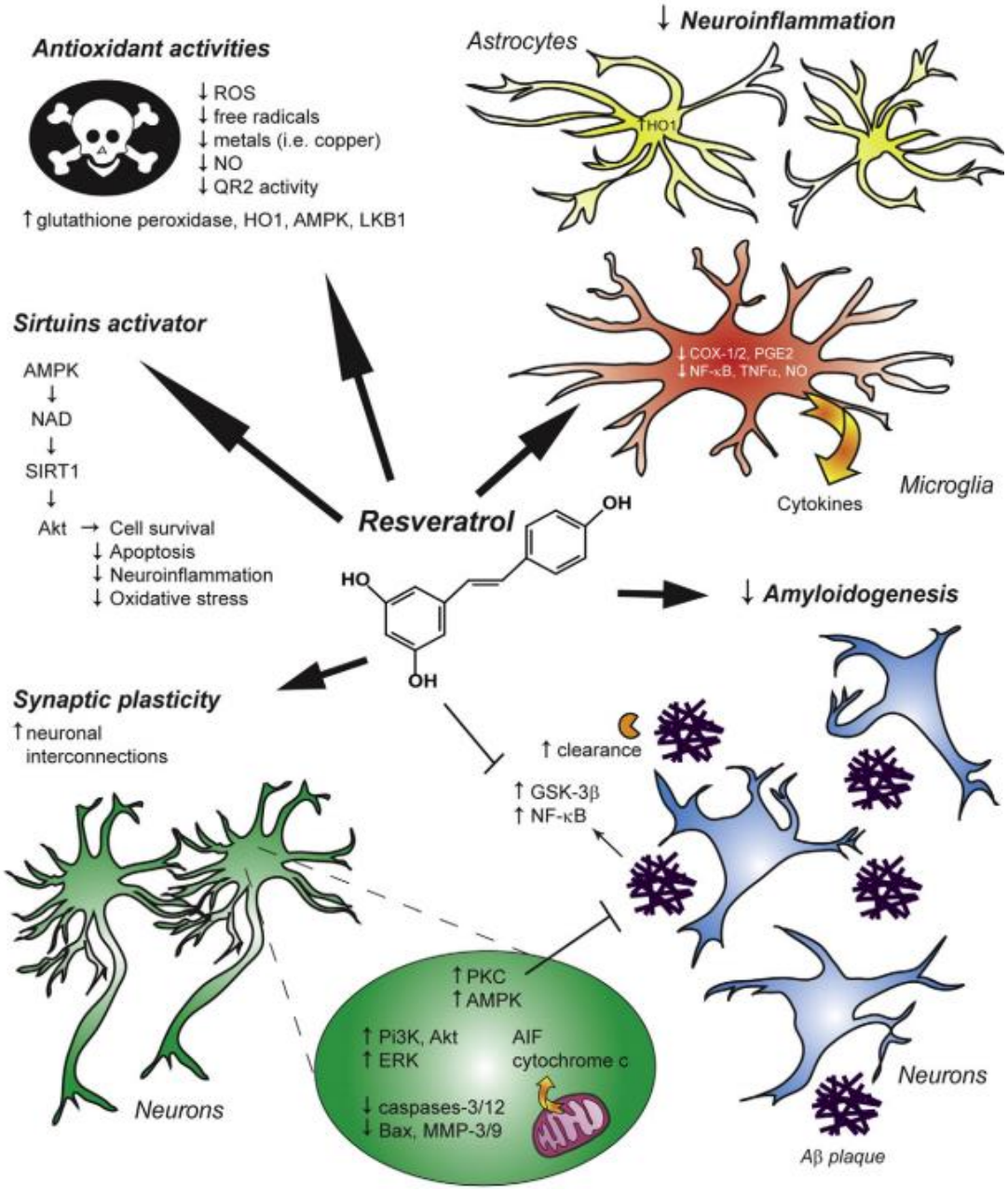


Figure 4 Summary of the neuroprotective action of resveratrol (Bastianetto *et al.* 2015).

1.2.4 Limitation of resveratrol and the improvement

Although, resveratrol has been applied for multipurpose use with their wide variety of benefits, there are still some limitations in the low water solubility. However, many researchers try to studying on how to improve their water-insoluble of resveratrol. Numerous methods has been reported. Examples as follow:

- The improvement of resveratrol solubility through micellar and liposomal incorporation was reported. The result was showed that keto derivatives of cholic acid have greater ability to solubilize resveratrol than cholic acid, and that this efficiency increases with the number of keto groups present in bile acid. Also, it has been shown that the efficiency of incorporation of resveratrol in liposomes increases with the amount of added resveratrol during preparation and the presence of vitamin C or E in the formulation (Atanacković *et al.* 2012).
- Because of the poor solubility of resveratrol circumscribes its therapeutic applications. They are suggest that the encapsulation offers a potential approach for enhancing the solubility of resveratrol, stabilizing it against *trans*-to-*cis* isomerization, and improving its bioavailability (Augustin *et al.* 2013).
- The preparation of resveratrol nanosuspensions using the antisolvent precipitation method was reported. The optimal formulation consisted of drug 29.2 (mg/ml), polyvinylpyrrolidone (PVP) K17 0.38%, and F188 3.63%, respectively. In comparison to raw material, resveratrol nanosuspensions showed significantly enhanced saturation solubility and accelerated dissolution rate resulting from the decrease in particle size and

the amorphous status of nanoparticles. Meanwhile, resveratrol nanosuspensions exhibited the similar antioxidant potency to that of raw resveratrol (Hao *et al.* 2015).

- An aqueous liquid formulation of resveratrol was developed in combination with a modified glucan. Due to the low water solubility of resveratrol, solvents such as ethanol and dimethyl sulfoxide are often used to dissolve. However, these solvents have adverse effects on cultured cells or *in vivo*. It was found that the combination of resveratrol with the carboxymethylated (1,3/1,6)-β-D-glucan (CM-glucan) matrix exerted a strong stabilizing effect in aqueous medium and increased resveratrol stability up to 12 months at 25 °C. These data provide evidence of a stable resveratrol formulation in liquid suspensions and support the possible development of pharmaceutical applications of this association in biopharmaceutics and drug delivery (Francioso *et al.* 2014).

As the recent study has shown many researches about improving water insolubility of resveratrol, not only the chemical synthesis but they also has a biosynthesis method in order to be safe, reduce the effect of solvent and energy consumption. Specifically the glycosylation, an important method for the conversion of water-insoluble and unstable organic compounds into the corresponding water-soluble and chemically stable derivatives (Shimoda and Hamada 2010). (Sato *et al.* 2014) report the glucosylation of resveratrol by cultured cells of *Phytolacca americana* to its 3- and 4′-β-glucosides and their radical scavenging activity. In addition, (Pandey *et al.* 2014) was successfully study on enzymatic biosynthesis of novel resveratrol glucoside and glycoside derivatives, resveratrol was used as an

acceptor and various rare nucleotide diphosphate sugars were used as sugar donor substrates to produce glycodiversified resveratrol derivatives that have not been previously described. a GT, YjiC (GenBank accession no. AAU40842.1) from *Bacillus licheniformis* DSM 13 was used to catalyzd the transglycosylation reaction.

1.3 Cyclodextrin glycosyltransferase (CGTase)

1.3.1 General information and structure of CGTase

Cyclodextrin glycosyltransferases (CGTases, EC 2.4.1.19) are an important industrial enzyme closely related to α-amylases family but has the unique ability to the cyclodextrins production (circular α-(1,4)-linked glucoses), produced by a wide *range* of microorganisms; especially *Bacillus* strains. CGTase is a 75 kDa enzyme that consists of five domains (Figure 5) (Uitdehaag *et al.* 2002). The A, B and C domains are commonly found in both α-amylase and CGTase, whereas D and E domains are two additional domains that are unique to CGTase. The A domain resembles a folded TIM barrel and is interrupted by B domain. The C domain has an anti-parallel β-sandwich fold and is involved in the starch binding. The D domain is only present in CGTase and its function is indeterminate. The E domain is known as raw starch binding domain (Lawson *et al.* 1994).

CGTases is an extracellular enzyme can catalyze four types of reactions; cyclization, coupling, disproportionation and hydrolysis (Figure 6) (Kelly *et al.* 2009) . The cyclization activity, the intra-molecular synthesis of cyclic ring structures consisting of 6, 7, or 8 glucose units from starch or similar α-(1,4)-linked glucose polymers; named α-, β- and *γ*-cyclodextrins, respectively. The main properties of cyclodextrins are given in Table 3. The coupling activity is the convert reaction of

cyclization, catalyzed the opening of cyclodextrin rings and transfer to acceptor molecules. While, the disproportionation activity (transfer of linear malto-oligosaccharides to suitable acceptors) of CGTases represent a valuable tool for the intermolecular glycosylation of acceptor molecules (Strompen *et al.* 2015). In addition, the CGTases can catalyze hydrolysis reactions, but this activity is usually quite low (Rather *et al.* 2015). Coupling and disproportionation reactions have been used to glycosylate a wide variety of substances to make new products. These modifications have been carried out to achieve increased solubility, stability, biodegradability, improved antitumor and insecticidal activity and decreased cytotoxicity (Leemhuis *et al.* 2010).

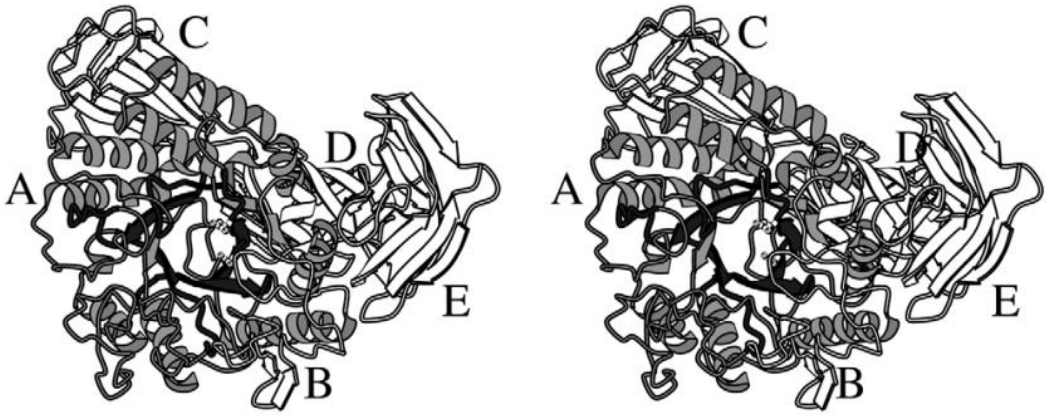


Figure 5 Stereo-view of the structure of CGTase from Bacillus circulans strain 251 in a maltose-dependent crystral form (Uitdehaag *et al.* 2002).

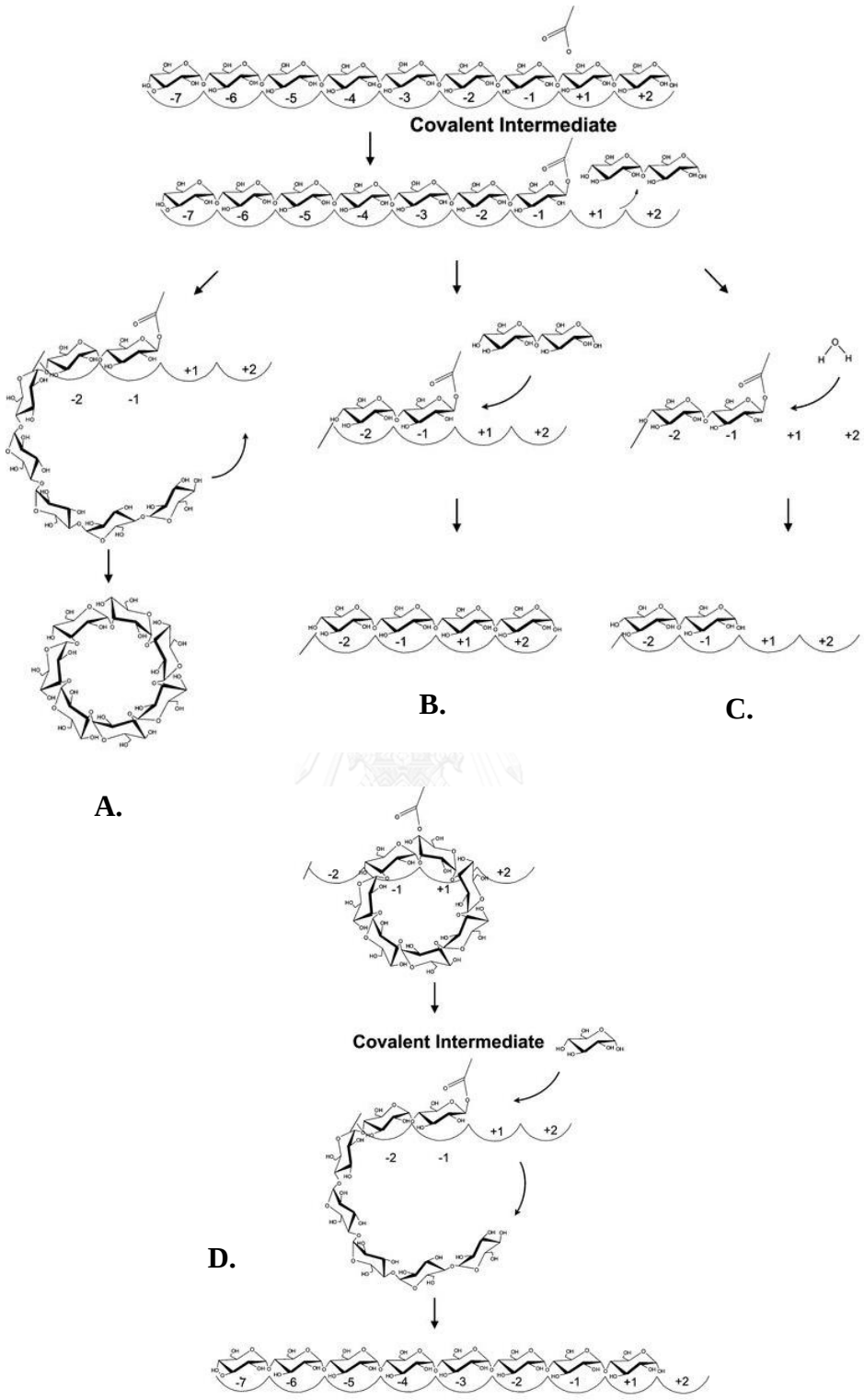


Figure 6 Schematic representation of the four reactions catalyzed by CGTase (Kelly *et al.* 2009): A.Cyclization, B.Disproportionation, C.Hydrolysis and D.Coupling reactions.

Table 3 The main properties of cyclodextrins. (Del Valle 2004)

Properties	**Cyclodextrin**		
	α	**β**	**γ**
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1,135	1,297
Solubility in water at 25 °C (%, w/v)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	174	262	427

1.3.2 Application of CGTase

In general, the application of CGTase is that it can produce CDs from glycosyl donor via cyclization reaction. The glycosyl donor used is not only starch but also most saccharides such as dextrins and oligosaccharides except monosaccharides (Jun *et al.* 2001). However, another field of application of CGTase is that it can synthesize derivatives of some compounds in order to improve their disadvantage properties such as low water solubility via disproportionation reaction. Studies on the transglycosylation reactions of CGTase have been reported. Examples are as follow:

Kometani and co-workers reported on the enzymatic synthesis of hesperidin mono-glucoside and a series of its oligoglucosides by CGTase with hesperidin as an acceptor and soluble starch as a donor. It was found that the solubility of both hesperidin mono- and diglucoside in water was about 300 times higher than that of hesperidin, and they were found to have a stabilizing effect on the yellow pigment crocin, from fruits of Gardenia jasminoides, against ultraviolet radiation (Kometani *et al.* 1994).

In 1996, Kometani also reported the enzymatic synthesis of naringin glucoside by CGTase. The solubility of naringin monoglucoside in water was also at least 1,000 times higher than that of naringin without altering its bitterness (Kometani *et al.* 1996).

Thanadolsathien (2007) performed a two-step of enzymatic synthesis of a novel vitamin E (Trolox) glycoside through the combination of α-glucosidase and CGTase enzymes to transfer glycosyl donor (maltose or β-cycloextrin) to an acceptor(Trolox, vitamin E derivative). It was reported that the water solubility of the glycoside product (α-D-glucopyranosyl-(1,4)-2-(α-D-glucopyranosyl) methyl-2,5,7,8-

tetramethylchroman-6-ol; TMG_2) was improved by $7x10^4$ times higher than that of the Trolox.

Several epicatechin glucosides (EC) were obtained through the transglycosylation reaction of CGTase, using β-CD as a glycosyl donor. The major product, EC-3'-α-D-glucopynosides, exhibited higher water solubility and stability towards UV radiation. However, the antioxidant activity was 1.5 folds lower. This derivative, however, was suggested as better food additive than the parental EC (Aramsangtienchai *et al.* 2011).

To synthesize dodecylglucoside derivative, β-glycoside or β-maltoside was incubated with CGTase from *B.mecerans* using either α-CD or starch as donor. The result showed that α-CD was advantageous for the synthesis of purified compound whereas starch was appropriate for producing the mixture of alkyl glycosides (Mathew and Adlercreutz 2012).

To examine whether anomeric configuration of acceptor might affect the production of glucoside derivatives, α and β anomers of p-nitrophenyl-glucopyranoside were incubated with CGTase from *B.mecerans* using β-CD as a glycosy donor. The result showed that the derivative with α-1,4 linkage was obtained only when α anomer substrate was used. A striking result was observed on β-anomer substrate. The derivatives with both α-1,3- and α-1,6-linkages were obtained. It was therefore possible to selectively produce the derivatives of interest, depending upon substrate configuration (Strompen *et al.* 2015).

Zehentgruber *et al*.(2011) reported the synthesis of dodecyl-β-maltooctaoside (DDMO) from dodecyl-β-maltoside (DDM) by CGTase from *Bacillus macerans*. In order to study the substrate complexation, α-CD and starch playing a role as glycosyl

donor were compared. The best glycosyl donor in the coupling reaction was free α-CD. (Zehentgruber *et al.* 2011)

Mathew *et al*. (2012) studied on the novel glycoside production. Piceid (3,4-5-trihydroxy stilbene 3-O-β-d-glucoside) was synthesized by transglycosylation reaction of CGTase from *Bacillus macerans*. Glucose, maltose, sucrose, maltotriose and α-cyclodextrin (α-CD) were used to analyze their ability to function as donor substrates. It was possible to achieve product yields up to 78.9% and 72.1% with α–CD and maltodextrin as donor substrates, respectively (Mathew *et al.* 2012).

Li *et al*. (2013) reported an achievement on stevioside derivative production to improve the edulcorant quality by lower substitution using corn starch hydrolysate and CGTase. The product was mainly composed of mono- and di-glucosylated stevioside and the highest stevioside conversion reached 77.11% (Li *et al.* 2013)

Svensson and Adlercreut (2011) reported the use of CGTase from *Bacillus macerans* to covalently immobilize on Eupergit C (a carrier consisting of macroporous beads for immobilizing enzymes) in a packed-bed reactor to investigate the continuous production of long-carbohydrate-chain alkylglycosides from α-cyclodextrin (α-CD) and n-dodecyl-(1,4)-β-maltopyranoside ($C_{12}G_2\beta$). They found that a yield of the primary coupling product n-dodecyl-(1,4)-β-maltooctaoside ($C_{12}G_8\beta$) was about 50%, with a ratio between the primary and the secondary coupling product of about 10 (Svensson and Adlercreutz 2011).

In 2011, Torres *et al*. reported on the glycosylation at 3-OH, 4’-OH or both 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. Glycosylated resveratrol also displayed surfactant activity with the critical micelle

concentration in range of 0.5 to 3.6 mM (Torres *et al.* 2011). Hamada and coworkers (2014) successfully synthesized 3- and/or 4'-glucoside resveratrol by glycosyltransferase from *Phytolacca americana*. The 3-glucoside resveratrol exhibited chemoprotectant activity against migration and invasion of oral squamous carcinoma cell line (KB cells) (Hamada *et al.*, 2014). When 3- and 4'-glucoside resveratrols were further glycosylated to maltoside resveratrols, slight free radical scavenging activity was observed. Meanwhile, other glucosides showed no radical scavenging activity at all (Sato *et al.* 2014). All glycoside derivatives were further subjected to the pharmacological activity investigated. It was found that the inhibitory activity toward histamine release was successfully improved when compared to that of unglycosylated resveratrol. This could be suggested that the inhibitory activity was improved by glucosylation and/or maltosylation(Shimoda *et al.* 2015). Besides, a series of resveratrol glycosides (3-glucoside, 4'- glucoside, 3, 5-diglucoside and 3,5,4'-triglucoside) was obtained by the action of UDP glucosyltransferase using nucleotide diphosphate (NDP) as sugar donor (Pandey *et al.* 2014). Of about 1700-fold water solubility was achieved on 3,5-β-D-diglucoside and it can retain most of the antioxidant activity (Lepak *et al.* 2015).

The starch and cyclodextrin research unit at the Department of Biochemistry, Faculty of Science, Chulalongkorn university has been working on the transglycosylation ability of CGTase from *Paenibacillus* sp. RB01. The thermotolerant *Paenibacillus* sp. RB01 had previously been screened and isolated from hot spring soil in Ratchaburi, Thailand and this CGTase strain was first partially purified by Tessana (2001), completely purified and characterized by Yenpech (2002). The optimum temperature and pH for transglycosylation reaction were at

40°C and pH 6.0 (Chotipanang *et al.* 2011). In previous study, CGTase from *Paenibacillus* sp. RB01 demonstrated a success in enzymatic synthesis of vitamin E glycoside (Thanadolsathien, 2007) *and* epicatechin glucosides (Aramsangtienchai *et al*., 2011) in order to improve their properties such as the low water solubility of the parent compound. In addition, in our research unit, short chain (Chotipanang *et al*., 2010) and medium chain alkylglycoside (Katelakha, 2012) as biodegradable detergents were also synthesized by transglycosylatin reaction using CGTase. In this work, to improve the solubility of resveratrol, resveratrol glycosides were then synthesized by the transglycosylation reaction from glycosyl donor to resveratrol by using CGTase from *Paenibacillus* sp. RB01.

The objectives of this research were:

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

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

III.) To purify and elucidate the structure of resveratrol glycoside product

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	:	Avanti™ J-301, Beckman Instrument Inc., USA
Electrophoresis unit	:	Mini-PROTEAN® 3 Electrophoresis, Bio-Rad, USA
Filter paper	:	No.1, Whatman®, England
Fraction collector	:	Model Frac-920, Amersham Biosciences, Sweden
High Performance Liquid Chromatography unit	:	Shimadzu Cooperation, Japan
Incubator	:	Gallenkamp, England
Incubator shaker	:	New Brunswick Innova 4000, USA
Lyophilizer	:	Labconco corporation, USA
Mass spectrometer	:	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 Basic™ , Bio-Rad, USA
Spectrophotometer	:	G10S UV-Vis Spectrophotometer, Thermo Scientific™,USA

Streamline vertical laminar flow cabinet : SCV-4A1, Esco, USA

TLC plates : Silica gel 60 F245, Merck, Germany

Vortex : GS60E, VORTEX-GENIE®, Scientific Industries Inc., USA

Water bath : Memmart , Germany

2.2 Chemicals

Absolute ethanol : Merck, Germany

Acrylamide : Merck, Germany

Agar : Scharlau, Spain

Ammonium persulfate : Pharmacia fine chemicals, Sweden

Aquacide II : Calbiochem, U.S and Canada

β-Cyclodextrin : Sigma, USA

Beef extract : Biomark Laboratories, India

Bovine serum albumin : Sigma, USA

Bromophenol blue : Merck, Germany

1-Butanol : Carlo Erba Reagents, Italy

Calcium chloride : Scharlau, Spain

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

Coomassie blue G-250,R-250 : Sigma, USA

Corn starch : Unilever, Thailand

2,2-diphenyl-1-picrylhydrazyl : Sigma, USA

di-Potassium hydrogen orthophosphate : Univar, Australia

Ethylenediamine tetraacetic acid (ETDA) : Univar, Australia

Glacial acetic acid : Mallinckrodt Chemicals, Thailand

Glucose	:	Univar, Australia
Glycerol	:	Merck, Germany
Glycine	:	Sigma, USA
Hydrochloric acid	:	Carlo Erba Reagents, Italy
Iodine	:	Merck, Germany
Magnesium sulfate	:	Scharlau, Spain
Maltose	:	Laboratorios CONDA, Spain
Maltotriose	:	Fluka, Switzerland
Maltoheptaoste	:	Hayashibara Biochemical Laboratories Inc., Japan
Methanol	:	Merck, Germany
85% Orthophosphoric acid	:	BDH, England
Peptone	:	Scharlau, Spain
Phenolphthalein	:	M&B Laboratory Chemicals, England
Potassium dihydrogen phosphate	:	Univar, Australia
Potassium iodine	:	Merck, Germany
Potato soluble starch	:	Scharlau, Spain
2-Propanol	:	Carlo Erba Reagents, Italy
Sodium carbonate	:	Univar, Australia
Sodium chloride	:	Carlo Erba Reagents, Italy
Sodium dodecyl sulfate	:	Sigma, USA
Standard molecular weight marker protein	:	GE Healthcare, England
TEMED (N,N,N',N'-tetramethylene-ethylenediamine)	:	Fluka, Switzerland
Tris (hydroxymethyl) aminomethane	:	Research Organics Inc., USA
Yeast extract	:	Scharlau, Spain

2.3 Bacteria

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

2.4 Media preparation

2.4.1 Medium I

Liquid medium I, containing 0.5 % (w/v) of beef extract, 1.0 % (w/v) of peptone, 0.2 % (w/v) of NaCl, 1.0 % (w/v) of potato soluble starch and 0.2 % (w/v) of yeast extract. Medium I was prepared and adjusted to pH 7.2 with 1 N NaOH and then 1.5 % (w/v) of agar was added for solid medium. Both liquid and solid medium I were sterilized by autoclaving at 121°C for 15 minutes.

2.4.2 Horikoshi's medium

For CGTase production, Horikoshi's medium was prepared as reported by Rutchtorn (1993) which was slightly modified from Horikoshi (1971). The medium I contained 0.5% of (w/v) of peptone from meat, 0.5% (w/v) of yeast extract, 1.0% (w/v) of potato soluble starch, 0.1% (w/v) of K_2HPO_4, 0.02% (w/v) of $MgSO_4 \cdot 7H_2O$ and 0.75% (w/v) of Na_2CO_3. The pH of the medium was 10.1-10.2. Medium was sterilized as described above.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

Paenibacillus sp. RB01 was streaked on solid medium I and inoculated at 37°C for 18 hours. Then colonies of bacteria were grown in 50 ml of starter medium I at 37°C with continuous shaking until A_{660} reached 0.3-0.5.

2.5.2 Enzyme production

Paenibacillus sp. RB01 starter in medium I was transferred to 300 ml of Horikoshi's medium in a 1,000 ml Erlenmeyer flask to obtain 1.0% (v/v) final concentration and was cultivated with 250 rpm incubator shaking at 40 °C for 72 hours. Then cells were harvested by refrigerated centrifugation at 5,000 rpm at 4°C for 15 minutes. Crude enzyme was collected and kept at 4°C for the purification in next step.

2.6 Partial purification of CGTase

CGTase was partially purified by starch adsorption technique Kato and Horikoshi (1984) with slight modification by Laloknam (1997).

A solution of crude enzyme was gradually sprinkled with 5% (w/v) of corn starch after oven dried at 120°C for 30 minutes and cooled to room temperature. After continuous stirring at 4°C overnight, the mixture was centrifuged to collect the starch cake at 4°C, 8,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl pH 8.5 containing 10 mM $CaCl_2$ (TB1). CGTase was eluted from the starch cake twice using 0.2 M maltose in TB1 (125 ml for 1 L of crude enzyme) with stirring at 4°C for

30 minutes. The supernatant was collected, dialyzed three times at 4°C with distilled water and concentrated with aquacide II.

2.7 Enzyme assay

2.7.1 Dextrinizing activity

The dextrinizing activity assay was used to analyze CGTase activity (Fuwa, 1954), this assay used iodine as an indicator to measure the amount of starch digestion by CGTase. The reaction was carried out by incubating 50 μL of enzyme sample with 150 μL of substrate (0.2% (w/v) potato soluble starch in 0.2 M phosphate buffer pH 6.0) at 40°C for 10 minutes. After that, the reaction was stopped by the addition of 2 ml of 0.2 M HCl and 250 μL of iodine reagent (0.02% (w/v) of I_2 in 0.2% (w/v) of KI) was added. The reaction was adjusted by distilled water to a final volume of 5 ml and the absorbance at 600 nm was measured. For the control, HCl was added before enzyme sample. One unit of enzyme was defined as the amount of enzyme which produced 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition.

2.7.2 Coupling activity

This method was used to analyze the coupling activity of CGTase (Goel and Nene, 1995). The assay was measured the disappearance of β-cyclodextrin in the reaction mixture by phenolphthalein solution. The reaction mixture of 250 μL of β-cyclodextrin as standard or sample reaction (CGTase was preincubated with β-cyclodextrin and resveratrol) and 750 μL of phenolphthalein solution were incubated for 15 minutes then the absorbance at 550 nm was measured. The decrease of the

absorbance at 550 nm was the result of the inclusion complex of phenolphthalein in the hydrophobic cavity of cyclodextrin. Relative coupling activity was calculated by conversion of $\triangle A_{550}$ to μmoles β-cyclodextrin from the β-cyclodextrin phenolphthalein curve (see Appendix C). The disappearance of β-cyclodextrin in the reaction mixture was calculated from the difference between β-cyclodextrin concentration at 0 and 24 hour incubation with CGTase and resveratrol.

2.8 Protein determination

Protein concentration was determined by Bradford's method (1976). This method was used to determine the amount of protein by using bovine serum albumin as standard (see Appendix D). One-hundred microliter of enzyme sample and 1.0 ml of Coomassie blue reagent (100 mg of Coomassie blue G-250, 50 ml of absolute ethanol, 100 ml of 85% H_3PO_4 , then the mixture was adjusted by distilled water to 1 L final volume and filtered with whatman® No.1 filter paper and kept in a dark bottle) were mixed. After incubation for 5 minutes, the reaction mixture was recorded at 595 nm.

2.9 Polyacrylamide Gel Electrophoresis (PAGE)

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

The gel was carried out with 7.5% (w/v) separating and 5.0% (w/v) stacking gels, under Tris-glycine buffer pH 8.0 as electrode (see Appendix A). Samples were treated with sample buffer and introduced into wells. 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 the dye reached the bottom of gel, the gel was cut in to 2 parts for protein (see section 2.9.3) and activity staining (see section 2.9.4).

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

The denaturing gel consisted of 0.1% (w/v) of SDS in 7.5% (w/v) separating and 5.0% (w/v) of stacking gels, with Tris-glycine buffer pH 8.0 containing of 0.1% (w/v) of SDS as an electrode buffer (see Appendix A). Samples were mixed 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 to awards anode. After running, proteins in the gel were visualized by protein staining.

2.9.3 Protein staining (Coomassie blue staining)

After electrophoresis gel was soaked in Coomassie blue staining solution (0.1 % (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) of methanol and 10 % (v/v) of acetic acid) at room temperature. After 60 minutes, the gel was destained with destaining solution (10 % (v/v) of methanol and 10 % (v/v) of acetic acid) for several times until the gel background was clear.

2.9.4 Dextrinizing activity staining

After electrophoresis, the gel was immersed in 10 ml of 0.2% (w/v) potato soluble starch solution in 0.2 M phosphate buffer pH 6.0 at 40°C for 10 minutes. After that the gel was rinsed several times with distilled water to remove any residual substrate before the addition of 10 ml of I_2 staining solution (0.2% (w/v) I_2 in 2.0%

(w/v) KI) for color development at room temperature. The clear zone on the dark background was shown as starch-degrading activity of the enzyme.

2.10 Effect of DMSO on CGTase stability

A 200 U/ml of CGTase was incubated with varying 0-50%(v/v) of DMSO concentration and the reaction was performed in phosphate buffer pH 6.0, 40°C for 24 hours with continuous shaking. The CGTase activity was analyzed by dextrinizing activity assay.

2.11 Donor specificity

To determine for an appropriate glycosyl donor, various glycosyl donors including 1% (w/v) of potato soluble starch, β-cyclodextrin and maltoheptaose were used to find an appropriate donor. The initial composition of the reaction mixture was 1% (w/v) of soluble starch, 1% (w/v) of resveratrol in 0-50% (v/v) DMSO and 50 U/ml of CGTase in 50mM phosphate buffer pH 6.0. The reaction was performed at 40°C for 24 hours with continuous shaking. The reaction was then stopped by boiling for 5 minutes. After that, resveratrol glycoside products were then analyzed by TLC.

2.12 Transglycosylation reactions

In order to investigate the CGTase transferring efficiency of glucose residues from glucosyl donor to resveratrol and an appropriate concentration of DMSO, CGTase was used as a biocatalyst in the transglycosylation reaction and DMSO was used as a co-solvent for the synthesis of resveratrol glycosides. The composition of

the reaction mixture and the transglycosylation reaction condition were the same as previously described (in section 2.11 with the optimized DMSO concentration). And reaction products were then analyzed by TLC.

2.13 Preliminary structure characterization of glycosides products

In order to be sure that the products observed were glycoside products by the action of CGTase, the preliminary characterization of glycosides products was analyzed. A 250 μL of the reaction mixture from 2.12 and were treated with 125 μL of glucoamylase from *A. niger* from stock solution of 90 U/ml to get final activity 30 units/ml and incubated at 40°C for 1 hour to remove all glucose residues sequentially from non-reducing end of glucoside moiety conjugated to resveratrol glucoside. After that, glucoamylase was inactivated by boiling at 100°C for 5 minutes. In addition, in order to check the linkage between resveratrol and glucose unit, A 150 μL of the reaction mixture were futher treated with 50 μL of 120 units/ml of α-glucosidase from *S. cerevisiae* to get final activity of 30 units/ml was added and incubated at 40°C for 3 hours. After inactivation of α-glucosidase by boiling for 5 minutes, the reaction products were investigated by TLC.

2.14 Resveratrol glycoside product determination

2.14.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was used to analyze resveratrol glycoside products by applying 10 μL of each sample on siliga gel 60 F_{245} aluminium sheets (Merck, Co.) in a solvent mixture consisting of 1-butanol : 2-propanol : water (10:5:1).

The compounds were visualized by spraying with conc. sulfuric acid : methanol (1:2) and heating at 120°C for 10 minutes. The amount of product was determined from spot intensities using Quantity One® software from Bio-Rad. The intensity of the product spots were measured and compared to the intensity of glucose spot (20 μg) on the same TLC plate. The intensity of glucose spot was set to 100%.

2.14.2 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was performed using a C18 reversed-phase column (5μm, 4.6x250 mm, SHOWA DENKO K.K., Tokyo, Japan) with UV detector, the eluent was monitored at absorbance of 308 nm with 30% (v/v) methanol in ultrapure water as a mobile phase, flow rate of 1.0 ml/min at controlled temperature of 40°C.

2.15 Optimization of resveratrol glycoside production

To obtain high production yield, the transglycosylation reaction condition was optimized. The reaction was performed by varying resveratrol, glycosyl donor, enzyme concentration and incubation time. The amount of products were analyzed by HPLC.

2.15.1 Optimization of resveratrol concentration

To determine an appropriate concentration of resveratrol, resveratrol concentration of 0.2-1.6% (w/v) was used. The reaction mixture consisted of resveratrol in different concentration and 1.0% (w/v) of β-cyclodextrin, which was incubated with 50 U/ml of CGTase in 50mM phosphate buffer pH 6.0 at 40°C for 24 hours with continuous shaking, then the reaction was stopped by boiling for 5 minutes. The optimum concentration of resveratrol was analyzed by HPLC.

2.15.2 Optimization of β-cyclodextrin concentration

In order to investigate the optimum β-cyclodextrin concentration, its concentration was varied from 0.5-1.8% (w/v). The reaction mixture which contained of varying concentration of β-cyclodextrin and the optimized concentration of resveratrol from section 2.15.1 was incubated with 50 U/ml of CGTase in 50 mM phosphate buffer pH 6.0 at 40°C for 24 hours with continuous shaking, then the reaction was stopped by boiling for 5 minutes. The product were analyzed by HPLC.

2.15.3 Optimization of enzyme concentration

For optimization of the synthesis reaction of resveratrol glycosides by CGTase, various enzyme concentrations were also investigated. The reaction mixture consisted of resveratrol in the optimum concentration from section 2.15.1 and β-cyclodextrin in the optimum concentration from section 2.15.2, and they were incubated with 100-600 U/ml final activity of CGTase in 50mM phosphate buffer pH 6.0 at 40°C for 24 hours with continuous shaking, then the reaction was stopped by boiling for 5 minutes. The appropriate enzyme concentration was judged from HPLC analysis.

2.15.4 Optimization of incubation time

To study the time course of production, it was established by using the optimum condition obtained from section 2.15.1-2.15.3 and the reaction mixture was incubated at 40°C for 24-120 hours with continuous shaking, then the reaction was stopped by boiling for 5 minutes. The products were further analyzed by HPLC.

2.16 Large scale production of resveratrol glycoside

To scale-up the resveratrol glycoside production, the synthesis of resveratrol glycosides was then performed under the optimized conditions and the volume was increased from 0.25 ml to a total 12.5 ml. After completion, the reaction mixture was concentrated to 2.5 ml by rotary evaporator at 40°C. The products were further purified by column chromatography in the next step.

2.17 Purification of resveratrol glycosides

The reaction mixture from 2.16 was applied on Sephadex LH-20 column (1.2 cm x 100 cm) equilibrated with distilled water. The resveratrol glycoside products were separated from sugars and enzyme by elution of the column with 2 L of distilled water with flow rate of 0.5 ml/min. Then the mobile phase was changed to 50% (v/v) of methanol for the resveratrol elution. The fractions containing resveratrol glycosides were concentrated and separated by HPLC. Each resveratrol glucoside peak was collected for further characterization.

2.18 Characterization of resveratrol glycoside products

2.18.1 Mass spectrometry (MS)

The optimum reaction mixture from section 2.15 was dried by lyophilizer (Labconco corporation, USA) to removed DMSO before subjected to molecular mass measurement by spectrometry. Electrospray ionization-time of flight mass spectrometry (ESI-MS) profile was performed by a micrOTOF at department of

chemistry, faculty of science, Mahidol University, Thailand. The compounds were ionized by electrospray ionization on positive-ion mode.

2.18.2 Tandem Mass Spectrometry (LC-MS/MS)

The resveratrol glycoside main product was collected from a peak fraction from HPLC, Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS) profile was performed by Quadrupole-time-of-flight mass spectrometry (Q-TOF MS) with the Luna® 5 μm C18(2) 100 Å, LC Column (150 x 4.6 mm; Phenomenex, USA) at science lab centre, faculty of science, Naresuan University, Thailand. The compounds were ionized by electrospray ionization on positive-ion mode.

2.18.3 Water solubility

In order to study the solubility in water, excess amount of resveratrol and resveratrol glycosides were dissolved with 200 μL of water and continuous shaking at 30°C for 15 minutes. The sample was filtered through a 0.45 μm membrane and the soluble concentration was analyzed by HPLC.

2.18.4 Antioxidant activity

To determine the antioxidant activity of resveratrol glycosides, DPPH radical scavenging assay was used. This method was previously described by Gülçin (2006a) with slightly modified by Gülçin (2010) in order to evaluate the DPPH• free radical scavenging capacity of resveratrol. A 0.5 ml of samples (dissolved in ethanol at various concentrations of 5-30 μg/ml) were treated with 1.5 ml of 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl in ethanol). The reaction mixtures were incubated at room temperature in the dark for 30 minutes, then the absorbance was measured at

517 nm. The DPPH radical scavenging activity was determined by analyzed the percentage decrease in the absorbance of sample compared with ethanol as a blank.

The capability to scavenge the DPPH• radical was calculated using the following equation:

$$\textbf{DPPH• scavenging effect (\%)} = [1-(A_S/A_C)] \times 100$$

A_C is the absorbance at A_{517} of the control (reaction mixture without resveratrol)

A_S is the absorbance at A_{517} of the sample

CHAPTER III

RESULTS

3.1 Partial purification of CGTase

Paenibacillus sp. RB01 was cultured in Medium I at 37°C until the A660 reached 0.3-0.5. Then, 1.0%(v/v) of the starter was transferred and cultured in Horikoshi's medium at 40°C for CGTase production. After 72 hours, the supernatant was collected by centrifugation. Cell free-supernatant was used as a crude enzyme and corn starch adsorption was used to partially purify a crude enzyme as previously described in section 2.6. The purification table is shown in Table 4. The % yield of CGTase and purification fold were 33% and 95, respectively. Specific activity of the partially purify enzyme was 11,783 U/mg protein which was calculated and expressed in terms of dextrinizing activity.

The purity and the activity of CGTase after partial purification with starch adsorption technique was checked by Native-PAGE. The protein staining revealed that the CGTase was successfully purified through starch adsorption technique since less protein bands were observed (Figure 7, A, Lane 2). The mobility of the active bands was determined by dextrinizing activity staining with starch-degrading property. The degradation of starch substrate by GCTase showed clear zones. Three active bands coincided with those 3 bands which were stained with Coomassie brilliant blue (Figure 7, B, Lane 2). This implies that the partial purified enzyme had 3 isoforms with different net charge and size. Furthermore, to check the size of obtained protein, SDS-PAGE was performed. It was found that the molecular weight of CGTase was estimated to be 70 kDa (Figure 8, Lane 2).

Table 4 Purification table of CGTase from *Paenibacillus* sp. RB01.

Purification step	**Volume (ml)**	**Total activity* ($Ux10^3$)**	**Total protein (mg)**	**Specific activity* (U/mg protein)**	**Fold**	**Yield (%)**
Crude enzyme	2,200	236	1,918	123	1	100
Partial purified enzyme	400	77	6.56	11,783	95	33

* Dextrinizing activity

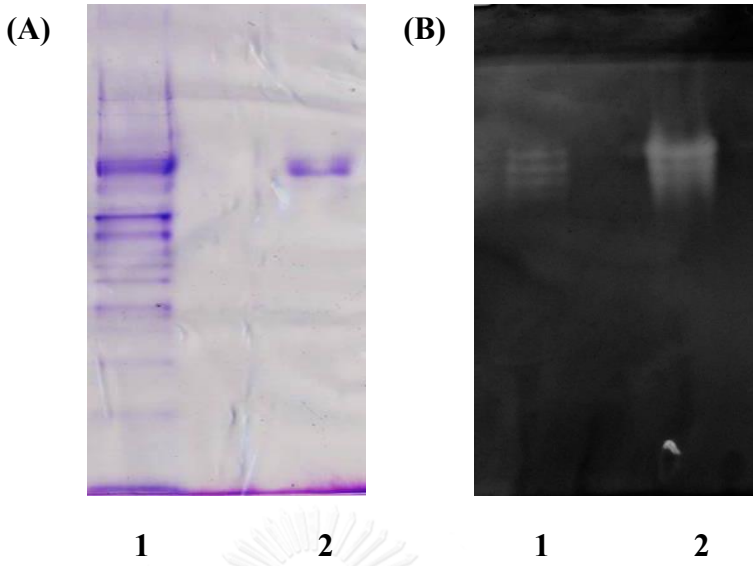


Figure 7 Native PAGE analysis of CGTase from *Paenibacillus* sp. RB01.

(A) Coomassie blue staining

Lane 1 : Crude enzyme (50 μg)

Lane 2 : Partial purified enzyme (20 μg)

(B) Dextrinizing activity staining

Lane 1 : Crude enzyme (0.05 U)

Lane 2 : Partial purified enzyme (0.05 U)

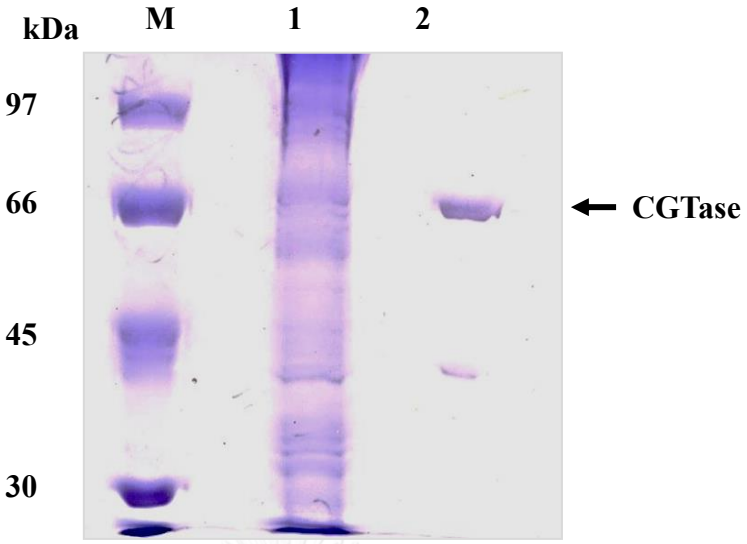


Figure 8 SDS PAGE analysis of CGTase from *Paenibacillus* sp. RB01.

M : Low molecular weight protein marker

Phosphorylase b (97 kDa)

Albumin (66 kDa)

Ovalbumin (45 kDa)

Carbonic anhydrase (30 kDa)

Lane 1 : Crude enzyme (50 μg)

Lane 2 : Partial purified enzyme (10 μg)

3.2 Effect of DMSO on CGTase stability

Dimethyl sulfoxide (DMSO) was used as a co-solvent due to the low water solubility of resveratrol. Previous studies on the enzymatic synthesis of flavonoid and stilbene(resveratrol) glycosides showed that DMSO had the best solvent properties in terms of substrate solubility and production yield (Bertrand A. *et al*, 2006)(Torres *et al.* 2011). However, the use of DMSO in enzymatic reaction was limited by the fact that it might cause enzyme inactivation. The DMSO concentration was varied from 0-50% (v/v) and the effect of DMSO on both coupling and dextrinizing activity of CGTase was investigated at the optimum pH (6.0) and temperature (40°C) of the enzyme for 24 hours. According to the low water solubility of resveratrol, the reaction mixture containing DMSO was separated into two phases. Hence, the reactions were performed with continuous shaking.

As shown in Figure 9 and 10, both relative coupling and dextrinizing activities of CGTase was dramatically decreased when DMSO concentration increased. Thus, the DMSO concentration had significant effect on CGTase activity.

For the next experiment, an attempt was made to synthesize resveratrol glycosides in various concentrations of DMSO.

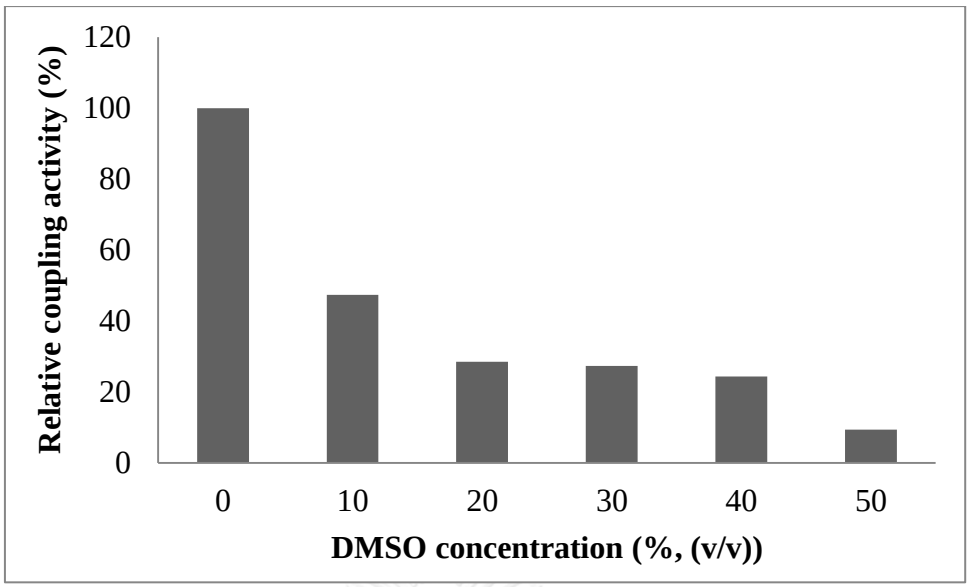


Figure 9 Relative coupling activity of CGTase in various DMSO concentrations

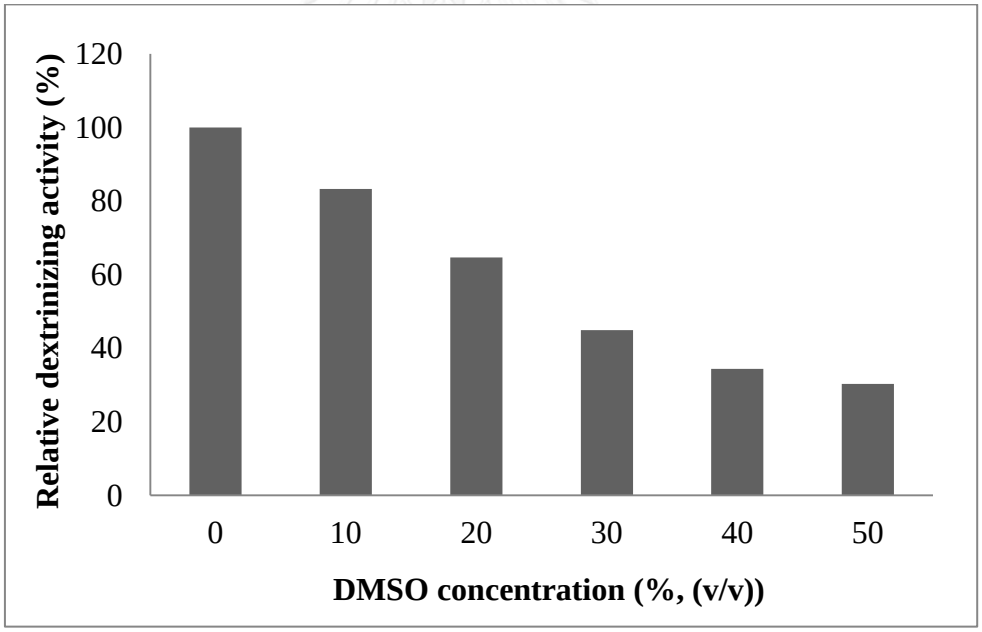


Figure 10 Relative dextrinizing activity of CGTase in various DMSO concentrations.

3.3 Effect of DMSO on resveratrol glycoside production

The transglycosylation reaction with various DMSO concentrations was further determined to select an appropriate DMSO concentration for resveratrol glycoside production by comparison of the product intensity on TLC.

The effect of DMSO concentration on resveratrol glycoside production by CGTase was determined. DMSO concentration of 10-50% (v/v) was varied.

The expected glycoside products should possess more polarity than the parent resveratrol due to the attachment of glucose moieties to the resveratrol. In the same way, the expected product should have less polarity than the standard sugars due to the presence of resveratrol. Hence, the expected products were believed to have Rf values between those of resveratrol and standard sugars which were 0.55 and 0.77 (Figure 11). The intensities of the product spots were measured by Quantity One program and were calculated as relative intensities to glucose (20 μg) as a standard on the same TLC plate (Figure 12). The intensity of glucose spot was set as 100%. It was found that the degree of intensity of resveratrol glycosides, which represented as the production yield, synthesized from reaction mixture containing 20% (v/v) of DMSO gave the highest product. At low concentration of DMSO, resveratrol cannot be well dissolved in the reaction mixture but excess concentration of DMSO had an effect on CGTase activity. Thus, DMSO concentration had to be compromised between appropriate concentration to dissolve a substrate and to maintain CGTase activity for the transglycosylation reaction.

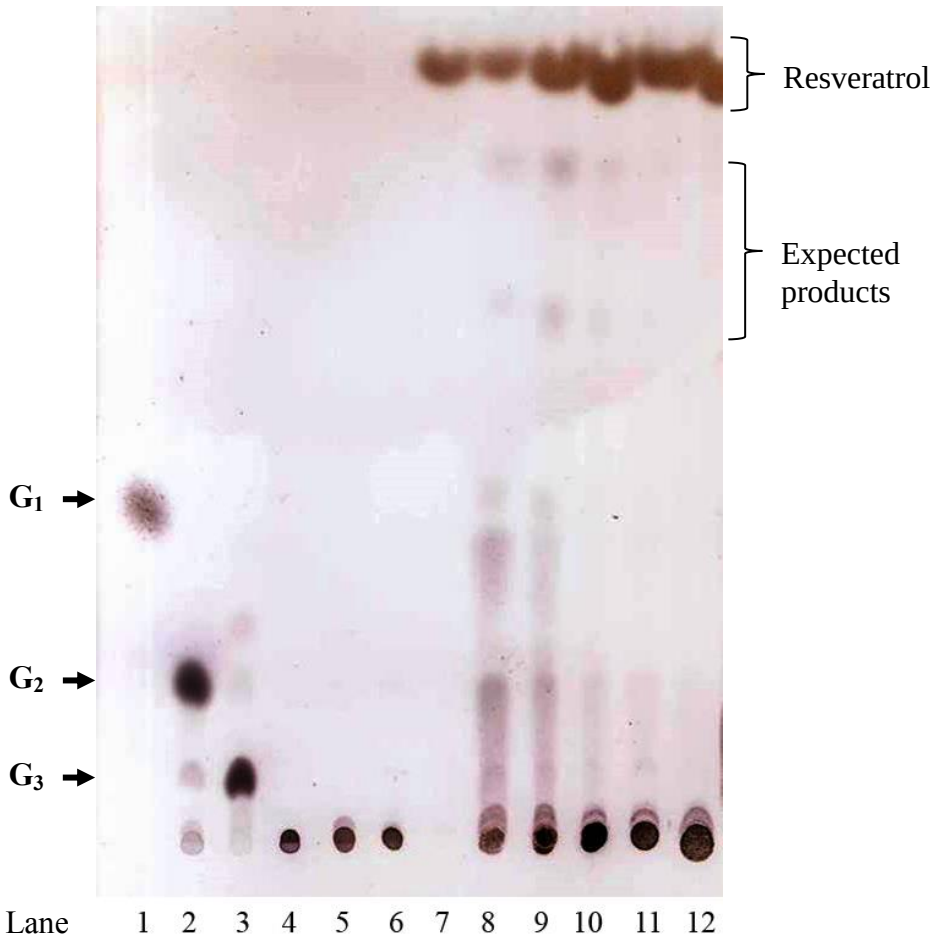


Figure 11 TLC analysis of the tranglycosylation reaction in various DMSO concentrations.

Lane 1-3: Standard glucose, maltose and maltotriose

Lane 4-6: Soluble starch , β-cyclodextrin and maltoheptaose

Lane 7: Resveratrol

Lane 8: Reaction mixture containing 10%(v/v) of DMSO (24 hours)

Lane 9: Reaction mixture containing 20%(v/v) of DMSO (24 hours)

Lane 10: Reaction mixture containing 30%(v/v) of DMSO (24 hours)

Lane 11: Reaction mixture containing 40%(v/v) of DMSO (24 hours)

Lane 12: Reaction mixture containing 50%(v/v) of DMSO (24 hours)

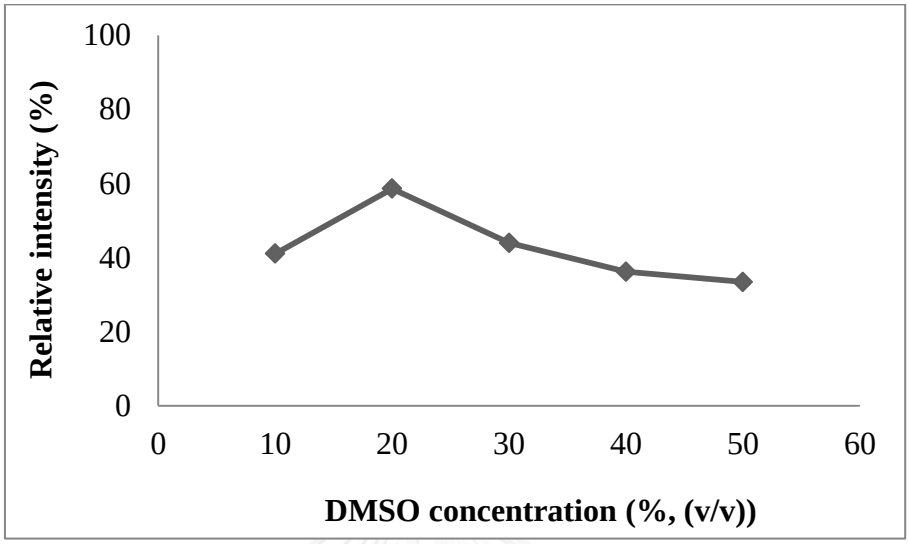


Figure 12 Relative intensity of resveratrol glycoside products in various DMSO concentrations.

3.4 Enzymatic synthesis of resveratrol glycosides

3.4.1 Preliminary structure characterization of glycoside products

Preliminary structure characterization of glycoside products was carried out with the use of two enzyme, namely glucoamylase and α-glucosidase. The TLC chromatogram of CGTase catalyzed the transfer of glucose from soluble starch to resveratrol and the action of glucoamylase and α-glucosidase on the glucoside products is shown in Figure 13. In order to be sure that the products observed as spots on TLC were glycoside products by the action of CGTase, the reaction mixture with 0 minute incubation with CGTase, the reaction mixture at 24 hours without either CGTase or resveratrol was used as control experiments. No products were observed in control experiments (Figure 13, Lane 6-8). The reaction mixture were further treated with glucoamylase to remove all glucose residues sequentially from non-reducing end of glucoside moiety conjugated to resveratrol glucoside and α-glucosidase to confirm the linkage between an acceptor and glucose (Figure 13, Lane 10-12). The result showed that after treatment with glucoamylase the amount of hydrolysis product disappeared, only the spot intensity of glucose and expected resveratrol monoglucoside increased due to the fact that the glycosidic linkage was hydrolyzed except those between resveratrol and glucose (Figure 13, Lane 10). In addition, after further treatment with α-glucosidase to elucidate the linkage between resveratrol and glucose, it was found that the intensity of the spot of the expected resveratrol monoglucoside decreased (Figure 13, Lane 11) which could be due to the fact that the α-linkage was hydrolyzed. Thus, it can be preliminary concluded that CGTase from *Paenibacillus* sp. RB01 was able to catalyze the transglycosylation reaction from

starch to resveratrol. The Rf values of standard and transglycosylated products were summarized in Table 5.

Furthermore, in order to achieve high production yield, various glycosyl donors were used to find an appropriate glycosyl donor for the resveratrol glycoside production in the next step.

3.4.2 Donor specificity

The three different glycosyl donors; potato soluble starch, β-cyclodextrin and maltoheptaose were used to find appropriate glycosyl donor for the resveratrol glycoside production. The concentration of all glycosyl donors was controlled at final concentration of 1% (w/v). The composition of the reaction mixture and the transglycosylation reaction condition were the same as previously described in section 2.11. As can be seen from Figure 14, same product types were observed with the same Rf values when each donor was used as a substrate. Semi-quantitative analysis was determined in order to differentiate the product spot intensities. The intensity of the product spots were measured and compared to the intensity of glucose spot (20 μg) on the same TLC plate. The intensity of glucose spot was set to 100%. It was found that starch was the best glycosyl donor as can be seen in Figure 15.

However, due to the limitation of a solution containing starch in HPLC analysis for the next experiment, β-cyclodextrin was then chosen as an appropriate glycosyl donor for the transglycosylation to resveratrol by CGTase.

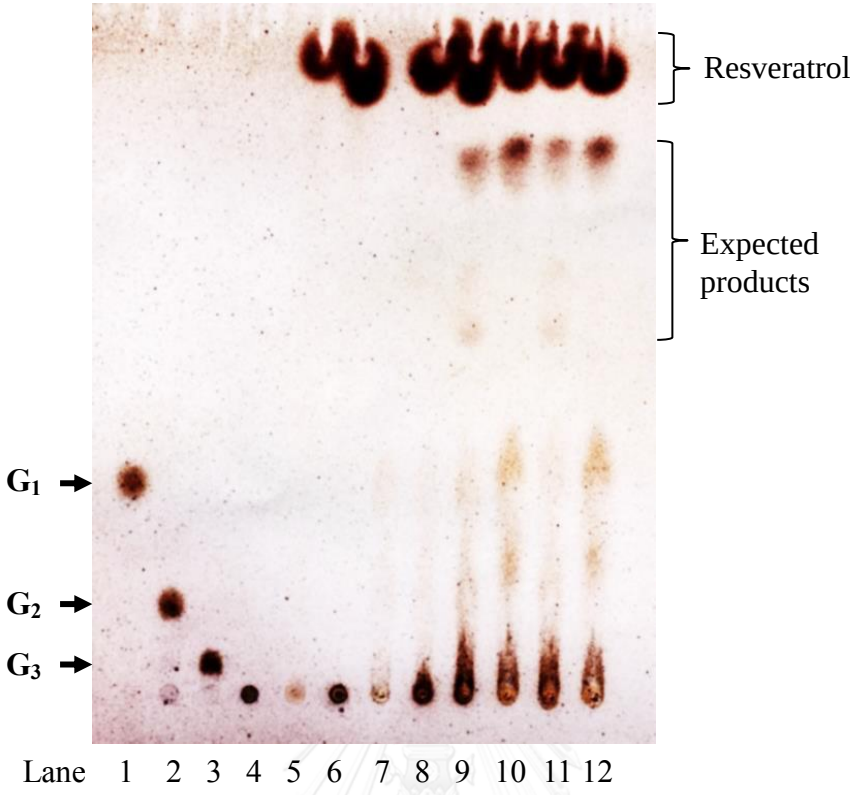


Figure 13 TLC chromatogram of amylolytic enzyme treatment of transglycosylation reaction products.

Lane 1-5: Standard glucose, maltose, maltotriose, soluble starch, resveratrol

Lane 6: Reaction mixture without CGTase

Lane 7: Reaction mixture without resveratrol

Lane 8: Reaction mixture at 0 minute incubation

Lane 9: Reaction mixture after 24-hour incubation

Lane 10: Reaction mixture treated with glucoamylase

Lane 11: Reaction mixture treated with α-glucosidase

Lane 12: Reaction mixture treated with glucoamylase and α-glucosidase

Table 5 Rf values from TLC analysis of resveratrol glycoside products.

Standards / Products		**R_f**
Standard	Glucose	0.38
	Maltose	0.18
	Maltotriose	0.07
	Soluble starch	0.00
	Resveratrol	0.89
Product	Resveratrol glycoside product I	0.77
	Resveratrol glycoside product II	0.55

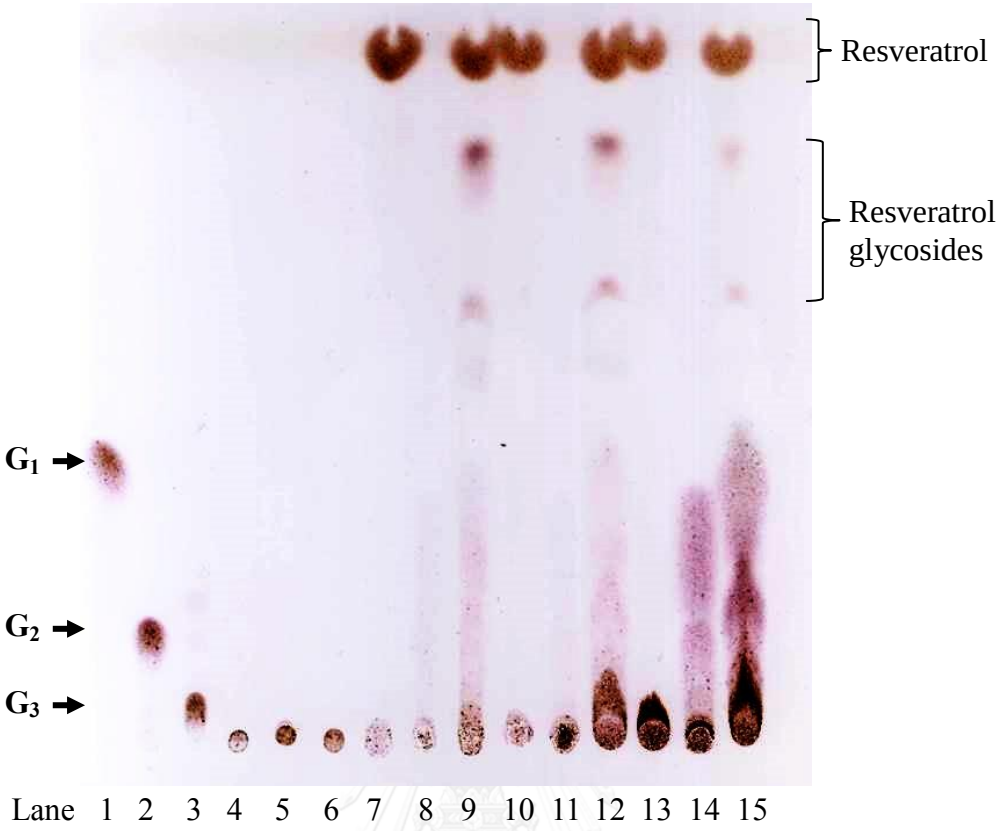


Figure 14 TLC chromatogram of transglycosylation reaction products of CGTase using soluble starch, β-cyclodextrin and maltoheptaose as a donors.

Lane 1-3: Standard sugars ; glucose, maltose, maltotriose

Lane 4-6: Standard glycosyl donors; soluble starch, β-cyclodextrin, maltoheptaose

Lane 7: Reaction mixture with starch as a glycosyl donor without CGTase

Lane 8: Reaction mixture with starch as a glycosyl donor without resveratrol

Lane 9: Reaction mixture with starch as a glycosyl donor

Lane 10: Reaction mixture with β-cyclodextrin as a glycosyl donor without CGTase

Lane 11: Reaction mixture with β-cyclodextrin as a glycosyl donor without resveratrol

Lane 12: Reaction mixture with β-cyclodextrin as a glycosyl donor

Lane 13: Reaction mixture with maltoheptaose as a glycosyl donor without CGTase

Lane 14: Reaction mixture with maltoheptaose as a glycosyl donor without resveratrol

Lane 15: Reaction mixture with maltoheptaose as a glycosyl donor

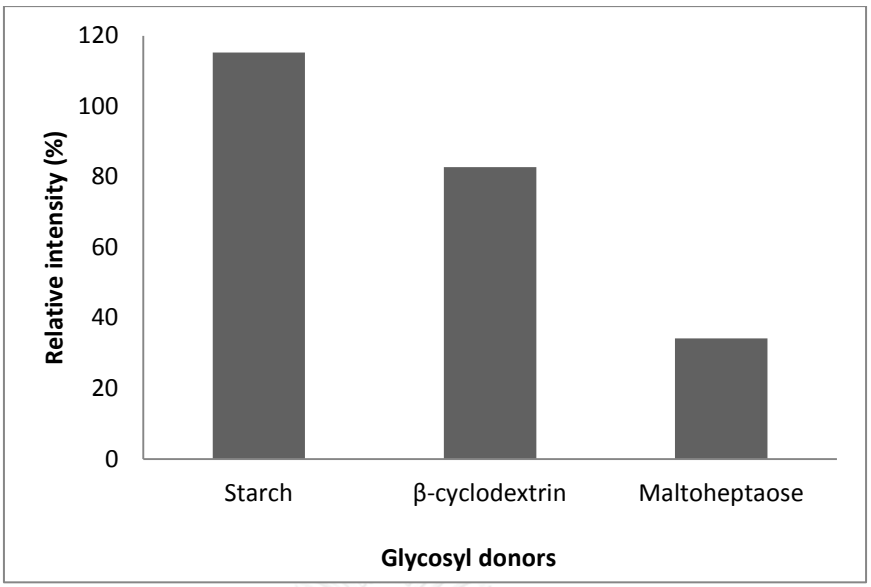


Figure 15 Relative intensity of transglycosylation products using various glycosyl donors.

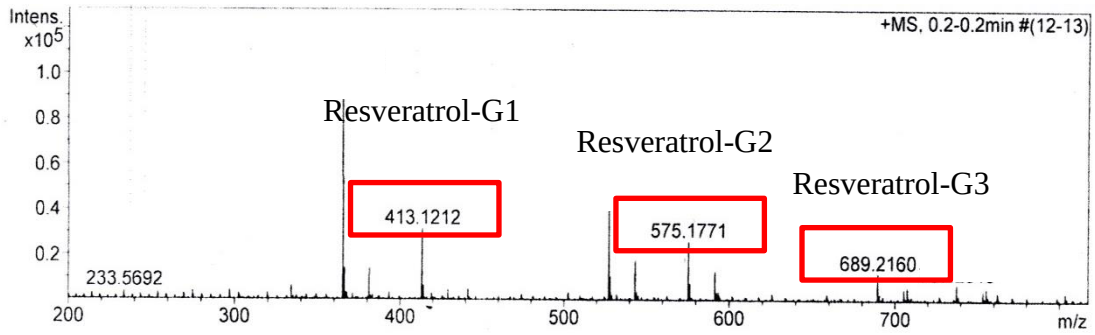
3.4.3 Mass spectrometry (MS)

To further confirm that procucts obtained were form the action of the transglycosylation reaction by CGTase, the molecular weight of resveratrol glucosides was then elucidated by mass spectrometry (MS).

Transglycosylation products were analyzed by mass spectrometry for their mass as described in section 2.14. The reaction mixture containing transglycosylation products was subjected to MS. It was found that the pseudomolecular ion with positive mode $[M+Na]^+$ of products gave 3 product peaks at m/z of 413 (390 plus 23 of sodium molecule), 575 (552 plus 23 of sodium molecule) and 737 (714 plus 23 of sodium molecule) (Figure 16, A). These corresponded with the molecular mass of resveratrol monoglucoside ($C_{20}H_{22}O_8$), resveratrol maltoside ($C_{26}H_{32}O_{13}$) and resveratrol maltotrioside ($C_{32}H_{42}O_{18}$), respectively.

To confirm that the products were glycoside derivatives, the transglycosylation reaction after 24-hour incubation was further treated with glucoamylase. After that the products were analyzed by MS (Figure 16, B). The results showed that after treatment with glucoamylase, the intensity of resveratrol maltoside was dramatically decreased and the peak of resveratrol maltotrioside was disappeared. Meanwhile, the intensity of resveratrol monoglucoside was increased. These results unconditionally confirmed that the products were resveratrol monoglucoside, resveratrol maltoside and resveratrol maltotrioside.

A.



B.

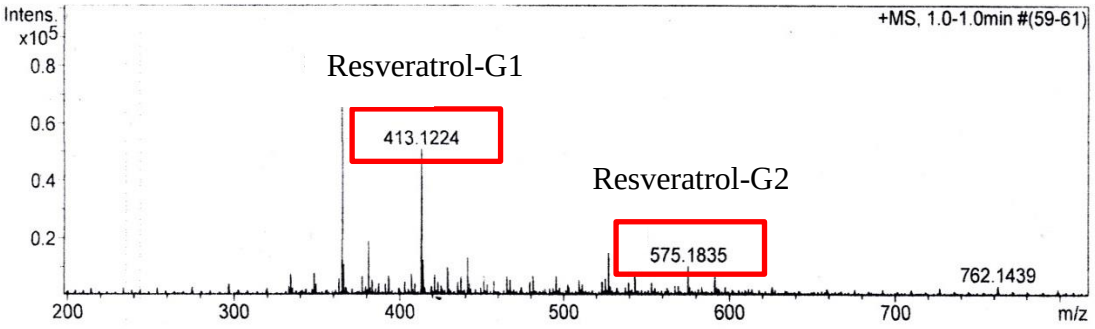


Figure 16 ESI-TOF mass spectra of resveratrol glycoside products.

A. Resveratrol glycosides in the transglycosylation reaction mixture

B. Resveratrol glycosides in the transglycosylation reaction treated with glucoamylase (final concentration of 30 U/ml)

3.5 Optimization of resveratrol glycoside production

To reach high production yield, various parameters affecting the yield including donor, acceptor and enzyme concentration were optimized and total product was analyzed by HPLC. The amount of product yield was analyzed as follow:

Product yield (%) = (Amount of product / Amount of resveratrol at t_0) x 100

And the peak area of transglycosylated product was also plotted. The reaction was performed under optimum pH (50 mM acetate buffer, pH 6.0) and temperature (40 °C) of the enzyme.

3.5.1 Optimization of resveratrol concentration

Resveratrol concentration of 0.2-1.6% (w/v) was used to determine an appropriate concentration of resveratrol. The condition of transglycosylation reaction was employed as described in section 2.16.1. The increase of resveratrol concentration increased the production of resveratrol glycosides. However, when resveratrol concentration was higher than 0.6% (w/v), the amount of the production yield decreased (Figure 17). Thus, 0.6% (w/v) of resveratrol was chosen to be the optimum concentration.

3.5.2 Optimization of donor concentration

In order to investigate the optimum glycosyl donor concentration, resveratrol glycoside synthesis was determined as described in section 2.16.2, β-cyclodextrin concentration was varied from 0.5-1.8% (w/v). The production yield increased when β-cyclodextrin increased from 0.5-1.4% (w/v). However, further increase in β-

cyclodextrin concentrations (>1.4% (w/v)) did not result in an increase of the product (Figure 18). Thus, the suitable concentration of β-cyclodextrin was at 1.4 % (w/v) and was further used in the next experiment.

3.5.3 Optimization of incubation time

To study the effect of incubation time on resveratrol glycosides production. The reaction mixture consisted of donor and acceptor in the optimum concentration was incubated with CGTase at 40°C for 0-120 hours. The result showed that the amount of production was slight increased until it reached 48 hours. However, when incubation time was higher than 48 hours the amount of the production yield decreased (Figure 19). Hence, the extension of incubation time did not increase resveratrol glycosides production.

3.5.4 Optimization of enzyme concentration

For optimization of the synthesis of resveratrol glycosides by CGTase, various enzyme concentrations were investigated. The reaction mixture consisted of 1.4% (w/v) of β-cyclodextrin, 0.6% (w/v) of resveratrol in 20% (v/v) DMSO was incubated with 100-600 U/ml of CGTase. The optimum amount of enzyme for the production of resveratrol glycosides was found to be 400 U/ml (Figure 20).

Thus, the optimum condition for transglycosylation reaction of resveratrol glycosides by CGTase from *Paenibacillus* sp. RB01 was to use 0.6% (w/v) of resveratrol in 20% (v/v) DMSO and 1.4% (w/v) of β-cyclodextrin incubated with 400 U/ml of CGTase at 40°C for 48 hours. After optimization, it was found that the total yield was increased by 3.71 times (Figure 21, A. and B.). and the ratio of resveratrol-G1 : resveratrol-G2 : resveratrol-G3 was 3 : 1 : 2 (Figure 21, B.).

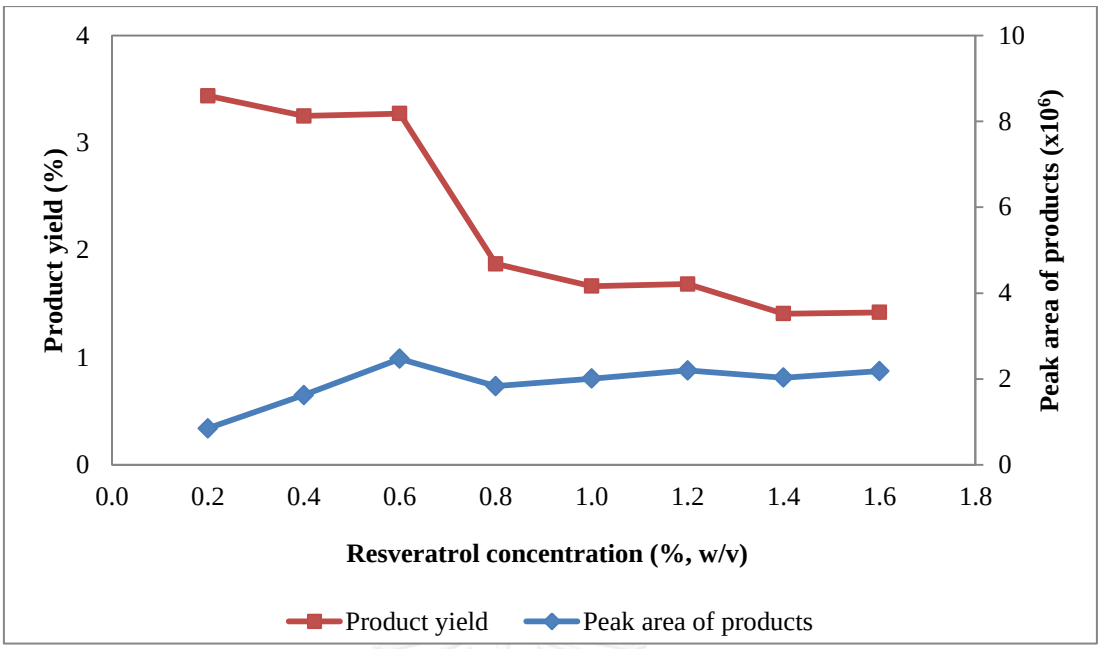


Figure 17 Effect of resveratrol concentration on resveratrol glycosides production by CGTase.

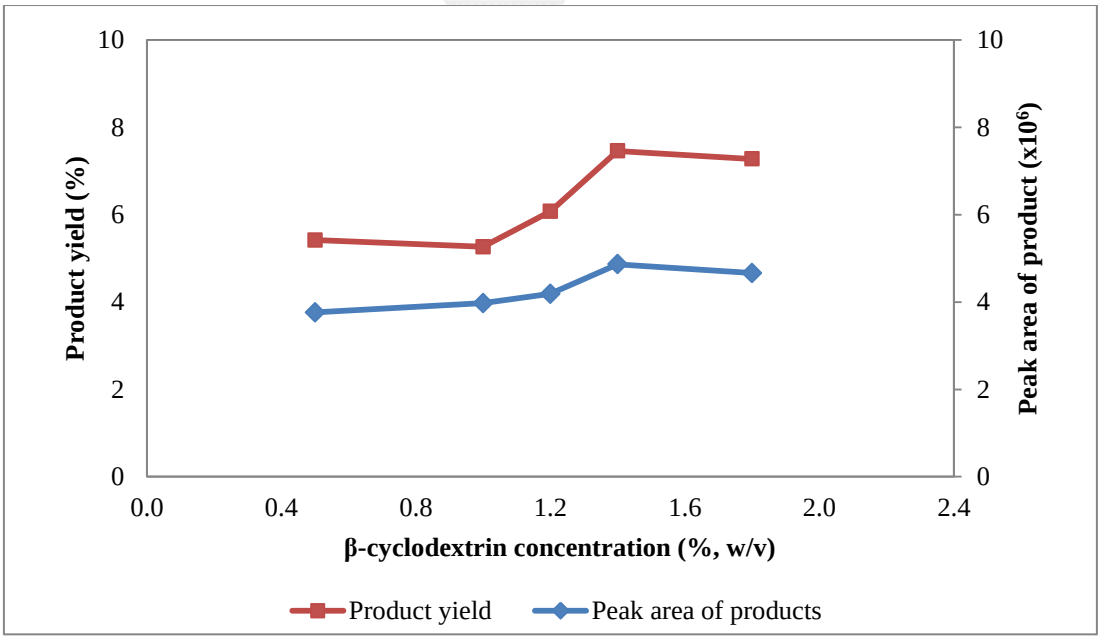


Figure 18 Effect of β-cyclodextrin concentration on resveratrol glycoside production by CGTase.

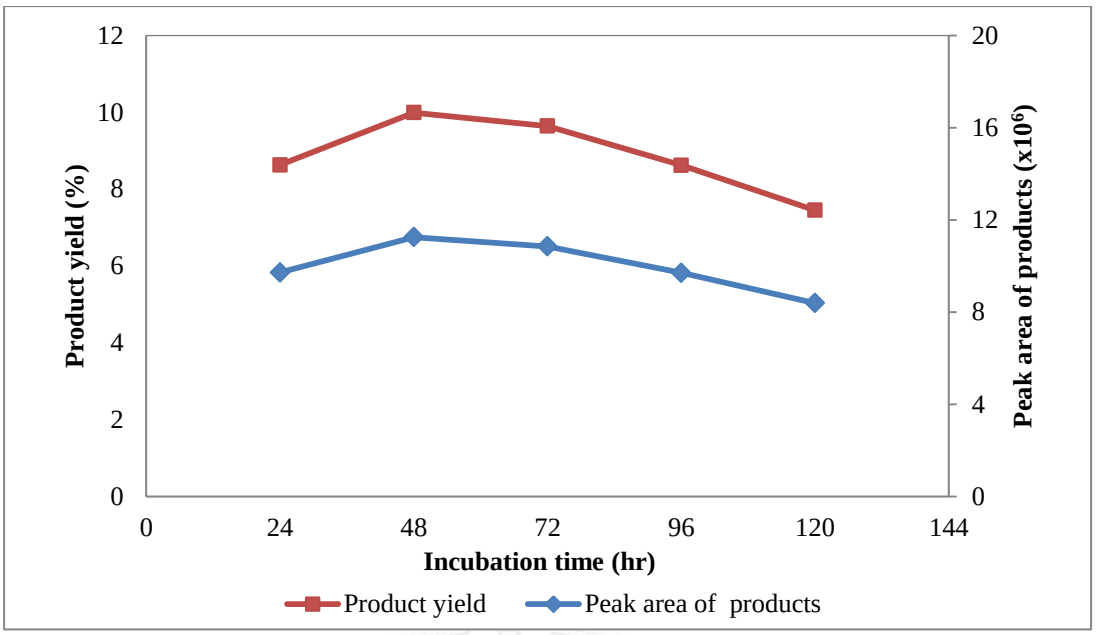


Figure 19 Effect of incubation time on resveratrol glycosides production by CGTase.

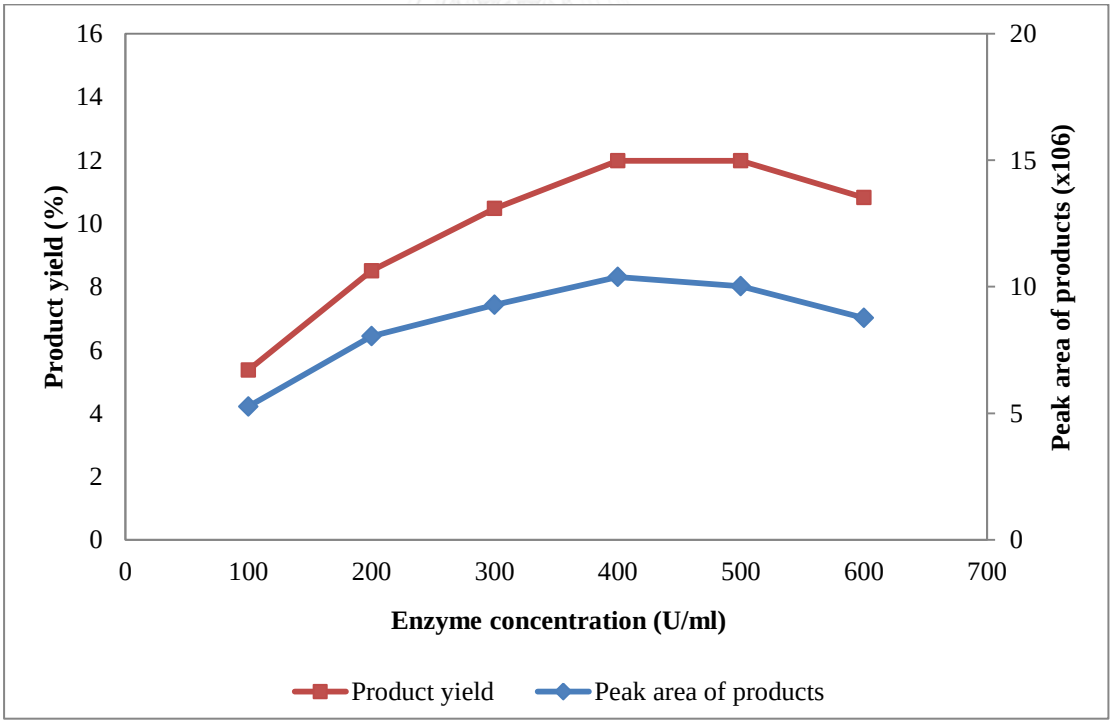
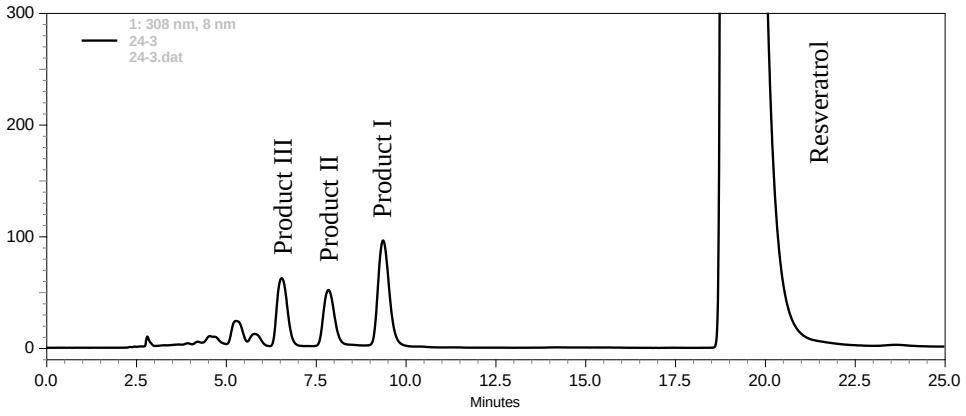


Figure 20 Effect of enzyme concentration on resveratrol glycoside production by CGTase.

A.



B.

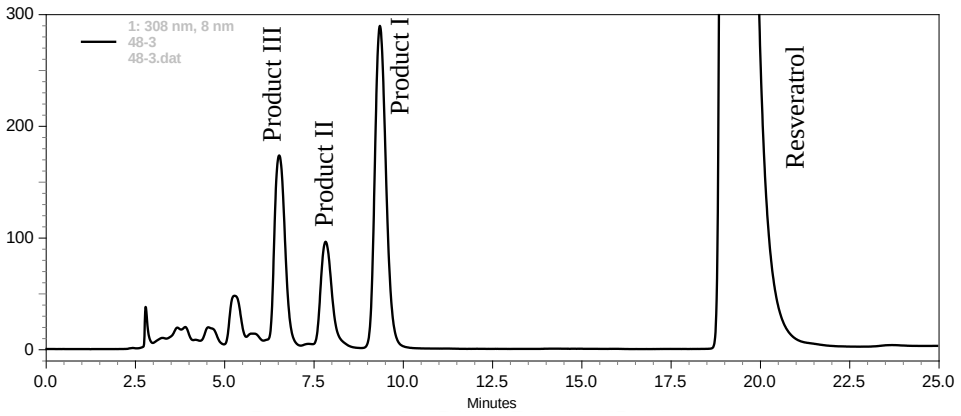


Figure 21 HPLC chromatogram of the resveratrol glycoside production before and after optimization (35% (v/v) of methanol was used as mobile phase).

A. Before optimization : 1.0%(w/v) of resveratrol, 1.0%(w/v) of β-cyclodextrin, 100 U/ml of CGTase and incubate for 24 hrs

B. After optimization : 0.6%(w/v) of resveratrol, 1.4%(w/v) of β-cyclodextrin, 400 U/ml of CGTase and incubate for 48 hrs

3.5 Large scale production and purification of resveratrol glycosides

3.5.1 Large scale production of resveratrol glycosides

Resveratrol glycoside production under optimum conditions (0.6% (w/v) of resveratrol in 20% (v/v) DMSO, 1.4% (w/v) of β-cyclodextrin, 400 U/ml of CGTase at 40°C, 48 hours) was scaled up 50 times. After 48 hours of incubation, the reaction mixture was concentrated to 1 ml solution by rotary evaporator at 40°C before subjected to column chromatography.

3.5.2 The purification of resveratrol glycoside products by column chromatography

3.5.2.1 Sephadex LH-20 column chromatography

To purify the resveratrol glycoside products, Sephadex LH-20 column was used as described in section 2.17, column was saturated with ultrapure water and eluted with 50% (v/v) of methanol and 100% of methanol, respectively. The fractions were collected and spectrophotometric method was used to measure A_{308} (λ_{max} of resveratrol) to construct the purification profile. From the Sephadex LH-20 column profile in Figure 22, fraction number 29, 40, 48, 275, 298, 469, 486 and 505 were identified by HPLC.

Figure 23 shows the HPLC chromatogram of fraction 29, 40, 48, 275, 298, 469, 486 and 505, It was found that when the column was flowed with ultrapure water, the column profile showed peaks at fraction number 29, 40, 48. However, when they were analyzed by HPLC, the products could not be observed in HPLC chromatogram (Figure 23, A-C). Meanwhile, when the mobile phase was changed to 50% (v/v) of methanol, fraction number 275

showed an overlapped peak at Rt = 3.3 and 3.6 minutes (Figure 23, D). While fraction number 298, peaks were found at Rt 3.7 and 9.8 minutes as shown in Figure 23, E. After that, the mobile phase was changed again from 50% to 100% (v/v) of methanol for the elution of resveratrol, four peaks were found at Rt 4.9, 5.8, 7.1 and 8.3 minutes in fraction number 469 (Figure 23, F). In fraction number 486, peaks at Rt 5.9 and 8.3 minutes (Figure 23, G) were observed and finally resveratrol was eluted in fraction number 505 as shown in Figure 23, H. From these results, Sephadex LH-20 column cannot be used for the purification of resveratrol glycosides since many peaks were observed in each fraction collected. In order to separate products with high purity, Bio-Gel P2 chromatography was then further used.

3.5.2.2 Bio-Gel P2 column chromatography

The reaction mixture of resveratrol glycosides was for on to biogel-P2 column (column size 1.2 x 92 cm). The typical fractionation range was between 100-1,800 Dalton. The column was equilibrated with distilled water with a flow rate of 8 ml/hr. From Figure 24, in order to preliminary check the purity of resveratrol glycosides, fraction number 305, 410, 515, 695, 866 were identified by TLC analysis (see section 2.14.1) compared with the reaction mixture before subjected to purification (Figure 25, lane 8). From these results, the products were again unsuccessfully purified by Bio-Gel P2 since all of the fractions collected were impure products as shown in Figure 25, lane 3-5, except for resveratrol in fraction 695 and 866 (Figure 25, lane 6-7).

3.5.2.3 Silica gel column chromatography

After two types of column chromatography were used for the purification of resveratrol glycoside products and it was unsuccessful. Then, the silica gel column chromatography was further employed. The reaction mixture of resveratrol glycosides was also applied on silica gel column with a flow rate of 60 ml/hr and eluted with n-butanol:2-propanol:water (10:5:1) at room temperature. From silica gel column chromatography profile in Figure 26, fraction number 15, 348, 419, 462, 518 were collected and further identified by HPLC analysis (Figure 27). The mobile phase used was the same as before but the percentage was changed in order to improve the separation of the product. The mobile phase used in this HPLC analysis was 15% (v/v) of methanol. All of the compounds were found to retain in the column longer since more polar eluent was used. All the fraction collected showed the peak at Rt of 58.7 minutes which was believed to be resveratrol. No other peaks were observed. So, resveratrol glycosides could not be separated under this condition.

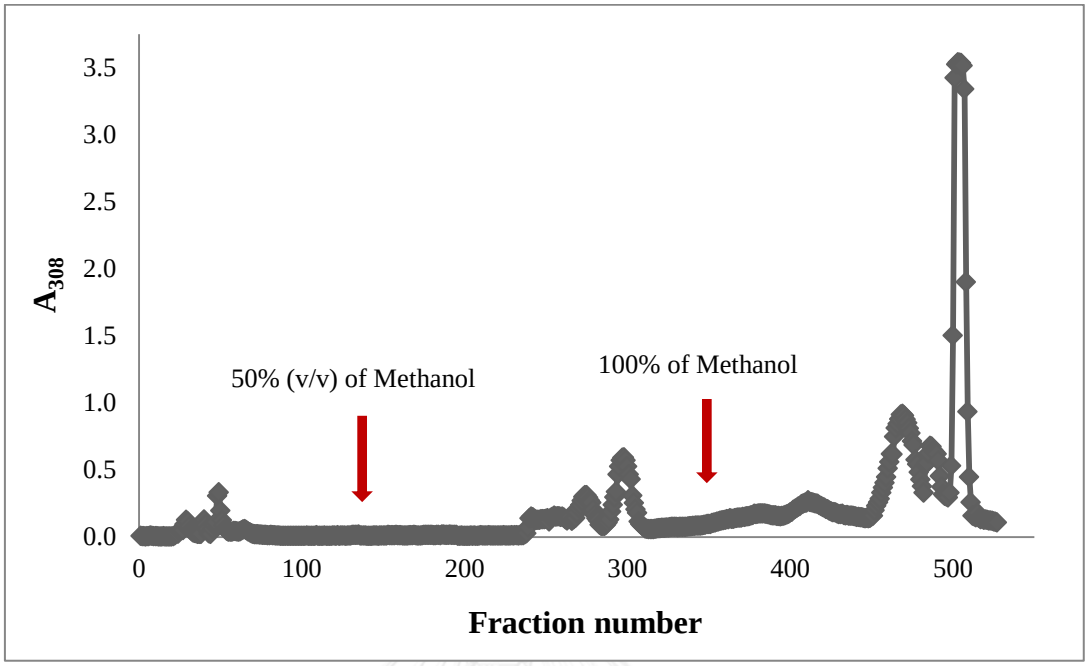


Figure 22 Sephadex LH-20 column chromatography profile of resveratrol glycoside products (column size 1.2x100 cm). Flow rate 30 ml/hr, fraction size 2 ml (water, 50% (v/v) of methanol and 100% of methanol were used as eluent).

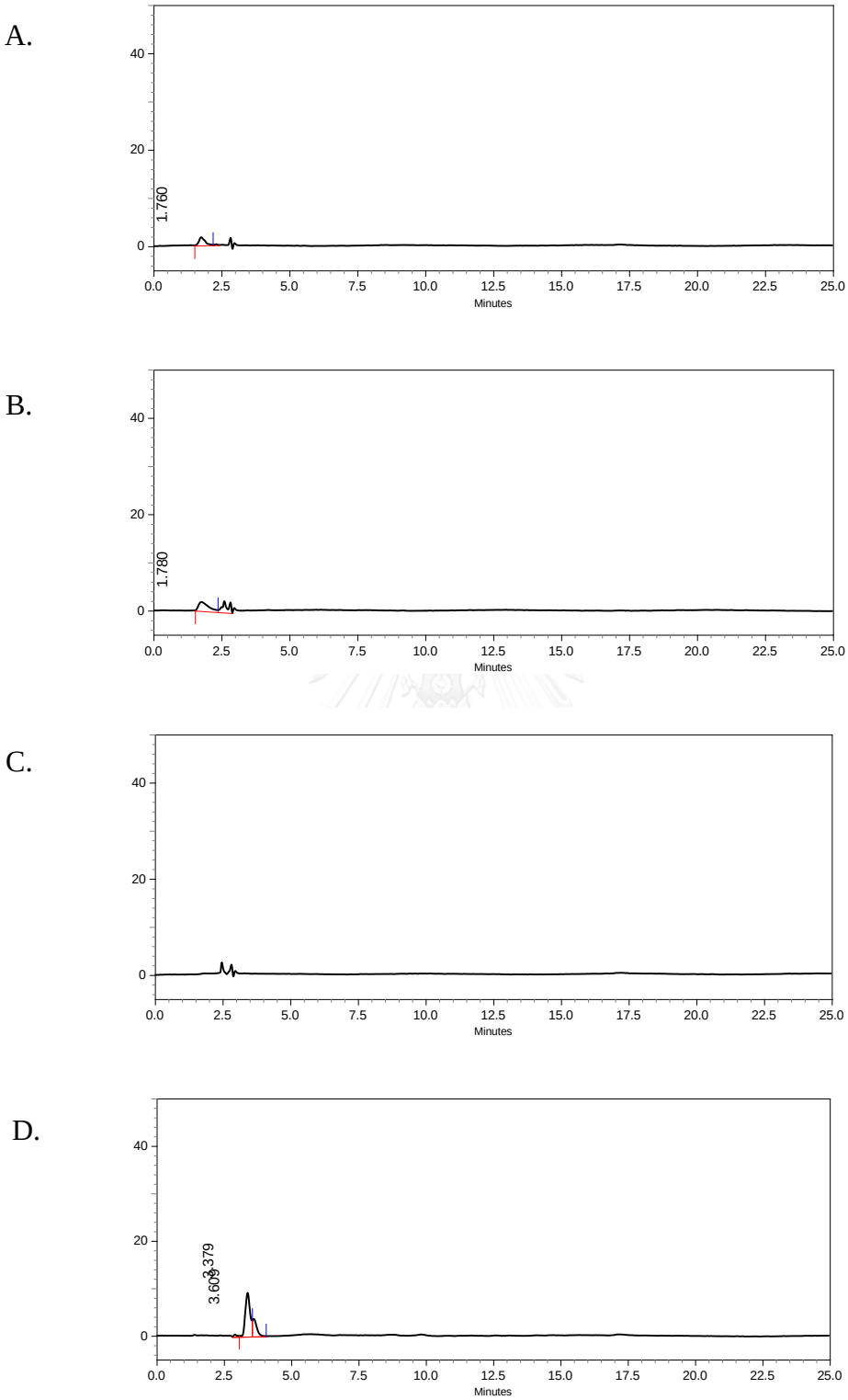
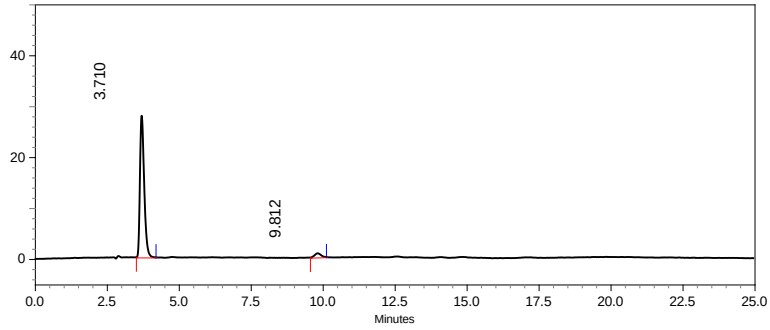
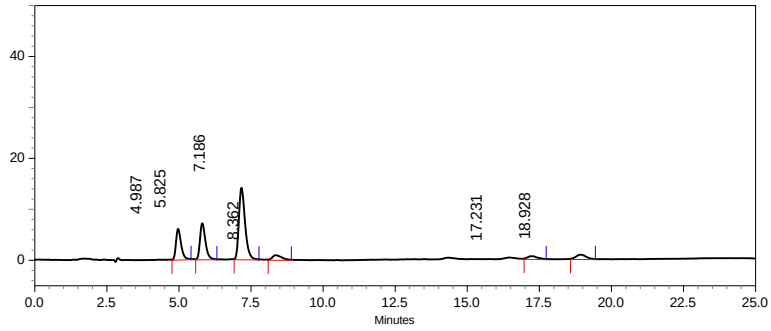


Figure 23 HPLC chromatogram of resveratrol glycoside products collected by Sephadex LH-20 column chromatography (A,B,C, D, E, F, G and H: fraction number 29, 40, 48, 275, 298, 469, 486 and 505, respectively) (30% (v/v) of methanol was used as mobile phase).

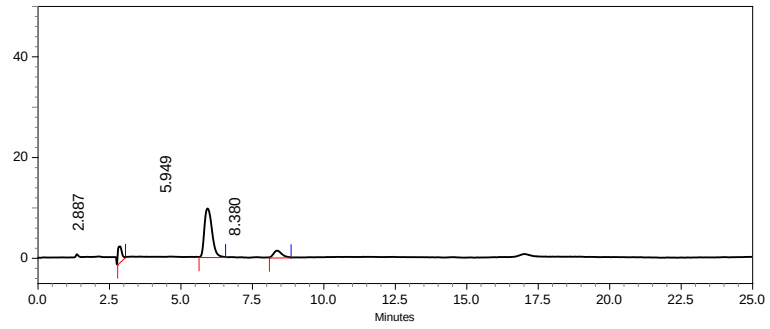
E.



F.



G.



H.

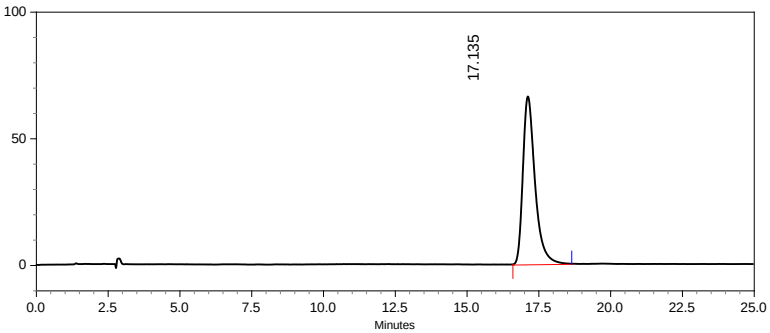


Figure 23(cont.) HPLC chromatogram of resveratrol glycoside products collected by Sephadex LH-20 column chromatography (A,B,C, D, E, F, G and H: fraction number 29, 40, 48, 275, 298, 469, 486 and 505, respectively) (30% (v/v) of methanol was used as mobile phase).

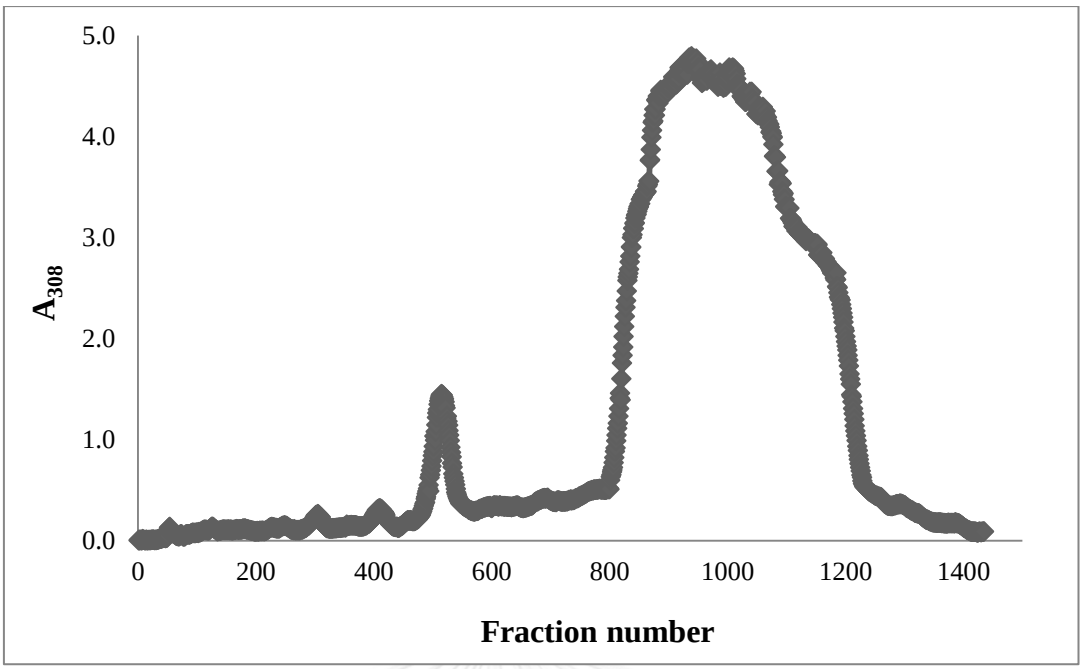


Figure 24 Biogel-P2 column chromatography profile of resveratrol glycoside products (column size 1.2 x 92 cm). Flow rate of 8 ml/hr, fraction size was 1 ml (ultrapure water at room temperature was used as eluent).

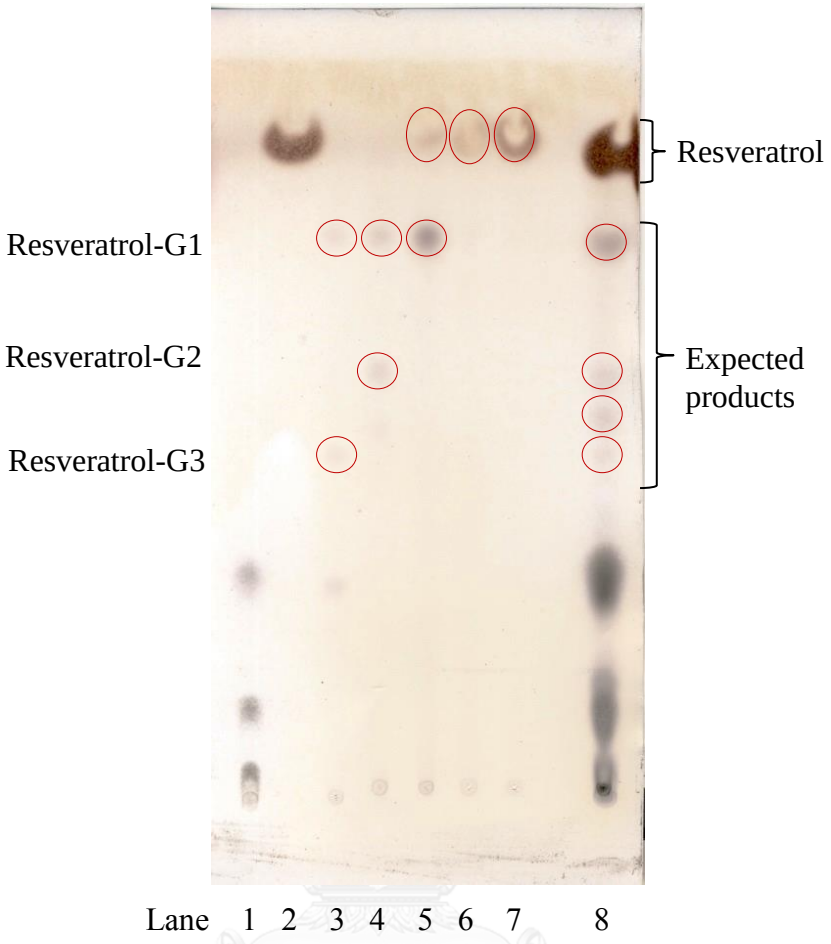


Figure 25 TLC analysis of the resveratrol glycoside products collected from Biogel-P2 column.

Lane 1: Standard glucose, maltose, maltotriose

Lane 2: Resveratrol

Lane 3: Fraction number 305

Lane 4: Fraction number 410

Lane 5: Fraction number 515

Lane 6: Fraction number 695

Lane 7: Fraction number 866

Lane 8: Transglycosylation reaction mixture before purification

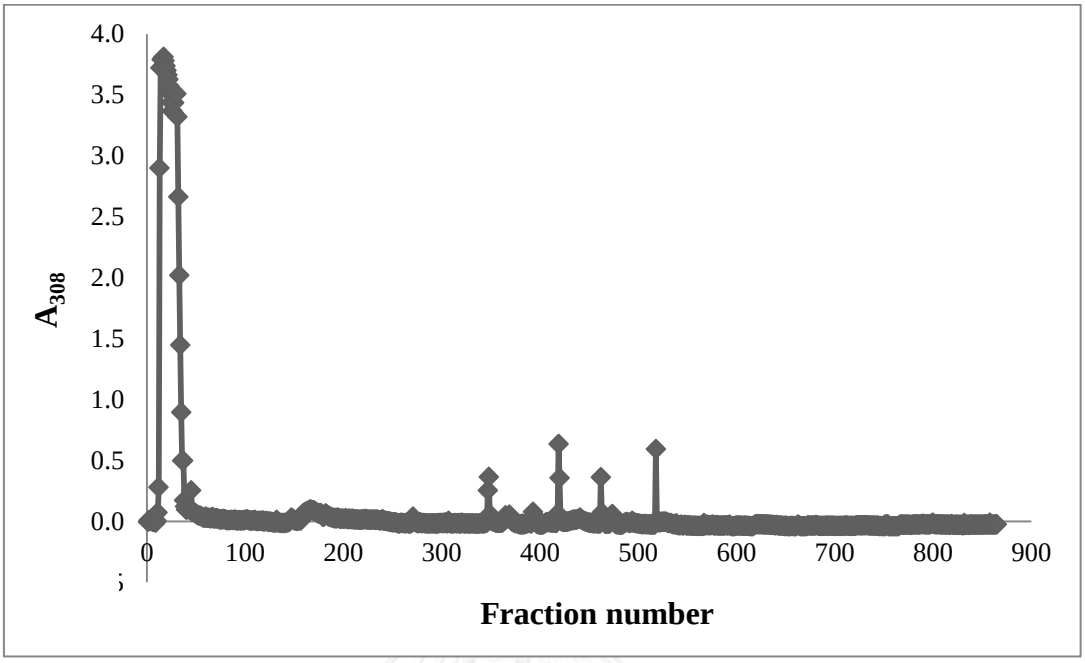


Figure 26 Silica gel column chromatography profile of resveratrol glycoside products (column size 2 x 50 cm). Flow rate of 60 ml/hr, fraction size was 1 ml (n-butanol : 2-propanol : water (10 : 5 : 1) at room temperature was used as eluent).

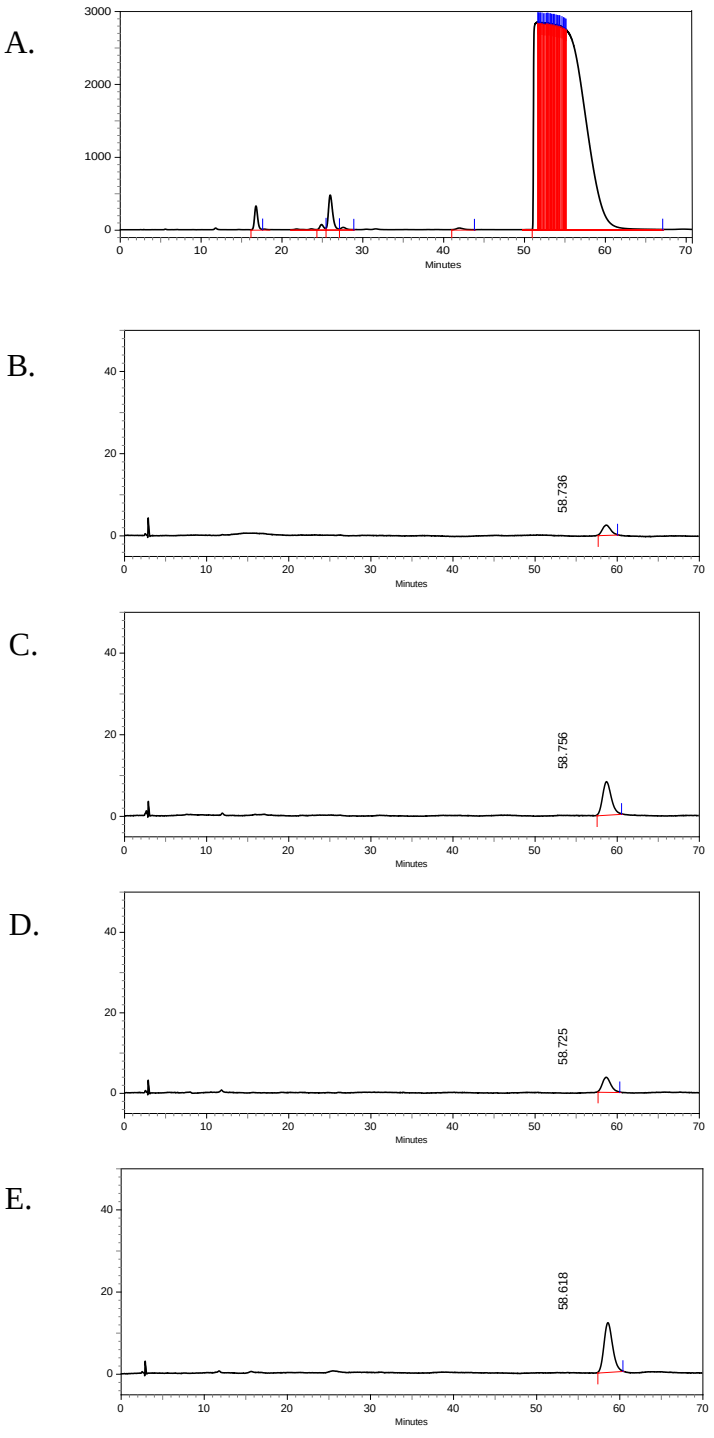


Figure 27 HPLC chromatogram of resveratrol glycoside products collected by silica gel column chromatography (A, B, C, D and E : fraction number 15, 348, 419, 462 and 518, respectively) (15% (v/v) of methanol was used as mobile phase).

3.5.3 The purification of resveratrol glycoside products by collecting fractions from HPLC

Various types of column chromatography were carried out to purify resveratrol glycosides. Among them were Sephadex LH-20, bio-gel P2 including silica gel column chromatography. Nevertheless, the purity of resveratrol glycoside products could not be achieved. So, in order to collect only the main product, glucoamylase was used in order to remove all glucosyl residues sequentially from non-reducing end of glucoside moiety conjugated to resveratrol glucoside. Then, HPLC was used to collect the resveratrol glucoside products. The percentage of mobile phase (methanol) was changed to 30% (v/v) for better separation of the products. Since the percentage of methanol was decreased from 35 to 30%, this maximized the time required to run the column and thus, resveratrol came out at Rt of 28 minutes. Resveratrol monoglucoside peak was found to be doublet at Rt of 12.8 and 13.6 minutes (Figure 28). Peak at Rt of 13.6 minutes was referred to as resveratrol glucoside I and at Rt of 12.8 minutes was referred to as resveratrol glucoside II were collected. The fraction of resveratrol glucoside I at Rt of 13.6 minutes was also used for structure elucidation (see section 3.6).

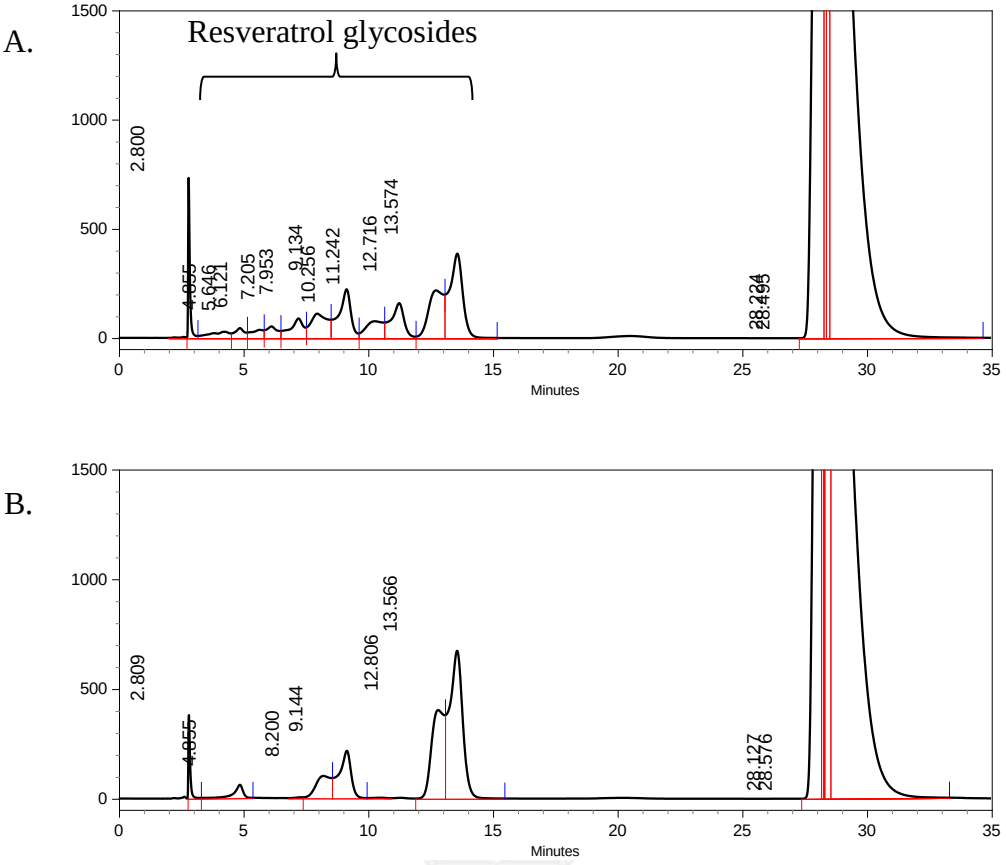


Figure 28 HPLC chromatogram of A. resveratrol glycoside in reaction mixture and B. resveratrol glycoside in reaction mixture treat with glucoamylase (30% (v/v) of methanol was used as mobile phase).

3.6 Characterization of resveratrol glycoside products

3.6.1 Tandem Mass Spectrometry (LC-MS/MS)

The use of LC with tandem mass spectrometry (MS/MS) in combination with collision-induced dissociation (CID) has obtained as a simple and sensitive method for detecting, identifying, and quantifying the molecular weight (MW) of samples.

For the investigation of MW and structure of resveratrol glucoside I, LC-MS/MS with positive mode was analyzed as described in section 2.19.1. The total ion current (TIC) is the sum of all the separate ion currents carried by the ions of different m/z contributing to a complete mass spectrum or in a specified m/z range of a mass spectrum. Figure 29 shows TIC chromatogram which presented the target peak of resveratrol glucoside I at Rt 11.6 minutes with m/z of 391. TIC has to be obtained first, this peak was then further analyzed by the first unit of MS.

As seen in Figure 30, A, $[M+H]^+$ ion of investigated product displayed a peak at m/z of 391 (390 plus 1 of H molecule) which was consistent with resveratrol monoglucoside. In addition, from ms/ms spectrum peak list support data (Appendix H), it was found that the spectrum also yielded fragment ions at m/z of 295, 271, 229 and 135, apart from m/z of 391.

From Figure 30, B, it can be seen that the resveratrol glucoside I can be splitted into the fragment ions at m/z of 229 and 135. As shown in a schematic diagram of the proposed fragmentation pathway (Figure 31), the fragment ion size at m/z of 229 was believed to form from 390 ($C_{20}H_{22}O_8$) after a loss of 162 ($C_6H_{10}O_5$) and plus 1 of H molecule. For the fragment ions at m/z of 135, it was formed from a further loss of 94 (C_6H_6O) from the m/z of 229 (Figure 31) Thus, these fragment sizes corresponded to the m/z value of resveratrol.

Furthermore, the fragment ions at m/z of 295 was formed from 390 ($C_{20}H_{22}O_8$) after a loss of 96 (C_6H_8O) and plus 1 of H molecule and at m/z of 271 was formed from 390 ($C_{20}H_{22}O_8$) after a loss of 120 (C_8H_8O) and plus 1 of H molecule. Hence, it was concluded that the glycosyl group was attached to resveratrol at the position of 3-hydroxyl group in resveratrol.

3.6.2 Water solubility

The water solubility of resveratrol was compared with that of resveratrol glucoside I and II. The standard curve of resveratrol was analyzed by HPLC then plotted in mg/ml (Appendix E). It was found that the water solubility of resveratrol glucoside I and II was 0.1151 and 0.0409 mg/ml, respectively. Whereas the water solubility of resveratrol was 0.0016 mg/ml. It can clearly be seen that the water solubility of resveratrol glucoside I was about 72 times whereas resveratrol glucoside II was 26 times higher than that of resveratrol. From these results, it was concluded that the water solubility of resveratrol can be increased by the attachment of glycosyl group to the original compound by enzymatic synthesis via transglycosylation reaction.

3.6.3 Antioxidant activity

The spectrophotometric DPPH radical scavenging assay is the most commonly applied in measuring the ability of antioxidants to scavenge free radicals. The DPPH radical scavenging activity was determined by analyzing the percentage decrease in the absorbance at 517 nm of sample compared with ethanol as a blank. The DPPH• scavenging effect (%) was calculated from DPPH assay as described in section 2.18.4. As can be seen in Table 6, The DPPH• scavenging effect (%) of both of resveratrol glucoside I and II was lower than those of free resveratrol and vitamin c.

Compound Label	*m/z*	RT	Algorithm
Cpd 1: 11.651	391.1236	11.651	Targeted MS/MS

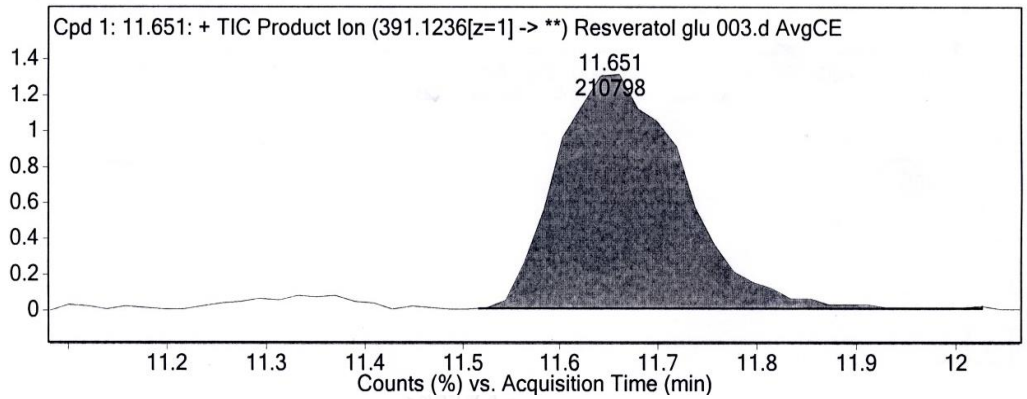
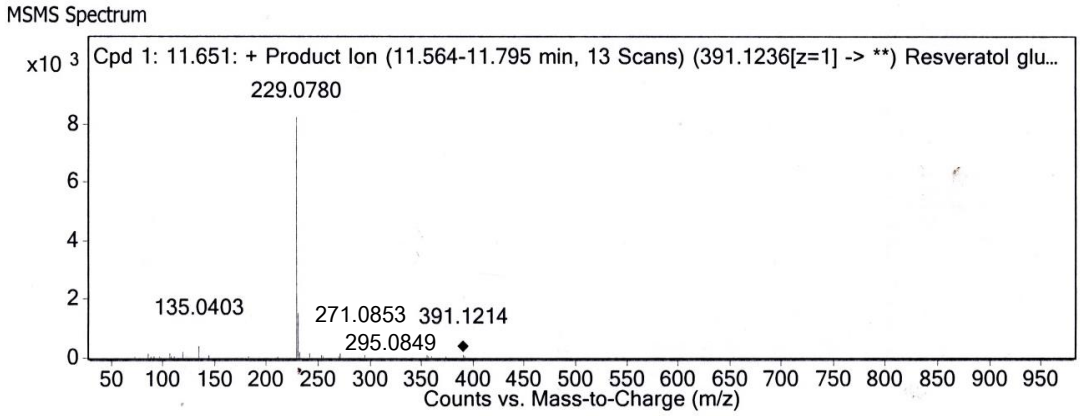


Figure 29 The total ion current (TIC) chromatogram of resveratrol glucoside I.

A.



B.

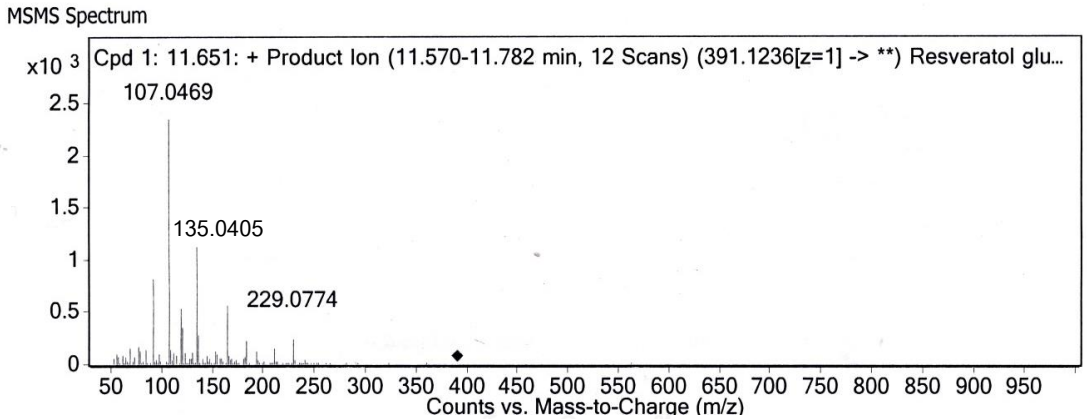


Figure 30 The LC-MS/MS spectra of A. resveratrol glucoside I (m/z 391) and B. its fragments.

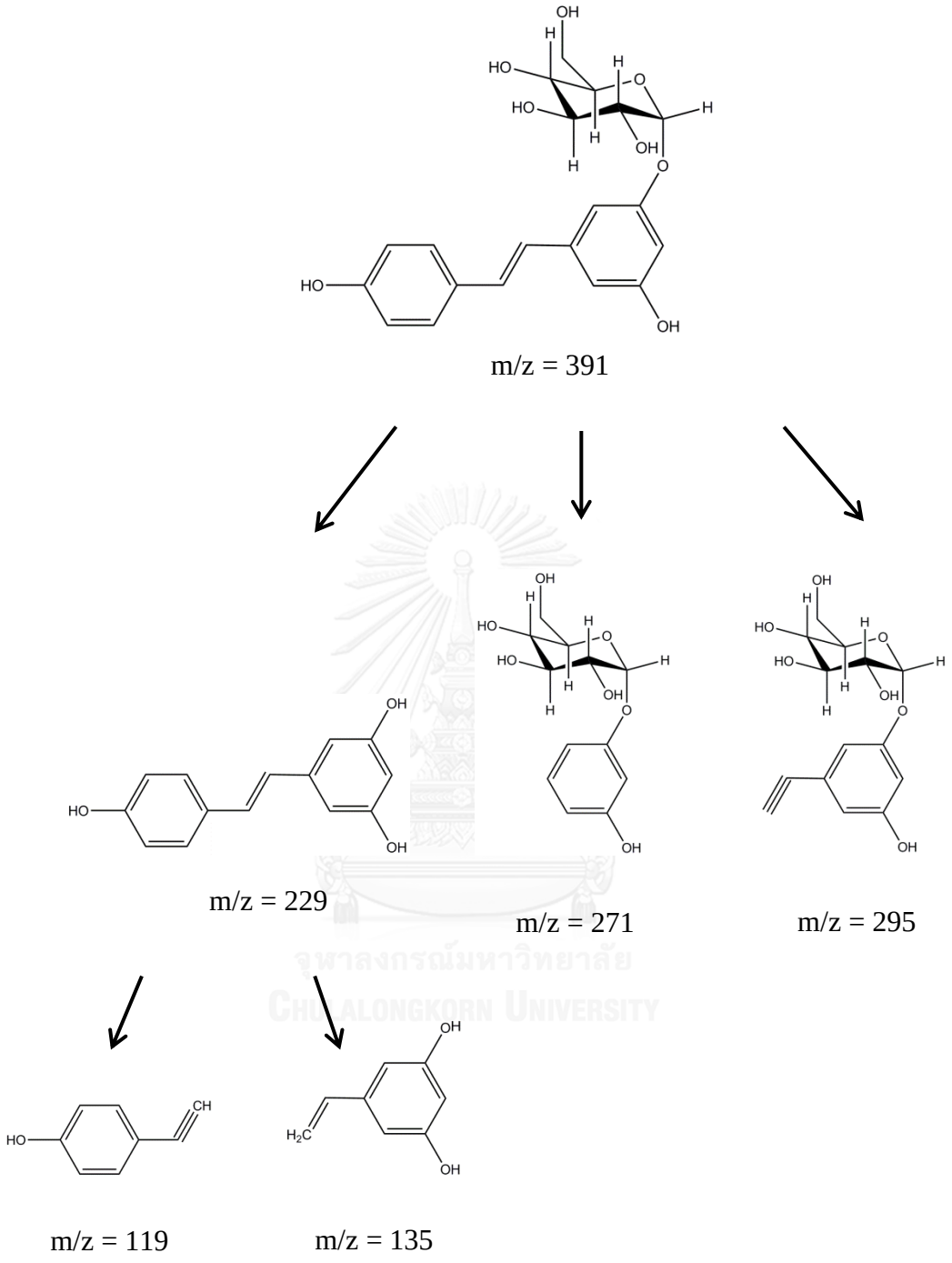


Figure 31 The proposed decomposition mechanism of the m/z 391 [M+H]+ ion of resveratrol monoglucoside

Table 6 The DPPH• scavenging effect (%) of ascorbic acid (vitamin c), resveratrol, resveratrol glucoside I and II at the concentration of 0-30 μg/ml.

Standards / Products	**DPPH• scavenging effect (%)**				
	Concentration (μg/ml)				
	0	**5**	**10**	**20**	**30**
Standard Ascorbic acid (vitamin c)	0	14	34	40	52
Standard Resveratrol	0	39	45	67	74
Resveratrol glucoside I	0	5	8	10	11
Resveratrol glucoside II	0	10	15	15	22

CHAPTER IV

DISCUSSION

In this research, transglycosylation reaction was investigated. Resveratrol was used as a substrate acceptor to study the enzymatic synthesis of transglycosylation reaction by cyclodextrin glycosyltransferase using β-cyclodextrin as a glycosyl doner to provide glucose moiety into the molecule. CGTase is one of the most important key for transglycosylation reaction responsible to the synthesis of glycosidic linkage, recognized as a glycosyltransferase and has widely been utilized for the purpose of glycoside formation for developing change in properties of many modified compounds.

Numerous reports on the transglycosylation to interesting compounds other than saccharides for the production of many useful glucosides using CGTase are available. They suggested that glycosylation usually offers the improvement of the bioavailability and physicochemical properties such as water solubility, absorption, and partition coefficient. For example, the synthesis of hesperidin glycosides (Kometani *et al*., 1994) and naringin glycosides (Kometani *et al*., 1996). They found that the glucoside derivatives of hesperidin and naringin were 300 and 1,000 folds more soluble than their parent molecules, respectively. In addition, the epicatechin glycosides catalyzed by CGTase has improved its water-solubility and stability against UV irradiation (Aramsangtienchai *et al*., 2011). Alcohol can also be used as an acceptor molecule for tranglycosylation reaction by CGTase. For example, the glycosylation of dodecyl glucooligosides by CGTase produced a biodegradable and non-ionic surfactant which is important in detergent industries (Mathew and

Adlercreutz, 2011). Chotipanang *et al*. (2010) reported the synthesis of alkyl glycosides from β-cyclodextrin and various soluble alcohols by CGTase and short-chain alkyl glycosides were successfully produced. In addition, Katelakha (2012) synthesized the medium-chain alkyl glycosides by CGTase. Isobutyl-α-monoglucoside and Isobutyl-α-maltoside were observed and showed emulsification activity of 11.4% and 41.1% of triton X-100, respectively.

Although resveratrol has drawn great interest as health-promoting such as antioxidant, anticancer, anti-aging, cardioprotective and neuroprotective activity, their applications are frequently limited because of low water solubility of resveratrol (less than 0.03 g/L). Thus, to improve its aqueous solubility, transglycosylation from glycosyl donor to resveratrol by CGTase should be a useful way to solve the problem.

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

CGTase from *Paenibacillus* sp.RB01; thermo-tolerant bacteria producing extracellular enzyme was first screened from hot spring soil area in Ratchaburi, Thailand (Tesana, 2001). CGTase was partially purified by a single step using starch adsorption technique. This technique is dependent on the interaction between commercial corn starch and enzyme (binding affinity of its substrate and enzyme). Crude enzyme was stirred with starch and centrifuged to remove other proteins in supernatant. Enzyme that can use the starch as a substrate would bind to starch, other enzymes would then be removed. Maltose solution was then added to resuspend starch cake in order to separate enzyme from starch because maltose had higher affinity for binding with CGTase more than starch. Accordingly, partial purified

CGTase that bound to maltose was separated from starch by centrifugation. Then, the supernatant was dialyzed against buffer at 4°C to remove maltose. The enzyme obtained was concentrated with aquacide II. From the purification table (Table 4), the yield of partial purified CGTase was 33 % with 95 purification fold and specific activity of 11,783 U/mg protein. From the previous research on partial purification by starch adsorption technique of CGTase from *Paenibacillus* sp.RB01, Chotipanang *et al.* (2010) and Katelaekha (2012) reported the yield and purification fold; the yield was 38.1 % and 50.6 % with 46.3 and 120 fold increase in purity, respectively. These results suggested that the amount as well as the purity of enzyme were dependent on the quality of person-performing purification process. In order to check the enzyme purity and activity, both Native- PAGE and SDS-PAGE were used. The native-PAGE was investigated by dividing the gel into two parts. The first part was determined with dextrinizing activity staining, the distinct clear zone bands on the gel showed the location of CGTase. Another section of the gel was stained with Coomassie brilliant blue, three bands were observed which showed the same position with clear zone bands in dextrinizing activity staining. Due to less bands of proteins were observed than in the first part, it can be concluded that CGTase was successfully purified through starch adsorption technique. These indicated that the partial purified enzyme obtained was CGTase with 3 isoforms in different net charge. In order to check the size of CGTase, SDS-PAGE was performed. SDS was added to the protein sample to provide negative charges to the polypeptide chain. Binding of SDS to polypeptide chain allows an even distribution of charge per mass. The enzyme showed one major and one minor band, the molecular weight of a major band was estimated to be 70 kDa which was approximate mass of CGTase and corresponded with the previous

work by Chotipanang *et al*. (2010) and Katelakha (2012). From these result, it was concluded that CGTase was successfully purified.

4.2 Effect of DMSO on the CGTase stability

Due to the low water solubility of resveratrol, organic solvents were used as a co-solvent in transglycosylation reaction. It was found that at low concentration of DMSO used, resveratrol could not be dissolved well so the resveratrol in the reaction mixture might be separated from aqueous phase. Meanwhile at high concentration of DMSO, although it gave a homogeneous reaction mixture but the DMSO might cause the loss of enzyme activity. Klibanov (1997) described that enzymes lose activity in organic solvents for many reasons such as conformational change and active center blockage (Klibanov 1997). While Park *et al.* (1998) explained that CGTase was stabilized in the presence of low concentrations of DMSO and it could inactivate enzymes at higher concentrations. The residual activity of the enzyme was significantly decreased in the presence of 30% organic solvents or more (Park *et al.* 1998). Moreover, Blackwood and Bucke (2000) reported that CGTase showed good performance in the presence of small amount of polar organic solvents (Blackwood and Bucke 2000). However, the highly polar organic solvents sometimes inactivated CGTase. The result showed that the reaction mixture containing 20% (v/v) of DMSO is an appropriate concentration in terms of reaction mixture solubility and gave the highest production yield. Similarly, Torres *et al.* (2011) reported that 20% (v/v) of DMSO was the optimum concentration in terms of total yield of resveratrol glycosides. DMSO concentration had to be compromised between the solubility of a

substrate and to still maintain the activity of the enzyme for the transglycosylation reaction.

4.3 Transglycosylation reaction and optimization of resveratrol glycoside production

In order to produce the water soluble resveratrol, many researches on resveratrol glycoside synthesis were carried out. Torres *et al.* (2011) demonstrated a success in enzymatic synthesis of α-glucosides of resveratrol with surfactant activity. Resveratrol was used as an acceptor and starch was used as a donor substrate, which was catalyzed by CGTase from *Thermoanaerobacter*. This CGTase was an α-CGTase and it was found that the antioxidant activity in Trolox equivalent to antioxidant capability assay (TEAC assay) was decreased substantially towards increasing number of glucosyl moieties. We then attempt to synthesize a variety of resveratrol glycosides by CGTase from *Paenibacillus* sp. RB01 which is a β-CGTase. The mechanism was proceeded by the glucose transfer reaction from β-cyclodextrin as glycosyl donor to resveratrol as an acceptor. β-cyclodextrin was used instead of starch in hope that new products would be achieved. Various parameters affecting the yield including donor, acceptor and enzyme concentrations were optimized to reach high production yield.

CGTase is a thermotolerant enzyme obtained from hot spring soil, the optimum temperature and pH for transglycosylation reaction was previously reported. It was found to be stable at 40°C and the pH optimum of 6.0 (Chotipanang *et al*., 2010). Thus, to investigate the effect of resveratrol acceptor, glycosyl donor on resveratrol production, the reaction for resveratrol glycoside production was

performed at 40°C and at pH of 6.0. Before subjected to HPLC analysis, a solution was lyophilized in order to remove DMSO.

To determine for the optimal resveratrol concentration, it was found that resveratrol concentration higher than 0.6% (w/v) did not increase production yield. Thus, 0.6% (w/v) of resveratrol was chosen to be the optimum concentration. The resveratrol production yield increased when β-cyclodextrin increased. However, further increase in β-cyclodextrin concentrations more than 1.4% (w/v) did not result in an increase of the product. Thus, the suitable concentration of β-cyclodextrin was at 1.4 % (w/v) and was further used in the effect of incubation time. Resveratrol glycoside synthesis was determined every 24 hour from 0 to 120 hours. The production yield was dramatically increased at the initial stage of the reaction time. After 48 hours, the yield obtained reached plateau. This could be caused by the fact that all proteins suffer denaturation, especially in solvent system and hence loss of catalytic activity with time. In addition, much of the substrate might be used up during the incubation. Hence, the appropriate incubation time for resveratrol glycoside production was 48 hours and the amount of CGTase at 400 U/ml (dextrinizing activity) was found to be an appropriate CGTase concentration. High amount of CGTase was used which could be due to the inactivation by organic solvent.

Figure 21 showed the HPLC chromatogram of the resveratrol glycoside production before and after optimization. The amount of transglycosylated products was found to be varied. And it was found that resveratrol glucoside product I was found to be the main product of the transglycosylation reaction mixture when compared with the other resveratrol oligoglucosides. While product II after optimization seemed to decrease. This was due to the different amount of glucose

molecules attached to resveratrol. It was previously shown that CGTase exhibited glucoamylase-like activity (Jun *et al.* 2001, Tanaka *et al.* 1991). Thus, the enzyme can remove glucose units sequentially from the non-reducing end of oligosaccharide conjugated to resveratrol. It might be concluded that CGTase from *Paenibacillus* sp. RB01 favored monoglucoside synthesis more than oligoglucosides.

4.4 Large scale of resveratrol glycoside production and isolation

Resveratrol glycoside production under optimum conditions (0.6% (w/v) of resveratrol in 20% (v/v) DMSO, 1.4% (w/v) of β-cyclodextrin, 400 U/ml of CGTase at 40°C for 48 hours) was scaled up 50 times. After 48 hours of incubation, the reaction mixture was concentrated to 1 ml by rotary evaporator at 40°C then applied onto Sephadex LH-20 column.

Sephadex LH-20 is a liquid chromatography medium designed for molecular sizing of natural products such as steroids, lipids, and low molecular weight peptides. Depending on the chosen solvents, this medium can also separate sample components by partition between the stationary and mobile phases. In previous report, Sephadex LH-20 had been successful on the purification of epicatechin glycoside (Aramsangtienchai *et al*., 2011). So Sephadex LH-20 was then chosen. After the result was analyzed, it was found that the resveratrol glycoside products could not be separated. The unsuccess at resveratrol glycoside purification might be caused by 1.) Because of the wide range of fractionation; due to the molecular weight of the synthesized products were predicted to be about ~1000 while the exclusion limit of Sephadex LH-20 was found to be not over than 5,000. 2.) As the very low solubility of resveratrol, methanol was used as elution buffer for purifying resveratrol glucoside

products but the approximate packed medium volumes of Sephadex LH-20 swollen in water (4.0 - 4.4 ml/g dry powder) was different from the swollen in methanol (3.9 - 4.1 ml/g dry powder) so it might cause bead size change and some bead volume loss.

The concentrate reaction mixture of tranglycosylation reaction was purified again by Bio-Gel P2. Bio-Gel P gels are porous polyacrylamide beads prepared by copolymerization of acrylamide and N,N'-methylene-bis-acrylamide. The gels are extremely hydrophilic and essentially free of charge, and provide efficient, gentle gel filtration of sensitive compounds. High resolution is assured by consistent narrow distribution of bead diameters and excellent molecular weight discrimination. Miscible organic solvents may be added to the eluents used with Bio-Gel P2. Alcohol up to 20% will not substantially alter the exclusion properties of the gel, and will in some cases enhance separation of complex mixtures of poorly water soluble small molecules such as nucleotides, peptides, and tannins. From previous study, Bio-Gel P2 had been successful for the purification of anticariogenic oligosaccharides (Saehu *et al.* 2013). The reaction mixture of resveratrol glycoside products was then applied on Bio-Gel P2 column (typical fractionation range 100-1,800 Daltons) equilibrated with distilled water with a flow rate of 8 ml/hr.

Bio-Gel P2 column purification profile of resveratrol glycoside products by Bio-Gel P2 column is shown in Figure 24. It was observed that resveratrol glycoside products could not be separated. During transglycosylation reaction, a series of resveratrol glucosides was obtained. As analyzed by TLC, the fractions eluted from Bio-Gel P2 were a mixture of products. At the elution volume of 305 ml, both resveratrol-G1 to -G3 were found in the eluted fraction. Meanwhile, the mixture from elution volume of 410 ml also contained both resveratrol-G1 and -G2. Unfortunately,

resveratrol-G1 could not be purified at longer time course elution because unglycosylated resveratrol was also eluted at the same fraction. This suggested that Bio-Gel P2 could not purify the resveratrol glucoside products into homogeneity. The unsuccess at resveratrol glycoside purification in Bio-Gel P2 column chromatography may be caused by many reasons. It could be due to the effect of temperature and the low water solubility of product. When the temperature was increased to 40°C (the reaction mixture temperature), the product seemed to be more dissolved than when they are at room temperature. The sample should be clear and completely dissolved in buffer without particles or solid contaminants before applying onto the column.

After using two chromatography columns for resveratrol derivative separation, the purification of purified resveratrol-G1 was not achieved. Then, the silica gel column chromatography was further employed. Silica gel column chromatography is a conventional preparative separation method for resveratrol and their derivatives. From previous study, it has been reported that resveratrol was efficiently separated from the *Morus alba L.* leaf extract and gave a higher sensitivity when the elution was performed by using chloroform and methanol (10:1) as the solvent. In addition, the purified resveratrol by the silica gel column chromatography can be advanced from 0.8 to 99.3% (Tang *et al.* 2011). The reaction mixture of resveratrol glycosides was also applied onto silica column with a flow rate of 60 ml/hr and eluted with n-butanol:2-propanol:water (10:5:1) as the same as TLC solvent system, at room temperature. Fractions number 15, 348, 419, 462 and 518, which were believed to be resveratrol derivatives obtained from silica gel column chromatography as shown by Figure 26 were further examined by HPLC analysis. The HPLC chromatogram of resveratrol glycoside products is shown in Figure 27. Resveratrol was eluted at Rt of

~58 minutes. The expected products were eluted at Rt of ~17 and ~26 minutes in fraction number 15, together with resveratrol at Rt of ~58 minutes (Figure 27, A). So, resveratrol glycosides could not be separated under this condition. The unsuccess of resveratrol glycoside purification using silica gel column chromatography may be derived from the close equivalence of mobile phase solvent and sample polarity. This made each of the products inseparable. The chromatogram of thin layer chromatography (TLC) and column chromatography applied the same stationary phase and solvent system revealed that it was quite difficult to separate the nearby product sharing the same polarity.

The transglycosylated products were then hydrolyzed by glucoamylase to get a single product. It was then purified by HPLC and the main product was further analyzed by LC-MS/MS system.

4.5 Characterization and properties of resveratrol glycoside products

4.5.1 Molecular weight and structure

In order to evaluate molecular weight and structure, both ESI-TOF-MS and LC-ESI-QTOF-MS/MS with positive mode were used.

The reaction mixture of resveratrol glycoside products was applied on ESI-MS. In electro spray ionization part, the reaction mixture was dissolved in methanol and the pseudomolecular ions with positive mode of products was addition of sodium $[M+Na]^+$. Then, the ions would pass through the mass analyzer of time of fight (TOF) part. After that the ions was then detected by time array detector. The pseudomolecular ion of products gave 3 product peaks at m/z of 413 (390 plus 23 of sodium molecule), 575 (552 plus 23 of sodium molecule) and 737 (714 plus 23 of

sodium molecule) (Figure 30, A). These were corresponded with the molecular weight of resveratrol monoglucoside ($C_{20}H_{22}O_8$), resveratrol maltoside ($C_{26}H_{32}O_{13}$), resveratrol maltotrioside ($C_{32}H_{42}O_{18}$), respectively. From previous study by Torres *et al*., (2011) they found three main products as resveratrol monoglucoside (3-O-α-d-glucosyl-resveratrol and 4'-O-α-d-glucosyl-resveratrol), resveratrol maltoside (3-O-α-d-maltosyl-resveratrol and 4'-O-a-d-maltosyl-resveratrol) and resveratrol maltotrioside (4'-O-α-d-maltotriosyl-resveratrol).

Tandem mass spectrometry (MS/MS) was a technique used to identify and quantify molecules in sample or compound mixtures. It was operated to break down selected ions into fragments then reveal aspects of the chemical structure of the precursor ion; the fragments are separated based on their individual *m/z* ratios in a second round of MS.

From Figure 32, schematic diagram of tandem mass spectrometry is shown. First, samples from LC unit are ionized (performed by ESI, MALDI, EI, etc.) to generate a mixture of ions, then the precursor ions of a specific mass-to-charge ratio (*m/z*) are selected (MS1) and then fragmented (MS2) to generate a product ions for detection. The selection-fragmentation-detection sequence can be further extended to the first-generation product ions. For example, selected product ions generated in MS2 can be further fragmented to produce another group of product ions (MS3) and so on.

In the QTOF (Quadrupole Time-of-flight), precursor ions are selected in the Quadrupole and sent to the collision cell for fragmentation. The generated product ions are detected by time-of-flight (TOF) mass. The limitation of this technique is to

analyze the complex mixtures or large molecules because the result will be show very complex and very difficult to interpret the data.

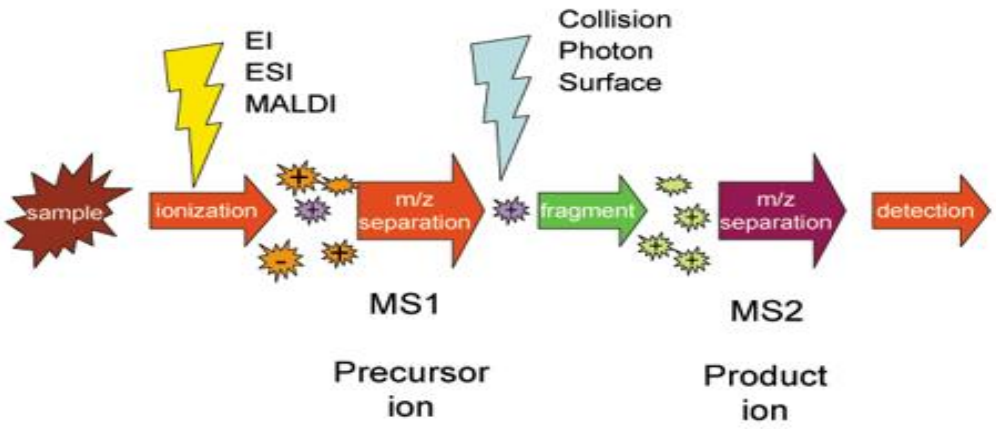


Figure 32 Schematic of tandem mass spectrometry

(http://en.wikipedia.org/wiki/Tandem_mass_spectrometry)

In this work, LC-MS/MS was used to analyze resveratrol glucoside I. The result showed that the molecular weight of the target product displayed at m/z 391 (The pseudomolecular ion with positive mode $[M+H]^+$ are 390 plus 1 of H molecule). From Figure 30, the MS/MS spectrum showed the fragment peaks at m/z of 295, 271, 229, 135 and 119. From these results, the structure of resveratrol glucoside I can be predicted. From Section 3.6.1, the structure of resveratrol glucoside I was proposed to be resveratrol-3-O-glucoside which corresponded to previous study (Torres *et al.*, 2011). They proposed five different patterns of resveratrol glycoside products (Figure 33). However, the transglycosylation of glycosyl donor can only be attached to hydroxyl group of resveratrol acceptor at the position of 3-, 5- and/or 4'. Resveratrol-3-O-glucoside was also found to be the main product in this study. None of the elucidated structures presented either Type III or Type V patterns, probably due to the

steric hindrance between two glucosyl moieties in the spatially close 3- and 5-positions.

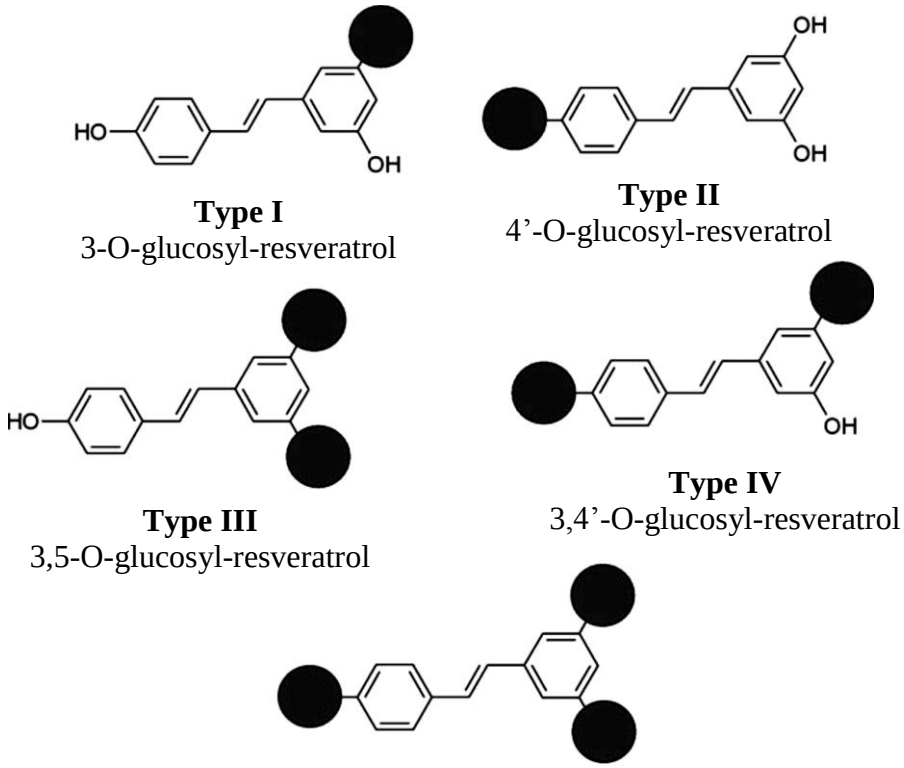


Figure 33 The possibility structure of resveratrol glycoside products (Torres *et al*., 2011).

4.5.2 Water solubility

In order to investigate the water solubility, the excess concentration of resveratrol and their glucoside molecules were dissolved in distilled water and filtrated. There are several reports investigating the transglycosylation reaction to improve some biological and phamarcological of interesting compounds. For examples, Kometani *et al*. (1994 and 1996) reported the enzymatic synthesis of hesperidin and naringin glucosides by CGTase. It was found that the water solubility of 4-α-D-glucopyranosyl hesperidin and 3-α-D-glucopyranosyl naringin were 300 and 1,000 times than the parent molecules. Aramsangtienchai *et al*. (2011) reported the enzymatic synthesis of epicatechin glycoside by CGTase. The water solubility of epicatechin-3'-O-α-D-glucopyranoside was 44 times higher than unglucosylated epicatechin. In addition, Lepak (2015) reported on the synthesis of resveratrol 3,5-β-D-diglucoside by UGT71A15 (a uridine 5'-diphosphate α-D-glucose-dependent glucosyltransferase from apple), it was found that this resveratrol derivative is more water-soluble than unglucosylated molecule, about 1,700 fold (~0.18 mM).

However, the water solubility of resveratrol glucoside I and II from our experiment were 0.1151 and 0.0409 mg/ml, respectively whereas the water solubility of resveratrol were 0.0016 mg/ml. In consequence, the water solubility of resveratrol glucoside I was about 72 times whereas resveratrol glucoside II was 26 times higher than that of resveratrol. According to the fact that generally glycosides are more polar than the aglycones and as a result glycoside formation usually increases water solubility.

4.5.3 Antioxidant activity

The free radical scavenging effect of products was measured by following the decrease of absorption of 2,2-diphenyl-1-picrylhydrazyl (DPPH•) at 517 nm because when a hydrogen atom or electron was transferred to the odd electron in DPPH•, the absorbance at 517 nm decreased correspondingly to the increase of non-radical forms of DPPH (Figure 34).

From section 3.6.3, it was found that the DPPH• scavenging effect (%) of both resveratrol glucoside I and II were lower than those of free resveratrol and vitamin C. In comparison with previous studies in two-step glucosylation of resveratrol by cultured cells of *Phytolacca Americana* and CGTase. Resveratrol-3-β-glucoside and resveratrol-3-β-maltoside showed low DPPH free radical scavenging activity, whereas other glucosides had no radical scavenging activity (Sato *et al*., 2014). In addition, Lepak (2015) explained 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". In conclusion, the attachment of glucosyl group to resveratrol had an effect on their antioxidant activity.

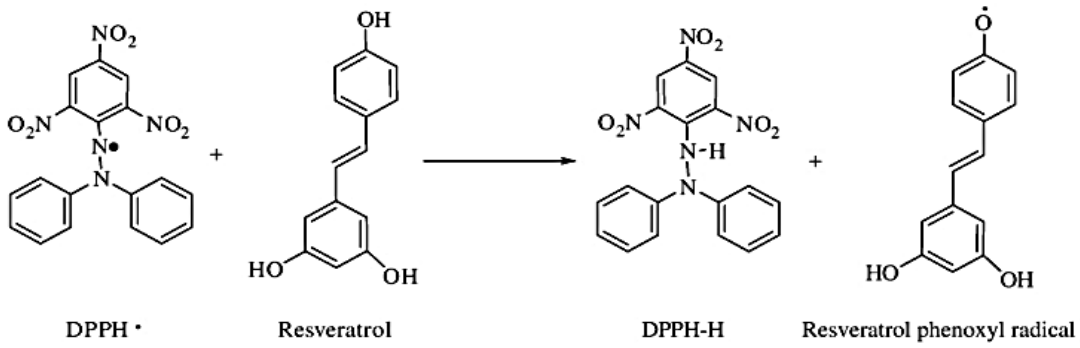


Figure 34 The reaction scheme between DPPH free radicals and resveratrol (Gülçin İ. ,2010).

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 33% and 95 with specific activity of 11,783 U/mg protein (in terms of dextrinizing activity).

2. The DMSO concentration had significant effect on CGTase stability. Both coupling and dextrinizing activities of CGTase were dramatically decreased when DMSO concentration increased.

4. Potato soluble starch was found to be the best glycosyl donor but because of the limitation of a solution containing starch in HPLC analysis, 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 20% (v/v) gave the highest production yield of tranglycosylation reaction of resveratrol glycoside production.

6. Under optimum condition (0.6% (w/v) of resveratrol in 20% (v/v) DMSO, 1.4% (w/v) of β-cyclodextrin with 400 U/ml of CGTase at 40°C for 48 hours) for transglycosylation reaction, the production yield of resveratrol glycosides increased by 3.71 times.

7. The molecular mass of resveratrol glucoside I (Rt ~13.6 minutes) was 390 Daltons having one monoglucosyl group attached to the position of 3-hydroxyl group of resveratrol (by tandem mass spectrometry technique).

8. The water solubility of resveratrol glycoside I (Rt ~13.6 minutes) and II (Rt ~12.8 minutes) was about 72 and 26 times higher than that of resveratrol.

9. The DPPH• scavenging effect (%) of both resveratrol glycoside I and II was lower than those of free resveratrol and vitamin C.

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APPENDICES

Appendix A : Preparation of polyacrylamide gel electrophoresis

I. Stock reagents

2 M Tris-HCl, pH 8.8

Dissolved 24.2 g of tris(hydroxymethyl)-aminomethane with distilled water. 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

Dissolved 18.17 g of tris(hydroxymethyl)-aminomethane with distilled water. 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

Dissolved 12.1 g of tris(hydroxymethyl)-aminomethane with distilled water. 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

Dissolved 6.06 g of tris(hydroxymethyl)-aminomethane with distilled water. Then, adjusted pH to 6.8 using 1 M HCl and adjusted volume to 100 ml with distilled water.

10 % (w/v) SDS

Dissolved 10 g of sodium dodecyl sulfate with distilled water and adjusted volume to 100 ml.

50 % (v/v) Glycerol

Mixed 50 ml of glycerol with distilled water and adjusted volume to 100 ml.

1 % (w/v) Bromophenol blue

Dissolved 100 mg of bromophenol blue with distilled water and adjusted volume to 10 ml. Then, solution was filtered to eliminate the aggregated dye.

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

Dissolved 0.5 mg of ammonium persulfate with distilled water and adjusted volume to 5 ml.

Solution A

40% (w/v) Acrylamide monomer solution containing 5% (w/v) of bis-acrylamide

Solution B

Mixed 75 ml of 2 M Tris-HCl pH 8.8 with 10 % (w/v) SDS 4 ml and adjusted volume to 100 ml with distilled water.

Solution C

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

Dissolved tris(hydroxymethyl)-aminomethane 3 g and glycine 14.4 g with distilled water and adjusted volume to 1 L (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) $(NH_4)_2S_2O_8$	25 μl
TEMED	3 μl

Sample buffer

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

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

Electrophoresis buffer

Dissolved 3 g of tris(hydroxymethyl)-aminomethane, 14.4 g of glycine and 1 g of SDS with distilled water, mix well and adjusted volume to 1 L (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

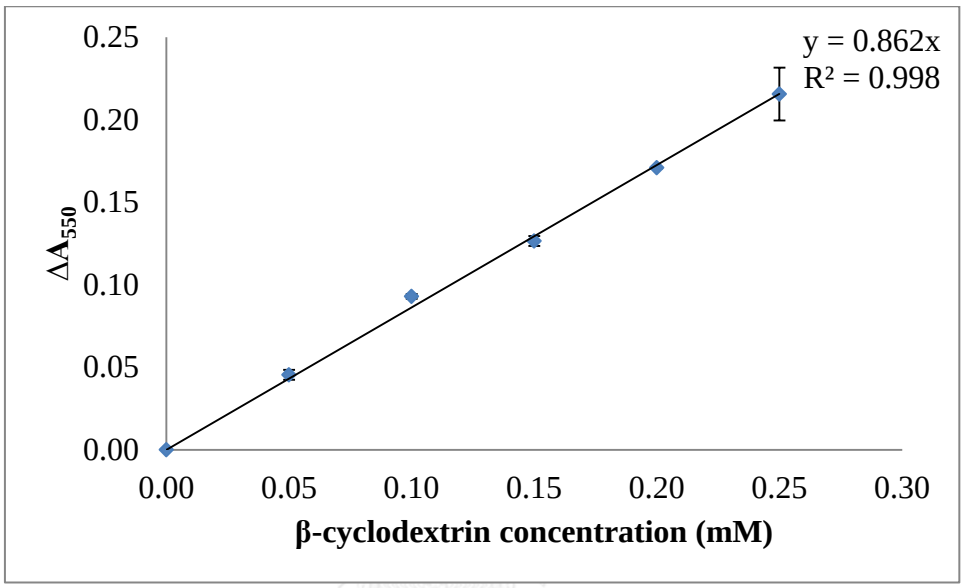
Used 50 mM KH_2PO_4 to adjust pH of 50 mM K_2HPO_4 to pH 6.0

50 mM Tris-glycine buffer, pH 8.0

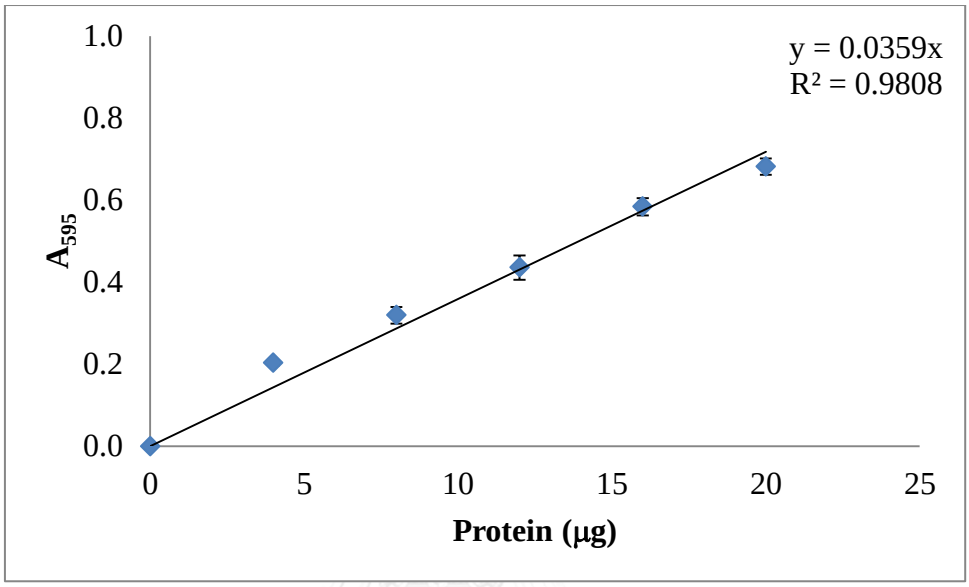
Tris(hydroxymethyl)-aminomethane	0.303 g
Glycine	0.188 g

Dissolved with distilled water adjusted pH to 8.0 by 1 M NaOH and adjusted volume to 50 ml.

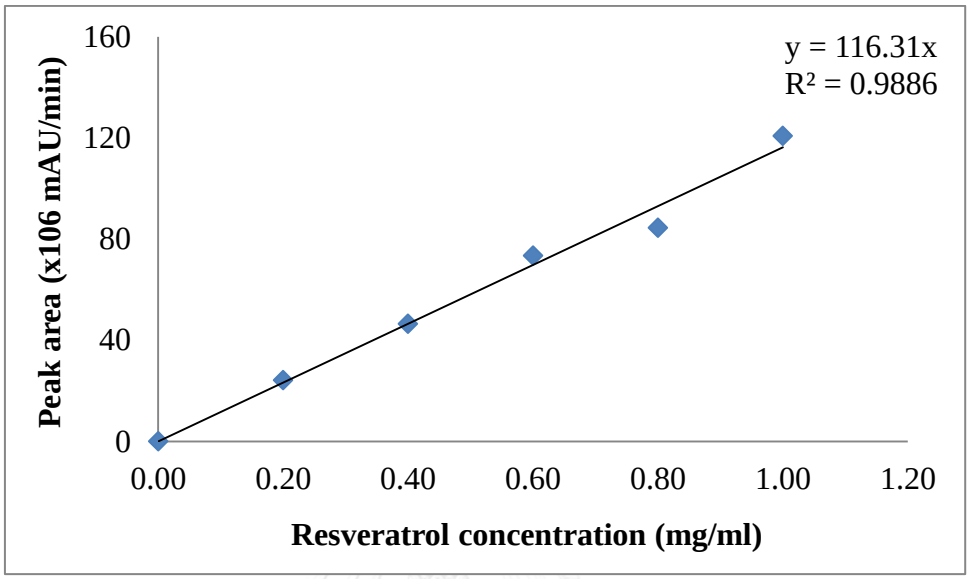
Appendix C : Standard curve of β-cyclodextrin determination by phenolphthalein method



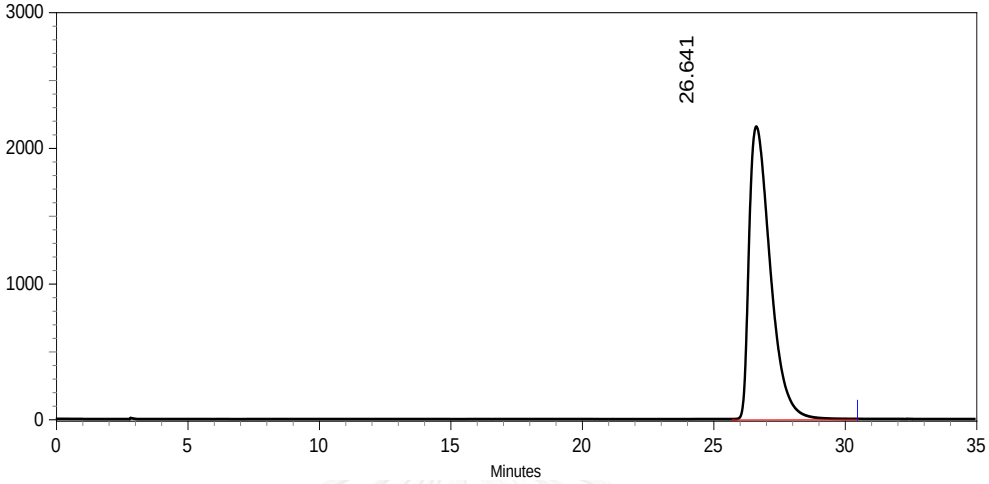
Appendix D : Standard curve of protein determination by Bradford's method



Appendix E : Standard curve of resveratrol concentration determined by HPLC technique

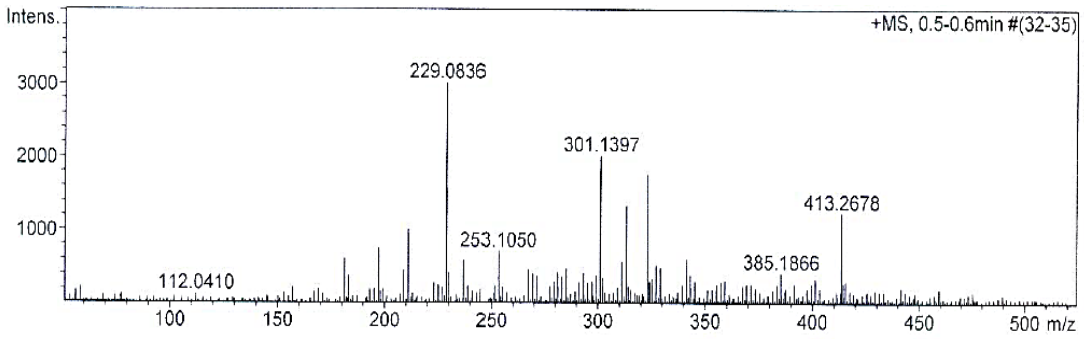


Appendix F : HPLC chromatogram of standard resveratrol



Appendix G :. ESI-TOF mass spectra of standard resveratrol

The pseudomolecular ion $[M+H]^+$ of resveratrol displayed at m/s 229 (228 plus 1 of H molecule)



Appendix H : MS/MS spectrum peak list

MS/MS Spectrum Peak List

m/z	z	Abund
85.0269		152.34
107.0467	1	137.59
119.0454	1	175.16
135.0403	1	413.81
229.078	1	8240.62
230.0808	1	1507.66
231.0834	1	208.55
241.0767	1	164.39
271.0853	1	138.25
295.0849		114.82
85.0265		282.41
91.0528		293
107.0467		769.36
111.0408		146.45
119.0464	1	477.86
135.0407	1	1380.93
183.0741		157.83
211.0682	1	202.28
229.0777	1	6010.58
230.0808	1	1234.4
91.0529	1	816.67
107.0469	1	2349.52
119.0461		536.54
120.0538	1	354.09
121.0254		220.31
135.0405	1	1123.51
136.0472	1	278.94
165.0645	1	555.72
183.0733	1	228.8

VITA

Miss Nattaya Anurutphan was born on December 14th, 1989. She graduated with the bacherlor's degree of science from faculty of science and technology at Thammasat University, majoring in biotechnology in 2012, and continued studying for the master degree of science in biochemistry and molecular biology program faculty of science at Chulalongkorn University.

Proceeding:

Nattaya Anurutphan and Manchumas Hengsakul Prousoontorn (2014). Enzymatic synthesis of resveratrol glycosides by cyclodextrin glycosyltransferase from Paenibacillus sp. RB01. The 26th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2014). 26th and 29th November 2014, Mae Fah Lunag University (School of Science), Chiang Rai, Thailand.