DEVELOPMENT OF SIMPLE METHOD FOR DETECTION OF STEVIOL GLYCOSIDES CONTENT FROM STEVIA LEAF

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Thesis
entitled

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ABSTRACT

In this study, enzymatic assay of stevioside content in *Stevia* samples was developed. Recombinant *BT_3567* gene was cloned from genomic DNA of anaerobic bacterium namely *Bacteroides thetaiotaomicron* HB-13 into pEt28a expression vector. Recombinant BT_3567 enzyme hydrolyses glucose moiety from stevioside at the position of C-13. Following with enzyme assay of glucose allowed stevioside quantitation. The steps of enzymatic stevioside determination are follows: 1) Incubate recombinant BT_3567 with *Stevia* sample in sodium acetate buffer, pH 6.0 at 37 °C for 10-20 min. 2) Stop reaction by heating at 80 °C for 5 min. 3) Measure glucose content using glucose oxidase and peroxidase assay. The molar amount of detected glucose was calculated as molar amount of stevioside presented in *Stevia* samples. Enzymatic assay developed in this study was validated and it exhibited high accuracy and precision as well as selectivity for stevioside determination in both of crude *Stevia* extract sample and the finished products of *Stevia*.

KEY WORDS: STEVIOSIDE DETECTION / METHOD DEVELOPMENT / RUBUSOSIDE / β-GLUCOSIDASE / ENZYMATIC METHOD

128 pages

การพัฒนาวิธีวิเคราะห์ปริมาณสารหวาน จากต้นหญ้าหวานอย่างง่าย
DEVELOPMENT OF SIMPLE METHOD FOR DETECTION OF STEVIOL GLYCOSIDES CONTENT FROM STEVIA LEAF

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บทคัดย่อ

ในการศึกษาครั้งนี้ ทำการพัฒนาวิธีการใช้เอนไซม์ ในการตรวจวัดปริมาณสตีวิโอไซด์ในตัวอย่างหญ้าหวาน โดยเริ่มจากโคลนนิ่งยีนลูกผสมบีที3567 จากดีเอ็นเอของแบคทีเรียกลุ่มที่ไม่อาศัยออกซิเจนในการหายใจที่มีชื่อเรียกว่า *Bacteroides thetaiotaomicron* HB-13 เข้าสู่พาหะนำยีน pET28a เอนไซม์ลูกผสมบีที 3567 สลายโมเลกุลกลูโคสตรงตำแหน่งคาร์บอนที่ 13 ของสตีวิโอไซด์ เมื่อทำการตรวจวัดกลูโคสหลังจากขั้นตอนนี้จะสามารถวัดปริมาณสตีวิโอไซด์ที่เกิดขึ้นได้ วิธีการใช้เอนไซม์ในการตรวจวัดปริมาณสตีวิโอไซด์มีขั้นตอนดังนี้ 1) บ่มเอนไซม์ลูกผสมบีที 3567 กับตัวอย่างหญ้าหวาน ในโซเดียมอะซิเตทบัฟเฟอร์ พีเอช 6.0 ในอุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 10-20 นาที 2) หยุดปฏิกิริยาโดยการให้ความร้อน 80 องศาเซลเซียส เป็นเวลา 5 นาที 3) วัดกลูโคสที่เกิดขึ้นโดยใช้วิธีการเอนไซม์ที่เรียกว่า กลูโคสออกซิเดสและเปอร์ออกซิเดส จำนวนโมลของสตีวิโอไซด์ที่วัดได้ในตัวอย่างหญ้าหวาน คำนวณได้จากจำนวนโมลของกลูโคสที่ตรวจวัดได้ในตัวอย่างนั้น วิธีการเอนไซม์ที่พัฒนาขึ้นในการศึกษาครั้งนี้ถูกตรวจสอบแล้วว่ามีความถูกต้องและแม่นยำสูง รวมถึงมีความจำเพาะต่อการตรวจวัดปริมาณสตีวิโอไซด์ทั้งในตัวอย่างสารสกัดหญ้าหวานและผลิตภัณฑ์หญ้าหวาน

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BSA	Bovine serum albumin
DAB	3,3′-diaminobenzidine
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
g	Gravity force
GPO	Glucose oxidase and peroxidase reagent
His	Histidine
ImRA	Impure rebaudioside A
ImSV	Impure stevioside
IPTG	Isopropyl-β-1-thiogalactopyranoside
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
*p*NPG	*p*-nitrophenyl-β-D-glucopyranoside
RA	Rebaudioside A
RT	Room temperature
RU	Rubusoside
SDS	Sodium dodecyl sulfate
SGE	Steviol glycoside extract
SGP	Steviol glycoside powder
SGS	Steviol glycoside syrup
SV	Stevioside
TEMED	N,N,N′,N-tetrametylethylene diamine
U	Enzyme Activity Unit

CHAPTER I
INTRODUCTION

1.1 Overview

Steviol glycosides, a group of high potency natural sweeteners, were found in the *Stevia rebaudiana* Bert. They possess a more preferential sweet taste than synthetic sweeteners such as aspartame, neotame, sucralose, saccharin, etc. Beyond sweetness, steviol glycosides were confidently accepted that have no effect on a human health. Although *Stevia* plant is native to the southeastern region of South America and is still growing wild in Brazil Paraguay and Uruguay, it can be cultivated in some climate conditions in the Thailand too. *Stevia* leaves have the most abundant concentration of stevioside (SV) and rebaudioside A (RA). The others minor steviol glycosides were rebaudioside C, dulcoside A, steviolbioside, rubusoside (RU) and so on. Both of RA and SV have recently gained importance as the best sweetener and also possess many therapeutic properties. Due to the demand of these valuable sweeteners, breeding of a high steviol glycosides-producing clones especially for producing of RA and SV was increased. In the part of manufacture, *Stevia* products were recently developed and served in sweetener market consequently the need of quantify amount of steviol glycosides for their quality control was also enlarged. For such purpose, it has been necessary to develop a simple and inexpensive method for sample determination.

At present date, HPLC is the standard method for screening and quantitative determination of steviol glycosides in *Stevia* plant. However, HPLC method is cost consuming due to requiring expensive instrument and maintenance. In addition, hazardous organic solvent usage in the HPLC experiment results in accumulation of a toxic chemical wastes which are make a poison for worker and an environment problem. Therefore, development of simple, fast and eco-friendly method used to determination steviol glycosides in *Stevia* sample was aimed of this study.

1.2 Literature review

1.2.1 *Stevia rebaudiana* (Bertoni) Bertoni

Stevia plant was classified by Moisés Santiago Bertoni in 1899 as the plant that belongs to the family of Asteraceae. Its original name is *Eupartorium rebaudianum* before was changed the name to *Stevia rebaudiana* (Bertoni) Bertoni [1]. *Stevia rebaudiana* is a short day plant, perennial herb, small branched bushy shrub as shown in Fig. 1.1; however, it can grow up to 1 m tall [2]. In addition, it has a branched root system, fragile stems and elliptic leaves [3]. In 1964, *S. rebaudiana* was firstly reported the commercial cultivation in Paraguay, a native habitat of *Stevia* plant located in South America [4, 5]. At present, *Stevia* plant was now widely grown in many countries such as Japan, Taiwan, Korea, Indonesia and also Thailand [6, 7].

Figure 1.1 *Stevia rebaudiana* (Bertoni) Bertoni

The leaves part of *Stevia* plant has more abundance of sweet compounds than the other organs especially stevioside (SV) and rebaudioside A (RA), two major steviol glycosides, which were approximately 4-10% and 2-4% (w/w) of dried leaves, respectively. Other two minor residues with the percentage lower than 2% belong to rebaudioside C and dulcoside A. In addition, traces steviol glycosides including of rebaudiside B, D, E and- F and steviolbioside, rubusoside were also found [1-2, 8]. The sweetness of *Stevia* sweet compounds has about 200 times of a sucrose by weight equivalent. RA has been considered to be the best taste and sweetest of *Stevia* sweetener. Beyond a high sweeting potency, steviol glycosides possess many pharmacologically properties and do not have mutagenic, teratogenic or carcinogenic

effects [1, 9]. For most importance, SV exhibits an anti-hyperglycemic action in type 2 diabetic patients [10], anti-inflammatory and immune modulator [11] and decreasing of blood pressure in hypertensive subjects [12, 13]. Recently, rubusoide (RU), a SV-hydrolytic product, was also reported that it had significant bioactivities such as antiangiogenic [14] and anti-allergic properties [15, 16]. Moreover, amphipathic RU can be used as a natural solubilizer to increase a solubility of pharmaceutical compounds which are hardly soluble in water such as paclitaxel, curcumin, capsaicin, cyclosporine, nystatin, and erythromycin [17].

In 1931, SV was first purified [18] and then its chemical structure was clarified. Similar with others member of steviol glycoside, it was built up from a diterpenoid compound, called steviol, which incorporated with sugar moieties at the position of C-13 and/or C-19. Almost of the sugar cooperate with steviol backbone belong to glucose. However, some of steviol glycosides member possesses rhamonose or xylose, at the position of C-13 instead of glucose such as rebaudioside C and -F. The chemical structures specifying of an individual steviol glycoside were described in Table 1.1

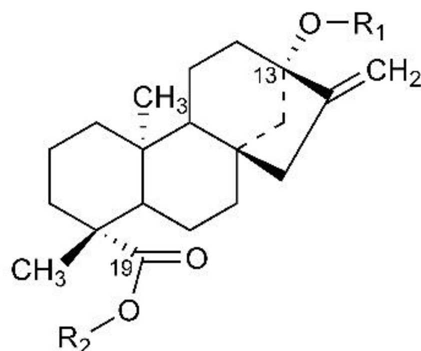


Table 1.1 Chemical structure of steviol glycosides.

Compound	R1	R2
Steviol (ST)	H	H
Steviol monoglucoside (SMG)	β-Glc	H
Steviol monoglucosyl ester (SME)	H	β-Glc
Rubusoside (Ru)	β-Glc	β-Glc
Steviolbioside (SB)	β-Glc-(2 → 1)-β-Glc	H
Stevioside (SV)	β-Glc-(2 → 1)-β-Glc	β-Glc
Rebaudioside A (RA)	β-Glc-(2 → 1)-β-Glc \ (3 → 1)-β-Glc	β-Glc
Rebaudioside B (RB)	β-Glc-(2 → 1)-β-Glc \ (3 → 1)-β-Glc	H
Rebaudioside C (RC)	β-Glc-(2 → 1)-α-Rha \ (3 → 1)-β-Glc	β-Glc
Rebaudioside D (RD)	β-Glc-(2 → 1)-β-Glc \ (3 → 1)-β-Glc	β-Glc-(2 → 1)-β-Glc
Rebaudioside F (RF)	β-Glc-(2 → 1)-β-Xyl \ (3 → 1)-β-Glc	β-Glc
Dulcoside A (DA)	β-Glc-(2 → 1)-α-Rha	β-Glc

Basically, each chemical or natural compound has its own characteristic which provides a specific procedure to monitor. In case of steviol glycosides, they mostly absorbed a UV spectrum with maximum wave length at 210 nm. Therefore, almost of chemical-base methods used to analyze a content of steviol glycosides were depended on a requirement of UV instrument.

1.2.2 Chemical-base analytical method of steviol glycosides determination

Many analytical methods have previously been reported to quantification and qualification of steviol glycosides in *Stevia* plant. These include thin-layer chromatography (TLC) [19-21], high-performance thin layer chromatography (HPTLC) [22-23], high-performance liquid chromatography (HPLC) [24-27], mass spectrometry [28-30] and near infrared reflectance (NIR) [31-32].

In general, TLC was used to qualification of steviol glycosides in the *Stevia* sample solution. Sometime pre-treatment of sample for TLC assay was required. In case of crude extract, it cannot be directly applied to TLC assay; since, plant matrix in the sample can interrupt the sugar development on TLC plate by occurrence a lot of brownish or greenish-color backgrounds. In 1992, Kusakabe *et al* observed transfer product from SV in the culture media of *Actinomycete* using TLC assay. At begin, SV and its transfer product were purified from culture media using affinity column. After that, purified samples were applied on the plate of TLC plate silica gel 60 and performed separation process with the solvent system of chloroform-methanol-water (90:65:15, v/v) [19]. This mobile phase system also was used in the experiment which was reported by Richman *et al* (2005). They monitor glucosyltransferase activity which was involved in systhesis of the major steviol glycoside in *Stevia* plant [20]. For more application of TLC method, in the experiment of Dacome *et al* they performed TLC assay with a mobile phase comprising with isopropanol-ethyl acetate-acetone-water (30:53:2:15, v/v) to monitor steviol glycosides which were pre-extracted from *Stevia* water extract [21]. Almost of TLC assay for steviol glycosides were finally performed the sample development step to visual brown spot of targeted compound in which comprised of sulfuric acid spraying and heating on the hot plate. Actually, human error for TLC experimental performance was frequently found in the step of sample application and development. To overcome

this problem, HPTLC technique was therefore developed from the basic knowledge of TLC and it now was accepted method for quantification analysis. For example, in report of Jaitak *et al* (2008) and Saifi *et al* (2014), simultaneous quantification of steviol glycosides, i.e. SV, RA and/or steviolbioside in *S. reabaudiana* leaves was carried out by this technique [22-23]. To perform HPTLC assay, there requires many instruments such as automatic TLC sampler, automated developing chamber and TLC scanner as well as the need of computer software for result analysis; therefore, cost of this requirement makes a limitation of the assay.

Basically, HPLC method has been widely used to determine steviol glycosides extracted from *Stevia* plant sample as well as other sample types. To analyze steviol glycosides in the *Stevia* sample, the extraction and purification of steviol glycoside from the sample should be performed if need. Commercial columns generally co-operated with HPLC machine are amino (NH_2) and reverse phase columns. Amino-bonded column can separate steviol glycosides well judged by retention time of individual analyst but the column cost is more expensive. Recently, in 2013, Tada *et al* reported that amino column has a limitation of used due to its instability after perform a conventional method resulted in inaccuracy of the assay. Thus, they attempt to resolve this problem by using of an octadecylsilyl column, C-18 reverse phase column, for determination of steviol glycosides [25]. Additionally, many reports were also established the development of HPLC method for determination of *Stevia* sample with diminishing of pre-treatment of sample by using of reverse phase column [24, 26-27]. It can be used well for determination array of steviol glycosides in crude extract of *Stevia* plant but not well for separation of some steviol glycosides due to close elution time between two major sweetening agents especially for SV and RA. However, HPLC method is now to be accepted method for quantification of steviol glycosides with accuracy and precision.

Beyond determination amount of sweet compounds from *Stevia* materials, mass spectrometry coupling with liquid chromatography method was used to qualify steviol glycosides in both of *Stevia* extract and commercial sweetener samples [28-29]. Base on molecular ion analysis, this method can be used to identify unknown sample when comparison with mass of reference sample. In 2012, Isabella *et al* reported direct analysis of steviol glycosides in *Stevia* leaves by ambient ionization mass

spectrometry. This method provided a fast and simple assay and without pre-treatment of sample and directly measured fresh *Stevia* leaves [30]. However, mass spectrometry analysis has a restriction upon very expensive instrument and people normally not familiar with this assay.

All methods describe above, they require complicated preparative procedures as well as exclusive of equipment and machine resulting in high cost of analysis. Currently, in China, near infrared spectroscopy (NIR) has been widely used for analysisof interested chemical compound base on physical property of substances. It has been used as a quick, accurate and no-destructive way of analyzing protein in feed and forages and any kind of crop productions. In 1992, Nishiyama *et al* established the quantitative analysis of SV in *Stevia* leaves using NIR technique comparison with analysis by HPLC method. The validation results were showed that NIR method gave a very low accuracy but it is a simple method for determination of SV [31]. Lately, in 2011, Yu *et al* [32] established the spectrum model for measuring of SV and RA in *Stevia* leaves which was more accuracy and precision than that previous study. Though NIR method is alternative easily method for assay amount of SV and RA without pre-treatment of sample, the most hardly and complication to work on it is to provide a corresponding spectrum and data gathering method for accurate for steviol glycosides content determination in the sample. This difficulty becomes a restriction of NIR analysis.

1.2.3 Enzyme-base method of steviol glycoside determination

In general, the major steviol glycosides found in *Stevia* plant are SV and RA. These two glycosides consist of glucose molecule as a sugar moiety both of a position at C-13 and C-19 residues. Hydrolysis of glucose from steviol backbone using enzymatic procedure makes a method for quantify amount of RA and SV by following the enzymatic hydrolysis process with another enzymatic glucose assay. As presented in 1982, Mizukami *et al*. reported the determination of SV in the extract of *Stevia* plant by enzymatic method [33]. They performed the assay by using of crude hesperidinase enzyme to hydrolyze glucose from SV and finally yielded 3 moles per mole of SV. Then, glucoses were subsequently detected using glucose oxidase and

peroxidase assay. A schematic principle of the enzymatic-base method of SV determination according to the report of Mizukami *et al* was showed in the Figure 1.2.

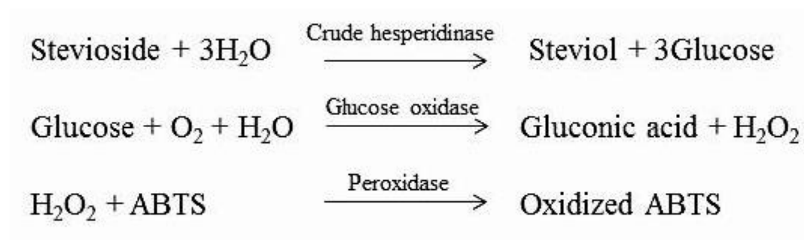


Figure 1.2 Schematic principle of the enzymatic-base method of stevioside determination (Mizukami, 1982).

However, crude hesperidinase not only specific hydrolyzes steviol glycoside but also hydrolyzes RA at the position of C-13 and then yielded one mole of rebaudioside B and glucose. However, crude hesperidinase can also degraded RA in less specific than hydrolysis of SV. The amount of glucose produced from RA was 3% of that produced from SV if they are present in equal amount in *Stevia* sample. It might be possible to make erroneousness for determination of SV in the *Stevia* sample contained varying of RA content or other glucosides. Additionally, this enzyme has not been isolated from impurity and also not been engineered to be a recombinant protein which was available for usage. For those issues, they are limitations of crude hesperidinase usage in SV determination assay. Nevertheless, enzymatic method possesses many advantages such as self-sufficiency, limit of organic solvent usage and minimize of cost and time consuming therefore it is necessary to increase a report of enzymatic method used for steviol glycosides detection. As described, development of enzymatic determination of these steviol glycosides required a novel enzyme which should be more specificity. Due to the previous study, many enzymes have been reported that it can hydrolyze steviol glycosides. The list of enzymes involved in steviol glycosides hydrolysis was shown in Table 1.2.

Table 1.2 List of enzymes involved in hydrolysis of steviol glycosides.

Sources of enzyme, protein	**Gene ID**	**Substrates**	**References**
Clavibacter michiganense β-glucosidase	ND[a]	*p*NPG, SV, RA, RU, SME, SB, SMG	Nakano, H. [34]
Flavobacterium johnsonae β-glucosidase	ND[a]	*p*NPG, SV, RA, RU, SME, SMG	Okamoto, K. [35]
Bacteroides sp.	ND[a]	RA, SV, SB	Gardana, C. [36]
Aspergillus sp. β-galactosidase	ND[a]	*p*NPG, SV	Wan, H. D. [37]
Aspergillus aculeatus β-glucosidase, SSGase	ND[a]	*p*NPG, SV, RU	Ko, J. A. [38]
Penicillium decumbens β-glucosidase, SPGase	ND[a]	*p*NPG, SV, RU, SME, SMG	Ko, J. A. [39]
Sulfolobus solfataricus β-galactosidase	ND[a]	*p*NPG, SV, RU, SMG	Chen, J. M. [40]
Thermus thermophilus β-galactosidase, rB-GLYPI	AY130254.1	*p*NPG, SV	Nguyen, T. T. H. [41]
Streptomyces sp. GXT6 β-glucosidase, BGL1	KJ958924.1	*p*NPG, SV, SB	Wang, Z. [42]
Kluyveromyces lactis β-galactosidase	ND[a]	*p*NPG, SV,	Chen, J. M. [43]

[a]not described

At beginning of this project, we screened the hydrolysis activity of SV and RA from many microorganisms which are available in our laboratory including of *Pichia pastoris*, *Bacillus* sp., *Enterococcus* sp. and *Bacteroides thetaiotaomicron* HB-13. In this study we found that *B. thetaiotaomicron* HB-13 can only hydrolyze SV not RA. We subsequently explored the unknown enzyme from *B. thetaiotaomicron* HB-13 which exhibited SV hydrolysis because there had no report of the enzyme involve in SV hydrolysis from this bacterium and the need of SV assay was also required. To identify an active enzyme from *B. thetaiotaomicron* HB-13, cloning of β-glucosidase gene was firstly performed. The result showed that the host harbored pET-28a/*BT*-

3567 can selectively hydrolyze SV to glucose and RU. Figure 1.3 showed the selective hydrolysis of SV by rBT_3567 from *B. thetaiotaomicron* HB-13. Characterization and kinetic study of recombinant BT_3567 expressed in an *Esherichia coli* strain were then reported. Afterwards, rBT_3567 was applied to develop enzymatic method for determination of SV content in crude *Stevia* plant extract and finished *Stevia* product.

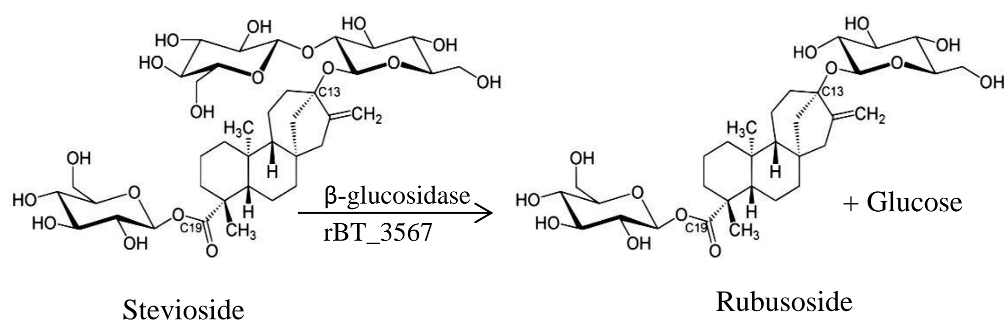


Figure 1.3 Stevioside hydrolysis by β-glucosidase rBT_3567 from *B. thetaiotaomicron* HB-13.

CHAPTER II
OBJECTIVES

1. Finding and biochemical characterization of a new enzyme specifically hydrolyze stevioside.

2. Development and validation of enzymatic-based approach for stevioside determination in crude *Stevia* plant extract and final product of *Stevia.*

CHAPTER III
MATERIALS AND METHODS

3.1Materials

3.1.1 *Stevia rebaudiana* materials

Steviol glycosides extract, standard purified stevioside and rebaudioside A and dried *Stevia* plant samples were obtained from Sugavia Co. Ltd.

3.1.2 *Bacteroides thethaitaomicron* HB-13

B. thetaiotaomicron HB-13 was kindly provided by Dr. Kwanrawee Sirikanchana (Translational Research Unit, Chulabhorn Research Institute, Bangkok Thailand).

3.1.3 Chromogenic and natural sugar substrates

*p*NPG, *p*NP, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-N-acetyl-β-D-glucosaminide, Ocyl-β-D glucopyranoside and isomaltose were purchased from Sigma Aldrich (St. Louis, United States).

3.2 General experiment methods

3.2.1 Partially purified steviol glycosides preparation

Partially purified stevioside and rebaudioside A were prepared by re-crystallization process. They were performed by dissolving of steviol glycoside extract in absolute methanol with the ratio of 1:5 (w/v). Then the mixture solution was then dissolved on the hot plate with stirred at 70 °C for 30 min. Firstly when the solution slightly cool down, partially purified rebaudioside A would be precipitated as white

powder and then solid phase was subsequently separated using Whatman filter paper. The remaining solution was later concentrated by warm evaporation until the white sediment of partially purified stevioside was observed and then it was colled down and filtrated to separate from the liquid solvent solution. Partially purified rebaudioside A and stevioside were subsequently air dried until no presence of solvent's smell. Both impure substances can be kept in air tight container at room temperature with light. The purity percentages of SV and RA contained in both of partially purified rebaudioside A and stevioside determined using HPLC method. Partially purified rebaudioside A contained RA (69%) as major component and SV (17%) as most contaminant. Partially purified stevioside contained SV (68%) as major component and RA (20%) as most contaminant. The HPLC results showed chromatogram of standard RA and SV and partially purified rebaudioside A and stevioside were presented in Appendix A1.

3.2.2 Thin-layer chromatography assay

Thin-layer chromatography (TLC) can be used to monitor the presence of steviol glycosides sample qualitatively. The sample concentration should to prepare in the ranging of 1-10 mg/mL. Firstly, 1 μL of sample solution was spotted to pre-coated silica gel 60 F_{254} plates and then let it to dry at room temperature. TLC plate was subsequently placed into the closed tank with presence of ethyl acetate/isopropanol/acetone/water (53:30:2:15). Then, sample spots would be separated along the moving of solvent on the silica TLC plate. After the solvent reached to mobile front line, silica plate was then pick up and then let the plate to dry. To develop separated compounds spots on TLC plate, the plate was firstly exposed to 5-10% sulfuric acid and then flaming or heating until the brown spots were observed. Retention factors (Rf) value of RA, SV and RU were 0.42, 0.53 and 0.64 respectively.

3.2.3 High performance liquid chromatography assay

High performance liquid chromatography (HPLC) can be used to monitor for both quantitative and qualitative of *Stevia* samples. Steviol glycosides extract, standard SV, RA and RU, partially purified rebaudioside A and stevioside and *Stevia* plant crude extract, should to filtrated using 0.2-0.45 μM syringe filter before subject

to HPLC machine. To reduce syringe filter usage in large amount of samples, centrifugation process at 13,000*xg* for 10 min can be performed before applied to HPLC machine.

HPLC system comprises with a model waters e2695 separation module system, 2487 dual wavelength detector (Waters Corporation, Milford, USA) and Acclaim 120 C18 column (Thermo Fisher Scientific, 4.6 mm×100 mm, 3 μm) was used. The temperature of the column oven was 60 °C. The mobile phase solvent contained with water (A) and acetonitrile (B) in an isocratic of 68% A and 32% B. Approximately 5 μL of samples were injected with flow rate of 0.75 mL/min. The UV absorption spectrum was measured at 210 nm.

A calibration curve of SV and RA used to calculate amount of these two compounds in *Stevia* sample were also prepare in some experiment. For that, concentration of these standard steviol glycosides should be ranged from 0.25-4 mg which was still in the linearity as shown in Appendix A2 and A3 respectively.

3.2.4 Hydrolytic activity assay

3.2.4.1 *p*NPG assay

In general, 50 μL of crude enzyme extract was incubated with 50 μL of 4 mM *p*NPG solution at indicated temperature and incubation time depending on the aim of experiment. The reaction was stopped by adding 100 μL of 0.2 M Na_2CO_3. The *p*-nitrophenol (*p*NP) product was determined at A_{405} by spectrophotometer (Shimadzu UV-2501 PC, Duisuburg, F. R., Germany). A standard curve of *p*NP was also performed and used to quantify the enzymatic product (see Appendix A4).

3.2.4.2 SV assay

In general, 50 μL of crude enzyme extract was incubated with 50 μL of 4 mM SV solution at indicated temperature and incubation time depending on the aim of experiment. The reaction was stopped by heating at 80 °C for 10 min. The enzymatic product was subsequently determined by another enzymatic glucose assay. The assay was performed by adding of 100 μL of glucose oxidase and

peroxidase reaction reagent (0.2 mg of glucose oxidase, 0.012 mg of peroxidase, 0.02 mg of *o*-dianisidine, in 50 mM sodium acetate buffer, pH 6.0). After that, sample was incubated at 37 °C for 15-25 min and then the reaction solution was stopped with 50 µL of 80% (v/v) sulfuric acid. Oxidized *o*-dianisidane, the final product from last step of glucose assay, presented in purple color, was then examined by reading the A_{540}. A standard curve of glucose was prepared and used to quantify the enzymatic product (see Appendix A5).

3.3 Culturing of microorganisms

3.3.1 Culturing of aerobic or facultative-aerobic microorganisms

In generally, unknown microorganisms as well as identified microorganisms such as *Bacillus* sp. and *Enterococcus* sp. were grown in the rich commercial Tryptic Soy Broth; TSB medium. Some experiment, M9 minimum medium was used instead of TSB medium. M9 consisted of 40 mL of 5X M9 salt pH 7.4 (64 g/L $Na_2HPO_4 \cdot 7H_2O$, 15 g/L KH_2PO_4, 2.5 g/L NaCl), 400 µL of 1 M $MgSO_4$, 20 µL of 1 M $CaCl_2$, 1 mL of 100 g/L NH_4Cl, 4 ml of 20% glucose and 160 mL of dH_2O. Cells cultures were then incubated at 37 °C with shaking for 220 rpm until testing of hydrolysis activity.

3.3.2 Culturing of *Bacteroides thetaiotaomicron* HB-13

B. thetaiotaomicron HB-13 was grown in the BRPM, rich medium for culturing of *Bacteroides* strain. BPRM consisted of 0.5 g/L L-cystein, 1 mL/L of 5% $CaCl_2$, 5 g/L NaCl, 0.12 g/L $MgSO_4$, 10g/L peptone, 10 g/L tryptone, 2 g/L yeast extract, 1.8 g/L glucose, 25 mL/L of 10% Na_2CO_3, 10 mL/L of 2N HCl, 10 mL/L of 0.1% (w/v) hemin in 0.02% NaOH, 100 mg/L Kanamycin, 100 mg/L Nalidixic acid. In some experiment, *Bacteroides* was cultured in minimum medium. The *Bacteroides* minimum medium consists of 1.36 g of KH_2PO_4, 0.0875 g of NaCl, 0.1125 g of $(NH_4)_2SO_4$ in 90 mL of distilled water and then adjust pH to 7.2 before supplement with 0.05 g of L-cysteine (free base), 100 µg/mL kanamycin, 100 µL of 1% hemin (in 0.02% NaOH), 100 µL of 0.1 M $MgCl_2$ solution (in water), 100 µL of 0.4 mg/ml

$FeSO_4 7 \cdot H_2O$ (in 10 mM HCl), 100 µL of 1 mg/ml vitamin K_3 solution (in ethanol), 100 µL of 0.8% w/v $CaCl_2$ solution (in water), 50 µL of 0.01 mg/ml vitamin B_{12} solution (in water). The medium was adjusted volume to 100 mL by adding of distilled water and then sterilized through 0.22 µM filter. Cells culture of *B. thetaiotaomicron* HB-13 was incubated with the condition of 37 °C under a CO_2 atmosphere without shaking.

3.4 Steviol glycosides hydrolysis activity test

3.4.1 Testing on unknown microorganisms

The unknown microorganisms were grown in 10 mL of M9 medium supplemented with SV and RA as a sole carbon source at 37 °C with shaking. At the end of incubation time, culture medium supernatant was collected by centrifugation. To monitor amount of SV and RA remaining in the cultured medium, the supernatant solution samples were then examined by either TLC or HPLC method.

3.4.2 Testing on *B. thetaiotaomicron* HB-13

B. thetaiotaomicron HB-13 was cultured in 10 mL of *Bacteroides* minimum medium plus SV and RA under a CO_2 atmosphere at 37 °C for 48 h without shaking. The medium supernatant was collected at the end of incubation time using centrifugation and then amount of SV and RA remaining in the cultured medium was examined using either TLC or HPLC method.

3.5 Molecular identification of unknown microorganisms by 16s rDNA nucleotide sequence analysis

An active microorganism was firstly isolated by streaking to single colony on rich TSB medium agar. Different colonies were marked and then picked up them from the medium plate. Individual selected colonies were incubated in new media. After grow for 1-2 days they were tested for observation of SV hydrolysis activity similar

with the experiment procedure described in section 3.4.1. Active microorganisms which exhibited SV-hydrolysis activity were then propagated for cryopreservation as well as performed gDNA extraction used as PCR template and then performed PCR technique to obtained 16s rDNA nucleotide element for nucleotide sequencing analysis. Steps for genomic DNA (gDNA) extraction were firstly performed by re-suspension of bacterial cells pellets with 100 µL of miniprep solution I (50 mM Tris buffer pH 8.0, 10 mM EDTA and 100 µg/mL RNase A). After that 200 µL of miniprep solution II (1% SDS, 0.2 M NaOH) was added into the tube and mixed by invert several times at temperature of 95 °C for 3 min. The cell lysate solution was next neutralized with 3 µL of glacial acetic acid. After that 300 µL of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) was added and mixed by vortex briefly. Solvent phase separation was then performed using centrifugation at 13,000*g* for 5 min. The solution in upper phase approximately 250 µL was collected and moved to a new tube. The next step, 750 µL of absolute ethanol to was added to precipitate gDNA. After centrifugation, the liquid solution was discarded. DNA pellet was redissolved in 200 µL of sterile water and reprecipitated with 300 µL of absolute ethanol and performed centrifugation again to collect gDNA. After discard ethanol solution sample was air dried until no presence any liquid occur in the tube. DNA pellet located at bottom of the tube was then dissolved by gently mixing with TE buffer (50 mM Tris buffer pH 8.0, 10 mM EDTA). Amount of gDNA was measured using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The amount of gDNA approximately 250-750 ng was used as template in setting of 25 µL of PCR reaction solution. Two degenerated primers, Uni340f: 5′-CCTACGGGRBGCASCAG-3′, and Uni1392r: 5′-ACGGGCGGTGTGTRC-3′were used to amplify the targeted 16sRNA gene from the gDNA with PCR conditions of annealing temperature at 46 °C. Then PCR products were sent to sequence by Macrogen Company. The 16sRNA nucleotide data obtained from the company were then analyzed by BLAST with NCBI database to identify microorganism species.

3.6 Cloning, expression, purification and characterization of recombinant β-glucosidase from *B. thethaitaomicron* HB-13

3.6.1 Cloning of recombinant β-glucosidases expression vector

B. thetaitaomicron HB-13 was cultured in 10 mL of *Bacteroides* medium at 37 °C under anaerobic atmosphere without shaking for 16-18 h. Cells were harvested by centrifugation and the pellet of cells was washed once with 1 mL of sterilized water and then performed centrifugation again to harvest the cells. Genomic DNA preparation and nucleotide content measurement was then performed as previously described in the section of 3.5.

All primers used for cloning of β-glucosidase genes of *B. thetaiotaomicron* HB-13 were designed base on the sequence of seven full length β-glucosidase genes obtained from data screening of the genome of a reference strain, *B. thetaiotaomicron* VPI-5482. Table 3.1 showed seven putative β-glucosidases primer sequences for amplification of *BT_1778, BT_1780, BT_1872, BT_2854, BT_3009, BT_3300* and *BT_3567* genes and the annealing temperature used in PCR reaction condition. The forward primer consisted of *NcoI* (CCATGG) site. The reverse primer consisted of *EcoRI* (GAATTC) site and still kept stop codon element at the end of full-length gene. Those restriction enzyme sites provided a compatible sticky region for cloning the gene product into *pET-28a* expression vector by T4 ligation procedure.

Table 3.1 List of primers and accession number. β-glucosidases retrieved in genome of *B. thetaiotaomicron* VPI-5842 was obtained from NCBI database and primers for the target genes in strain.

Gene	Accession number	Primer sequence (5' →3')	Ta (°C)
BT_1778	NP_810691.1	F: TATACCATGGGCATGATCATGATGAAAAGGTATAAAAAG R: CGAATTCTATAAATCAATGACTGTTTGC	56
BT_1780	NP_810693.1	F: TATACCATGGGCATGAAATGGATATTGGCAGTAGG R: CGAATTCTACATAGACACCATCTTGTATTTG	57
BT_1872	NP_810785.1	F: TATACCATGGGCATGAAAATAAGAAATCTGGTGTTTGG R: CGAATTCTTATTCCGCAATAAAGCGCTTTG	57
BT_2854	NP_811766.1	F: TATACCATGGGCATGACACTGAGAAATATAACTTG R: CGAATTCATTTTCCCTTCACAGTTATC	58
BT_3009	NP_811921.1	F: TATACCATGGGCATGAAAAAAGTACTGTTAACTTTAAG R: CGAATTCTACCGAAGTGTTACACTCA	58
BT_3300	NP_812212.1	F: TATACCATGGGCATGAAACAAAAAAGACTTACGTTTTTAC R: CGAATTCTATAGTGTATATTGAAATTTTTTTG	55
BT_3567	NP_812479.1	F: TATACCATGGGCATGATAAATAAGAAAATATTTTTTTCAC R: CGAATTCTATTTTAGCGTGAATCGTG	56

After perform PCR process, gene product were visualized and separated by running the sample with Novel juice DNA staining dye (GeneDirex, Taiwan) using electrophoresis technique on 0.7% agarose gel and then excised the gel in a piece at the interested position of expected size using DNA marker for estimation. The gene product was purified from gel by using universal DNA purification kit (TIANGEN Biotech, Beijing). Genes and vector were cut using double digestion of *NcoI* and *EcoRI* to prepare the compatible element for sticky end ligation. Amount of insert: vector for ligation reaction setting was 1:3 molar ratios. The ligation product was then transformed into *E. coli* DH5α strain. Transformants were grown on LB medium agar contained 50 µg/mL Kanamycin. They were then screen for searching of target gene inserted on expression vector by colony PCR with T7 promoter primer and reverse primer of the gene. The clone of *E.coli* with presence of positive PCR result was randomly picked up to grow in LB culture medium broth contained 50 µg/mL Kanamycin. After that, *E. coli* cells was harvested and performed plasmid extraction using Presto^{TM} mini plasmid kit. The plasmid was sent for sequencing by Macrogen Company. The correct sequenced plasmid then transformed into *E. coli* Rosetta2 (DE3) strain. Transformant was subsequently grown on the LB plate contained 34 µg/mL Chloramphenical and 50 µg/mL Kanamycin. After incubation for 16-18h, 4-5 transformant colonies were randomly selected and performed a small scale expression procedure by inoculation of them with 5 mL LB broth at 37 °C with shaking for 3-4 h. Then recombinant cells were induced by adding of 200 µM isopropyl-β-1thiogalactopyranoside (IPTG). Induced cells were harvested by centrifugation and then re-suspended in 100 µL of lyses buffer solution (50 mM sodium acetate buffer (pH 6.0), 0.2 mg/mL of lysozyme, 0.1 µg/mL of RNase A, 1 U/mL of DNase I, 50 mM of $MgCl_2$ and 1 mM PMSF) and incubated at 37 °C for 30 min. Supernatant residue of protein was subsequently collected after centrifugation at 13,000x*g* for 10 min. Then the induced protein extract solution approximately 50 µL was incubated with 50 µL of substrate solution (the concentration of 4 mM SV in 50 mM sodium acetate buffer, pH 6.0) for 1-2 h. at 37 °C. After that, the enzyme-substrate reaction solution was stopped by heating and the enzymatic hydrolysis activity was monitored by either HPLC or TLC assay.

Clone of expressed gene in Rosetta 2 (DE3) which possessed SV-degradation activity in the protein extract and SV reaction solution was selected to perform further experiment. The recombinant plasmid from this clone was firstly extracted and named pBT-3567. It was used as template for vector re-construction to produce a new plasmid version of C-terminal 6xHis taq framing by homologous recombination. *BT_3567* gene in pBT_3567 was amplified by PCR using two primers, T7 promotor: 5′-TAATACGACTCACTATA-3′ and Bt3567EcoRIHisr: 5′-GTGGTGGTGGTGGTGGAATTCTTTTAGCGTGAATCGTGC-3′ (*EcoRI* site underlined) with annealing temperature of 49 °C. The reward primer, Bt3567EcoRIHisr, was designed without stop codon to frame with C-terminal 6xHis tag. The homologous recombination was performed by transformation of cut vector (pET28a with *NcoI* and *EcoRI* digestion) and *BT_3567* gene into *ccdB* cells. Transformant was then screened on LB medium agar contained 34 µg/mL Chloramphenical and 50 µg/mL Kanamycin. It was then performed colony PCR examination and propagated in LB medium. Positive clone contained insert was performed plasmid extraction. Restriction enzyme digestion assay using *NcoI-Xho* restriction enzymes was also examined to check that *BT_3567* gene was re-constructed to be the version of C-terminal 6xhis-tag. After that plasmid version of C-terminal 6xhis-tag of pBT_3567 was named pBT_3567His. It was tested to confirm to have the hydrolysis activity on SV. Afterwards, DNA sequencing was performed by Macrogen Company to obtain the nucleotide sequence data of recombinant *BT_3567-7*gene in pBT_3567His. In brief, the cloning process to generate pBT_3567, and pBT_3567His were shown in Figure 3.1-3.3 respectively.

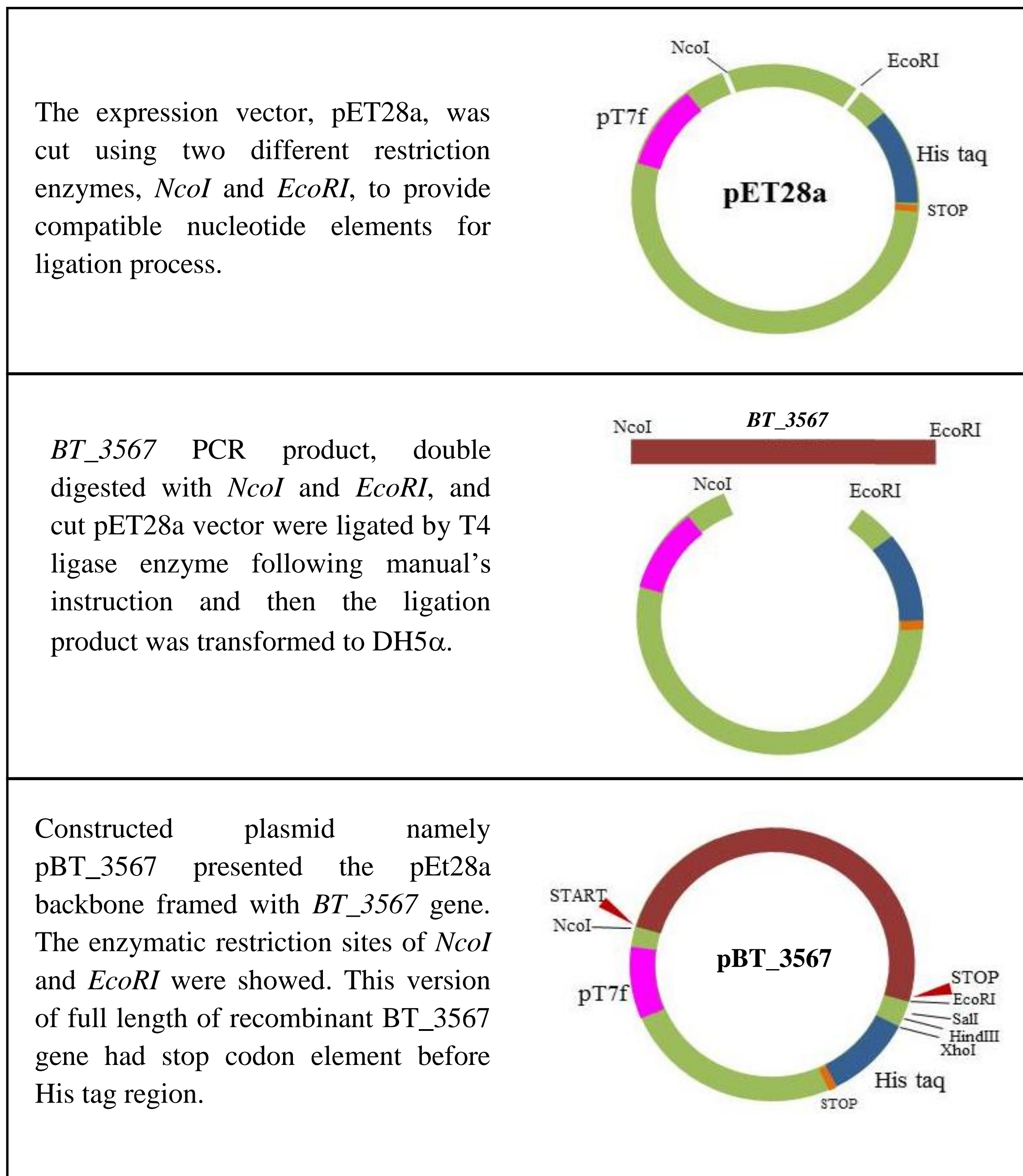


Figure 3.1 The overview process of pBT_3567 construction

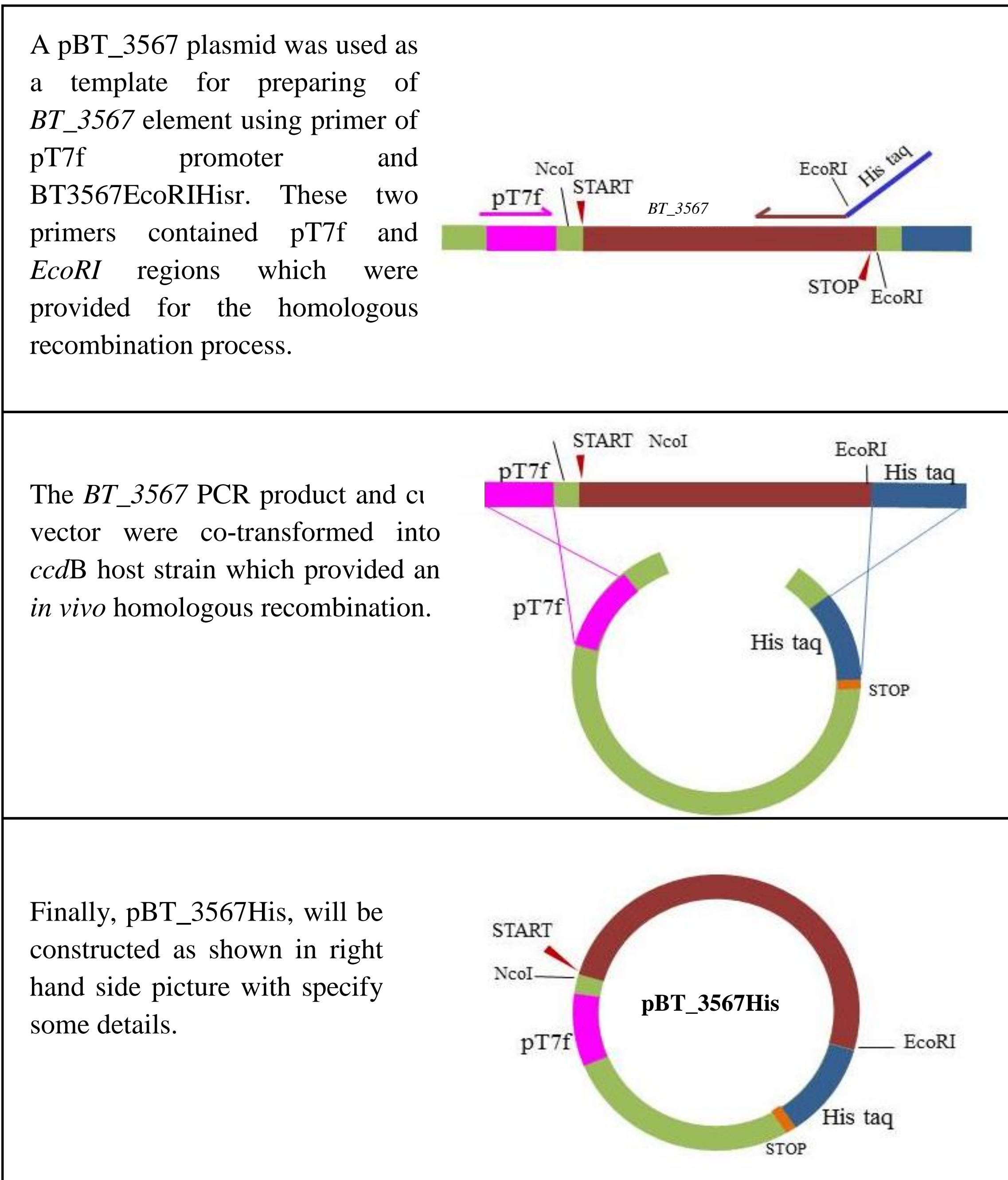


Figure 3.2 The overview process of pBT_3567His construction

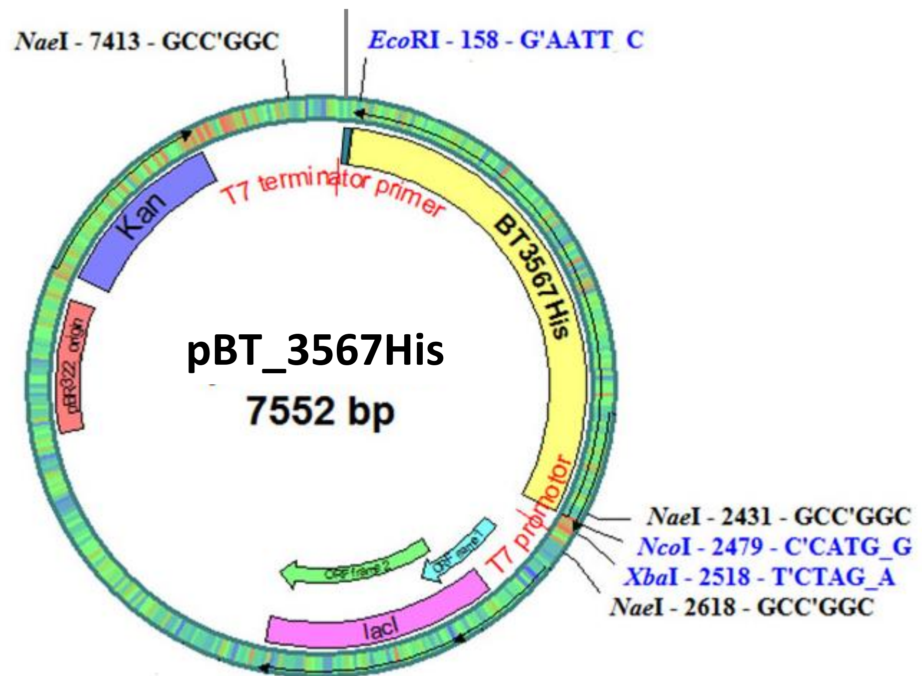


Figure 3.3 Plasmid map of pBT_3567His. It was labeled of available cut site and some annotated genes.

3.6.2 Molecular bioinformatic analysis of recombinant BT_3567

3.6.2.1 DNA/protein sequence analysis

BT_3567 gene sequence from the reference strain and our strain were compared to verify their identity by using the BLAST program online available from NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/).

3.6.2.2 Conserved domain analysis

The amino acid sequence of rBT_3567 was analyzed by SMART server (http://smart.emblheidelberg.de/) (Letunic et al., 2012) to accomplish conserved domain and classify β-glucosidase family of the recombinant protein.

3.6.2.3 Identification of catalytic sites

Other six β-glucosidases protein sequences from *B. thetaitaomicron* VPI-5482 and rBT_3567 from *B. thetaitaomicron* HB-13 were all aligned. The conserve regions were assumed to be proton donor and catalytic nucleophile residues. Multiple sequence alignment was performed by ClustalW2.

3.6.3 Expression and purification of the recombinant BT_3567

Firstly, pBT_3567His was transformed to *E. Coli* Rosetta2 (DE3), an expression host strain. One colony of transformant was picked up and then incubated with 10 mL LB broth contained 34 μg/mL Chloramphenicol and 50 μg/mL Kanamycin. Incubation was performed at 37 °C for 16-18 h with 220 rpm shaking. Five milliliter of the culture was subsequently inoculated into 100 mL of a new LB broth contained the same those two antibiotic drugs and continue growing for 3-4 h then pour this culture into 1 L of a new medium prepared same previous step. The cell culture was grown until the OD reach to 0.8-1.0 at 600 nm. Then cells were induced by adding of isopropyl-β-1 thiogalactopyranoside (IPTG) at a final concentration of 200 μM. Cells were grown continuously overnight at 25 °C with shaking. Induced cells were harvested by centrifugation and wash one time with sterile water then re-suspended in lyses buffer solution (50 mM sodium acetate buffer (pH 6.0), 0.2 mg/mL lysozyme, 0.1 mg/mL RNase A, 1 U/mL DNase I, 50 mM $MgCl_2$, 300 mM NaCl).

Cells suspension was then incubated in 37 °C for 30 min. Protein extract solution was collected by centrifugation at 13,000*xg*, 4 °C for 15 min. To purify rBT_3567 protein from impurity, affinity chromatography using His tag protein purification was performed. Approximately 2 mL of Ni-NTA resin was pre-equilibrated with 2 mL of Buffer A: 50 mM sodium acetate buffer (pH6.0) and NaCl (300 mM). The extracted protein was then loaded into the Ni^{2+} column and let it on refrigerator or room temperature for a while to allow His-tag protein bound with the Ni-NTA resin. After that gravity flow purification with a stepwise elution of imidazole (75-150 mM) in buffer A was performed. Each fraction was collected separately and monitored enzymatic activity on hydrolysis of either *p*NPG or SV. To concentrate these fraction and making of buffer exchange, 30 K cut off centrifugal filter (Amicon Ultra Centrifugal Filters, Millipore) was used. The protein concentration was determined by the Bradford assay using BSA as a standard [44]. Purified rBT_3567 was then analyzed by 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue R-250 staining.

3.6.4 Internal peptide sequence analysis by mass spectrometry

The purified rBT_3567 was then analyzed to confirm the identical propertity using mass spectrometry peptide sequences analysis. The single band of purified rBT_3567 on 10% SDS–PAGE was excised from the gel carefully with avoidance of human keratin contamination. A gel piece was kept in 1% of acetic acid until sent to analyze by LC-MALDI MS/MS at the Central Laboratory of Mahidol University, Thailand.

3.6.5 Measurement of optimum pH and stability

3.6.5.1 Optimum pH

To determine the optimum pH, 100 µL of enzyme solution was incubated with 700 µL of 2.38 mM substrate solution of either *p*NPG or SV in 50 mM of Britton-Robinson buffer (pH 3-12) at 45 °C for 10 min. Then the reaction of *p*NPG substrate assay was stopped by adding 100 µL of 0.2 M Na_2CO_3 and then *p*NP products was then determined at A_{405} nm. For the reaction of SV substrate assay, the

reaction was stopped by heating at 80 °C for 5 min following of glucose assay using glucose oxidase and peroxidase assay. SV-hydrolytic product, glucose, was then determined at A_{450} nm. *Note that*: BR buffer contains a mixture of 0.05 M H_3BO_3, 0.05 M H_3PO_4 and 0.05 M CH_3COOH that has been titrated to the desired pH with 0.25 M NaOH.

3.6.5.2 pH stability

In this experiment, the stability of enzymatic hydrolysis activity in varying of pH buffer values was performed using *p*NPG as substrate. The enzyme solution (10 µL) was incubated at 25 °C for 2 h in 90 µL of 50 mM Britton-Robinson buffer in pH ranging 3-12. And then, it was incubated with 700 µL of *p*NPG substrate solution (2.38 mM), pH 6.0, at 45 °C for 10 min. After that the reaction was stopped and performed *p*NP assay mentioned above.

3.6.6 Measurement of optimum temperature and stability

3.6.6.1 Optimum temperature

The temperature suitable for enzymatic activity was determined by incubation of 100 µL of enzyme solution 700 µL of 2.38 mM substrate solution of either *p*NPG or SV at varying of temperature ranged 25-65 °C for 10 min. Before that substrates solution were pre-heated at each individual temperature for 2 min. Then the reaction of *p*NPG substrate assay was stopped by adding 100 µL of 0.2 M Na_2CO_3 and then *p*NP products was then determined at A_{405} nm. For the reaction of SV substrate assay, the reaction was stopped by heating at 80 °C for 5 min following of glucose assay using glucose oxidase and peroxidase assay. SV-hydrolytic product, glucose, was then determined at OD_{450} nm.

3.6.6.2 Thermal stability

In this experiment, the stability of enzymatic hydrolysis activity in varying of temperature was performed using *p*NPG as substrate. The enzyme solution (100 µL) was pre-incubated at 25- 65 °C for 0-160 min in the same buffer. At the end of incubation time, samples were then let cool at room temperature

for 2 min. And then, it was incubated with 700 µL of *p*NPG substrate solution (2.38 mM) at 45 °C for 10 min. After that the reaction was stopped and performed *p*NP assay mentioned above.

3.6.7 Effect of buffer types on hydrolysis activity

Four buffer types, Tris-HCl, Britton-Robinson, sodium phosphate and sodium acetate buffer which are the same pH of 6.0 were tested for recombinant BT_3567 enzymatic activity on *p*NPG. The enzyme solution (100 µL) was incubated with 700 µL of 2.38 mM substrate solution (*p*NPG) at 45 °C for 20 min. After that the reaction was stopped and performed *p*NP assay mentioned above.

3.6.8 Effect of metal ions on enzymatic activity

To examine the inhibitory effect of metal ions on rBT_3567 activity, 75 µL of purified rBT_3567 (at final concentration as 50 µg/mL) was incubated with 75 µL of either $BaCl_2$, $CaCl_2$, $CoCl_2$ $CuCl_2$, KCl, $FeCl_3$, $MgCl_2$, $MnCl_2$, NaCl, $ZnCl_2$, $HgCl_2$ at the concentration of 2 mM in 50 mM sodium acetate buffer (pH 6.0). After incubate for 30 min at room temperature, 25 µL of 14 mM *p*NPG was added to the enzyme-metal ion solution and then incubated at 45 °C for 10 min. The reaction was then stopped by adding 25 µL of 0.8 M Na_2CO_3 and then *p*NP products were determined by measurement of A_{405} nm. A standard curve of *p*-nitrophenol (*p*NP) was prepared and used to quantify the product (see Appendix A4).

Note: The amount of enzyme required to release 1 µmole of *p*NP or glucose per minute was defined as one unit (U).

3.6.9 Substrate specificity assay

3.6.9.1 Chromogenic substrates

Fifty microliter of enzyme solution was incubated with 50 µL of 4 mM *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP -N-acetyl-β-D-glucosaminide in 50 mM sodium acetate buffer pH 6.0 at 45 °C for 10 min. After incubation, the reaction was stopped and then *p*NP products were determined as mentioned in section 3.2.4.1.

3.6.9.2 Natural sugar substrates

Fifty microliter of enzyme solution was incubated with 50 µL of 4 mM amygdalin, arbutin, ocyl-β-D-glucopyranoside, cellobiose, lactose, sucrose, trehalose and isomaltose in 50 mM of sodium acetate buffer pH 6.0 at 45 °C for 10 min. The reactions were later stopped by heating at 80 °C for 10 min. The liberated glucose was subsequently determined as mentioned in section 3.2.4.2.

3.6.10 Kinetic study

3.6.10.1 Examination of rBT_3567 on *p*NPG

The initial velocities of recombinant rBT_3567 were determined using various concentrations of 0.1-5 mM *p*NPG as substrate and were measured at 45 °C and pH 6.0. The reactions were prepared and performed hydrolysis activity as mentioned in section 3.2.4.1.

3.6.10.2 Examination of rBT_3567 on SV

The kinetic study of rBT_3567 with SV was performed using SV concentrations ranging from 0.1-6 mM at 45 °C and pH 6.0. The reactions were prepared and performed hydrolysis activity as mentioned in section 3.2.4.2.

3.6.10.3 Kinetic parameters analysis

Michaelis-Menten kinetic parameters of recombinant BT_3567 on *p*NPG and SV were determined by fitting data to nonlinear regression analysis. Plotting was performed using Graphpad Prism 6.0 software (GraphPad Software, San Diego, CA, USA).

3.7 Identification of the hydrolyzed product

3.7.1 Preparation and purification of hydrolyzed product

Partially purified SV (10 mg/mL) was incubated with the purified rBT_3567 in 50 mM sodium acetate buffer (pH 6.0) at 37 °C for 24 h. The reaction was applied to TLC silica gel 60 F_{254} plates with a mobile phase consisting of ethyl acetate/isopropanol/ acetone/water (53:30:2:15 v/v). The TLC plate was partially cut and sprayed with 5% sulfuric acid followed by heating at 100 °C until the brown spots of standards and the product were observed. The region of the TLC plate containing the hydrolyzed product was scraped and material was eluted using methanol extraction. The product was evaporated and a bright-yellow crystal was obtained. Approximately 5 mg of the compound was used for structure elucidation by mass spectrometry and NMR analysis.

3.7.2 Molecular mass analysis

Approximately 1 mg of pure product was used to perform mass analysis utilized electrospray ionization-time of fight (ESI-TOF) spectroscopy with a MicrOTOF (Bruker, Breman, Germany) in the positive mode. Parameters for mass acquisitions are as follows: scan start 50 *m/z*, scan end 3000 *m/z*, capillary exit 180 V, hexapole RF 400 V, skimmer 1 70 V, hexapole 1 25 V, corrector fill 50 V, pulsar pull 337 V, pulsar push 337 V, reflector 1300 V, flight tube 9000 V and detector TOF 2295 V. Data was acquired using Bruker Daltonics Data Analysis software version 3.3.

3.7.3 NMR spectra analysis

Approximately 3 mg of pure product was used to performed NMR analysis. The ^{1}H and, ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a NMR Bruker Ascend 400 MHz spectrometer (Bruker AVANCE 400 MHz Spectrometer, Germany) at 25 °C operating at 400 MHz for proton and carbon. The mass and ^{13}C spectrum of aglycone product were compared with that of previously report.

3.8 Development of enzymatic method for SV determination

3.8.1 Sample extraction procedure

To compare the stevioside extraction method from dried *Stevia* plant, samples were set up by combining the following components in the table below.

Table 3.2 Experimental setting for testing yield from different leaf extraction process

Recipes	**Tube#**		
	1, 2	**3, 4**	**5, 6**
Dried *Stevia* leaves	10 mg		
Distilled water	1 mL	-	-
30% ethanol	-	1 mL	-
70% ethanol	-	-	1 mL

Tube#1, 3, 5 were heated at 70 °C for 10 min while tube#2, 4, 6 were heated at the same temperature for 20 min. After that, all tubes were allowed to reach the room temperature. They were then performed centrifugation at 13,000*xg* for 20 min. The supernatant of each was removed and then diluted 10 times with deionized water. Samples were filtrated with 0.22 µM syringe filter before applied to HPLC.

3.8.2 Method optimization

3.8.2.1 Examination of co-substrates and optimal pH

Standard glucose samples were set up by combining the following components in table below.

Table 3.3 Experimental setting for testing effect of different chromogenic substrates and pH of reagent buffer on glucose oxidase assay

Recipes	**Volume (μL)**											
	Tube#											
	1 Blk	**2**	**3**	**4**	**5 Blk**	**6**	**7**	**8**	**9 Blk**	**10**	**11**	**12**
1 mM glucose[a]	-	10	20	40	-	10	20	40	-	**20**	**40**	**60**
50 mM NaOAc pH 6.0	110	100	80	60	110	100	80	60	110	**80**	**60**	**40**
100 mg/mL glucose oxidase[b]	10											
10 mg/mL peroxidase[b]	10											
10 mg/mL ABTS[b]	10				-	-	-	-	-	-	-	-
6 mg/mL *o*-dianisidine[b]	-	-	-	-	10				-	-	-	-
10 mg/mL DAB[b]	-	-	-	-	-	-	-	-	10			
50 mM NaOAc[b]	**660**											

[a]Recipes were prepared in 50 mM sodium acetate buffer pH 6.0.

[b]Recipes were prepared in 50 mM sodium acetate buffer pH 3.0, 4.0 or 6.0

Table 3.4 Experimental setting for testing effect of crude *Stevia* extract on different chromogenic substrates in glucose oxidase assay

<table>
<tr><th rowspan="3">Recipes</th><th colspan="6">Volume (µL)</th></tr>
<tr><th colspan="6">Tube#</th></tr>
<tr><th>1
Blk</th><th>2
Test</th><th>3
Blk</th><th>4
Test</th><th>5
Blk</th><th>6
Test</th></tr>
<tr><td>5 mg/mL crude extract[a]</td><td colspan="6">100</td></tr>
<tr><td>3 mM glucose[a]</td><td>-</td><td>10</td><td>-</td><td>10</td><td>-</td><td>10</td></tr>
<tr><td>50 mM NaOAc pH 6.0</td><td>110</td><td>-</td><td>110</td><td>-</td><td>110</td><td>-</td></tr>
<tr><td>100 mg/mL glucose oxidase[b]</td><td colspan="6">10</td></tr>
<tr><td>10 mg/mL peroxidase[b]</td><td colspan="6">10</td></tr>
<tr><td>10 mg/mL ABTS[b]</td><td colspan="2">10</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>6 mg/mL *o*-dianisidine[b]</td><td>-</td><td>-</td><td colspan="2">10</td><td>-</td><td>-</td></tr>
<tr><td>10 mg/mL DAB[b]</td><td>-</td><td>-</td><td>-</td><td>-</td><td colspan="2">10</td></tr>
<tr><td>50 mM NaOAc[b]</td><td colspan="6">660</td></tr>
</table>

[a]Recipes were prepared in 50 mM sodium acetate buffer pH 6.0.

[b]Recipes were prepared in 50 mM sodium acetate buffer pH 3.0, 4.0 or 6.0.

All standard and test samples were then incubated at 37 °C for 60 min. After that, 200 µL of 80% H_2SO_4 was added to all tubes. To monitor oxidized ABTS, *o*-dianisidine and DAB, samples were measured absorbance at 405, 540 and 450 nm, respectively. The calibration curves of glucose with different of co-substrates adding were constructed and then used to calculate amount of glucose presented in *Stevia* crude extract.

3.8.2.2 Crude *Stevia* extracts concentration test

Table 3.5 Experimental setting for testing effect of different volume of crude *Stevia* extract on DAB in glucose oxidase assay

Recipes	Volume (µL)							
	Tube#							
	1 Blk	2 Test	3 Blk	4 Test	5 Blk	6 Test	7 Blk	8 Test
10 mg/mL crude extract[a]	2.5		5		12.5		25	
1 mM glucose[a]	-	30	-	30	-	30	-	30
50 mM NaOAc pH 6.0	97.5	67.5	95	65	87.5	57.5	75	45
GPO-DAB[b]	700							

[a]sample was prepared in 50 mM NaOAc pH 6.0

[b]GPO-DAB contained 0.1 mg of peroxidase, 1 mg of glucose oxidase and 0.1 mg of DAB in 50 mM NaOAc buffer, pH 3.0.

All samples were then incubated at 37 °C for 60 min. After that, 200 µL of 80% H_2SO_4 was added to all tubes, mixed well and then measured A_{450}.

3.8.3 Time course analysis of SV in crude extract

3.8.3.1 SV hydrolysis assay

To monitor SV hydrolysis over time, 400 µL of crude extract (5 mg/mL) was incubated with or without 40 µL of rBT_3567 solution (0.05 U) in 50 mM of sodium acetate buffer (pH 6.0) at 37 °C for 5, 10 and 15 min. The reactions were then stopped by heating at 70 °C for 5 min. Insoluble materials were removed by centrifugation at 13,000*xg* for 10 min. The amount of SV in samples incubated with rBT_3567 was monitored by HPLC analysis as described in section 3.2.3.

3.8.3.2 Glucose assay

The time for glucose analysis was evaluated using 110 µL of crude *Stevia* extract incubated with or without rBT_3567 for 15 min from the previous experiment (above). Experimental procedures for the determination of glucose were performed by adding of 690 µL of GPO reagent (pH 3.0) containing DAB at 37 °C for 1 h. The reaction was stopped by addition of 80% (v/v) sulfuric acid, as described above, and the product of oxidized DAB was measured at 450 nm.

3.9 Method Validation

3.9.1 Method comparison

In this experiment, 16 samples of *Stevia* plant were randomly measured by HPLC method and enzymatic assay which was developed in this study. Samples were extracted with heating in water as described previously and the diluted to final concentration of 5 mg/mL. All samples were beforehand filtered by 0.22 µM syringe filter then applied to each of assay.

For HPLC assay, 5 µL of 5 mg/mL crude extract samples were injected to the machine. The standard curve of stevioside ranging from 0.25-1 mg/mL was prepared to calculated amount of SV in the sample.

For enzymatic assay, 100 µL of 5 mg/mL crude extract was incubated with 10 µL of rBT_3567 in 50 mM NaOAc buffer pH 6.0 at 37 °C for 20-30 min. The enzyme was heated inactivation at 80 °C for 5 min. After that, all tubes were allowed to reach the room temperature. It was then performed centrifugation at 13,000xg for 5 min to remove pellet of precipitated residue. Then supernatant was removed to a new tube and subsequently incubated with 690 µL of GPO-DAB at 37 °C for 60 min. After that, 200 µL of 80% H_2SO_4 was added to all tubes, mixed well and then measured A_{450}. The standard curve of glucose ranging from 20-80 µM was prepared to calculated amount of glucose in the sample.

3.9.2 Method precision and accuracy assay

In this study, precision and accuracy were determined by achieved the enzymatic assay on SV which was added into crude extracts with 3 known concentrations in µM range. The experiment includes repeatability assay (within day) and intermediate precision (between days). The accuracy of method was expressed by accuracy range while precision was expressed by %Co-efficient variation = (S.D./mean)x100. Acceptable accuracy range 95-105% while %CV should be not more than 5%.

3.10 Stevioside determination in *Stevia* product

In this experiment, analysis of SV content in *Stevia* products, including steviol glycosides extract powder and syrup form, were performed. *Stevia* products were dissolved and diluted 50 mM sodium acetate buffer (pH 6.0) to make a final concentration which responded to the standard curve of each individual experiment. Standard SV was prepared using serial dilutions with final concentrations ranging from 0.25-4 mg/mL. HPLC analysis was performed using 10 µL of *Stevia* product samples, or SV standards. A standard curve for SV was generated to quantify the amount of SV in *Stevia* samples. HPLC method was performed as described previously.

Enzymatic SV analysis was performed using 100 μL of *Stevia* products samples incubated with or without 10 μL of rBT_3567 solution (0.05 U) at 37 °C for 15 min. Samples were heated to inactivate the enzymes and then clarified by centrifugation. The supernatant was transferred to a new tube followed by the addition of 690 μL of GPO-DAB. After incubation at 37 °C for 45 min, the reaction was stopped with 80% sulfuric acid and color change was monitored using spectrophotometry. Samples without rBT_3567 were used as individual sample blanks. In this assay, five standard glucose solutions with concentrations between 5-160 μM were prepared as described previously to generate a standard curve for measurement of liberated glucose in *Stevia* product samples.

CHAPTER IV
RESULTS

4.1 Screening of microorganisms on steviol glycosides hydrolysis

In this experiment, microorganisms from Tua-Nao or TN, a fermented soy bean of North-Thailand, were examined for the ability to degrade steviol glycosides. The microorganisms were propagated in rich media to increase their population. After that, they were grown in minimal media supplemented with stevioside and rebaudioside A as a carbon source instead of glucose. To monitor amount of stevioside and rebaudioside A remaining, cultured medium supernatants were collected and performed TLC analysis. Stevioside degradation activity was found in cultured medium of TN microorganism as shown in Figure 4.1 (A)

In addition, the contaminant microorganisms from *Stevia* extract (or CM) were also tested on steviol glycoside hydrolysis. Similar with TN microorganisms, CM microorganisms were propagated in rich media before performed hydrolysis activity on steviol glycosides. TLC result showed that both of stevioside and rebaudioside A band disappeared in CM-cultured medium as shown in Figure 4.1 (B).

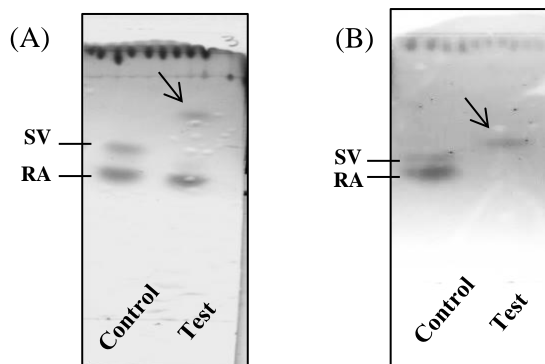


Figure 4.1 TLC analyses of rebaudioside A and stevioside hydrolysis activity of TN and CM microorganisms. Culture media of TN (A) and CM (B) contained stevioside (SV) and rebaudioside A (RA) as carbon source. Control, was medium only. Test, was medium contained microorganisms. (→) Hydrolysis product.

Microorganisms of TN were isolated to test hydrolysis activity on both of rebaudioside A and stevioside. TN microorganisms were characterized as 2 isolates namely TN1 and TN2. They individually incubated with minimum medium contained rebaudioside A and stevioside. After 3 days of incubation, media supernatants were collected then performed TLC assay. As shown in Fig 4.2 (A), both of TN1 TN2 cannot degrade any steviol glycosides. The incubation of TN microorganisms in medium contained steviol glycosides was continuing performed until 1 month. At the end, rebaudioside A and stevioside degradation by TN1 can be observed using TLC assay as shown in Fig 4.2 (B).

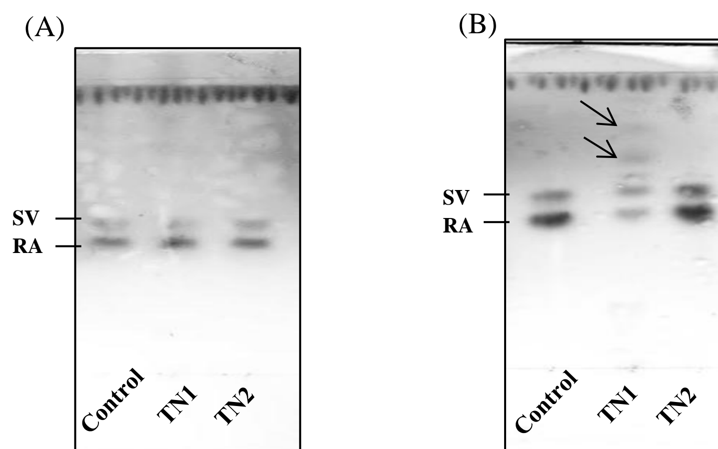


Figure 4.2 TLC result of rebaudioside A and stevioside hydrolysis activity test of 2 isolates from TN microorganisms. Cultured media were removed and replace with a new one every 3 or 4 days. Culture of TN1 and TN2 after 3 days (A) and 1 month (B) of incubation. Control, was medium only. Test, was medium contained microorganisms. SV and RA was refered to stevioside and rebaudioside A, respectively. (→) Hydrolysis product.

CM microorganisms were then isolated as 4 isolates namely CM1, CM2, CM3 and CM4. They were individually incubated with minimum medium added steviol glycosides. After 3 day of incubation, media supernatants were collected and performed TLC assay. As shown in Fig 4.3 (A), CM1 and CM4 can completely degrade stevioside while others two isolates did not. CM microorganisms were continuing incubated in medium contained steviol glycosides for 1 month. During time of incubation, the cultured medium supernatant was changed every 3 or 4 days. At the end, hydrolysis of steviol glycosides was monitored using the previous assay and the result showed that CM1 degraded both of SV and reabaudioside A as shown in Fig 4.3 (B).

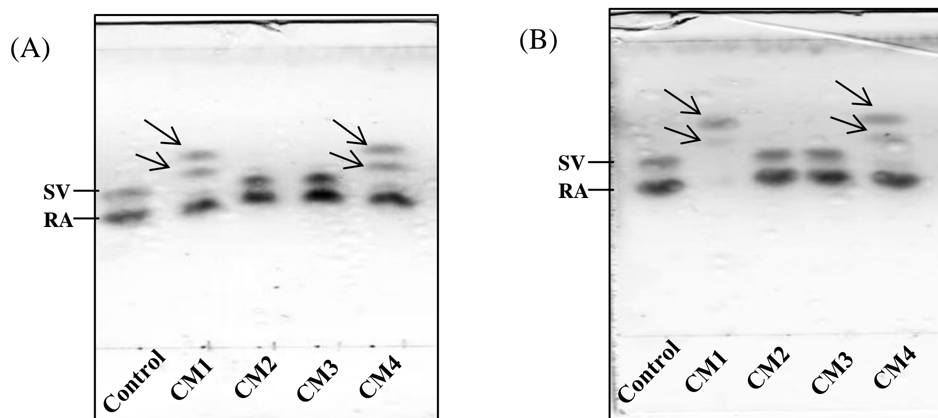


Figure 4.3 TLC result of rebaudioside A and stevioside hydrolysis activity test of 4 isolates from CM microorganisms. Cultured media were removed and replace with a new one every 3 or 4 days. Culture of CM1 to CM4 after 3 days (A) and 1 month (B) of incubation. Control, was medium only. Test, was medium contained microorganisms. SV and RA was refered to stevioside and rebaudioside A, respectively. (→) Hydrolysis product.

All active microorganisms, TN1, CM1 and CM4 were subsequently identified using 16sRNA sequence analysis. TN1 was identified as *Bacillus* sp. Both of CM1 and CM4 were identified as *Enterococcus casseliflavus.* Partial nucleotide sequences of 16s rDNA of *Bacillus* sp and *Enterococcus* sp. obtained from Macrogen company were shown in Appendix B.

At the same time during studying of microorganism from *Stevia* contaminant and Tua-Nao, the study of hydrolysis activity of *Bacteroides thetaiotaomicron* HB-13 on rebaudioside A and stevioside was performed. *B. thetaitaomicron HB-13* was cultured in minimal media supplemented with steviol glycosides for 2 days and then medium supernatant was collected and performed TLC assay. The result showed that *B. thetaitaomicron HB-13* had no ability to hydrolyze rebaudioside A however, *B. thetaiotaomicron* HB-13 showed strong hydrolysis activity on stevioside and results were shown in Fig. 4.4 (A) and (B), respectively.

The efficiency of *B. thetaiotaomicron* HB-13 on stevioside hydrolysis seemed better than of those *Enterrococcus* and *Bacillus* from CM and TN, respectively. Moreover, the result of SV hydrolysis by this bacterium was rapid and easily repeated. For further study, stevioside-hydrolytic enzyme from *B. thetaitaomicron* HB-13 was considered to reveal.

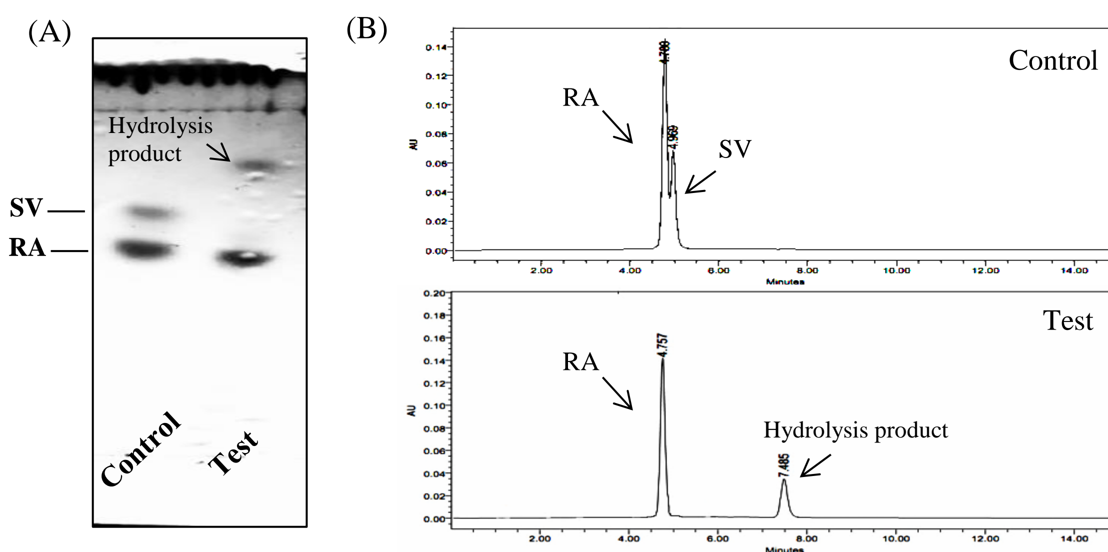


Figure 4.4 Results of rebaudioside A and stevioside hydrolysis activity test of *B. thetaiotaomicron* HB-13. After culture of *B. thetaiotaomicron* HB-13 with partially purified RA for 2 days, medium supernatant was collected and monitored rebaudioside A and stevioside hydrolysis by TLC (A) and HPLC (B) assays. Control, was medium only. Test, was medium contained microorganisms. SV and RA was refered to stevioside and rebaudioside A, respectively.

4.2 Screening for stevioside hydrolysis activity from seven candidate β-glucosidases genes

Due to the chemical structure of stevioside, the steviol the backbone component of steviol glycosides linked with glucose moieties by β-glycosidic bond at the position of C-13 and C-19. To explore enzyme involved in stevioside hydrolysis from *B. thetaiotaomicron* HB-13, β-glucosidase was thought to be candidate enzyme. Presently, β-glucosidase genes of *B. thetaiotaomicron* HB-13 were still not elucidate whereas the genome sequence data of *B. thetaiotaomicron* VPI-5842 was available in NCBI database in which 7 β-glucosidase genes, *BT_1778, BT_1789, BT_1872, BT_2854, BT_3009, BT_3300 and BT_3567* were already annotated. Although *B. thetaiotaomicron* strain of HB-13 in this study was not guaranteed to have identical genome sequence as a reference strain of VPI-5842 in the database, the strains belong to same species should have high genetic similarities. All primers were therefore designed based on nucleotide sequence of reference strain, *B. thethiotaomicron* VPI-5842 encoded full length of those 7 β-glucosidase genes.

After perform cloning assay, 3 β-glucosidase genes: *BT_1778, BT_1872* and *BT_3567* were successfully amplified and cloned to pEt28a expression vector. However recombinant BT_1778 and BT_1872 did not showed stevioside-hydrolysis activity. In contrast with recombinant BT_3567, it exhibited stevioside-hydrolytic activity. The results of PCR and cloning assay as well as stevioside-hydrolytic test on two substrates: *p*NPG and stevioside were all summarized Table 4.1.

Table 4.1 Summary of the seven β-glucosidase gene construction and the enzymatic activity test.

Gene	PCR product[a]	Clone	Substrate[b]	
			*p*NPG	stevioside
BT_1778	S	S	-	-
BT_1780	S	U	ND	ND
BT_1872	S	S	+	-
BT_2854	U	U	ND	ND
BT_3009	U	U	ND	ND
BT_3300	U	U	ND	ND
BT_3567	**S**	**S**	+	+

[a]gene amplification was performed

[b]enzymatic assay was performed

U = Unsuccessful

S = Successful

ND = Not detected

4.3 Construction of pBT_3567 and pBT_3567His

As previous result, β-glucosidase encoded by *BT_3567* gene had stevioside hydrolysis activity. The pBT_3567 was not engineered to tag with *6xHis* gene used for protein purification step. Therefore pBT_3567His contained *BT_3567 gene* frame with *6xHis gene* was generated. Here, the results of pBT_3567 and pBT_3567His construction were explained.

Firstly, *BT-3567* gene was amplified using gDNA of *B. thetaiotaomicron* HB-13 as a template. Some of PCR product was then analyzed on agarose gel as shown in Fig 4.5 (A). The PCR product was digested with *NcoI* and *EcoRI* restriction enzymes and cloned into pET28a vector cut with the same enzymes. Ligation reaction was performed according to T4 DNA ligase procedure and transformed to the strain of DH5α *E. coli*. Colony PCR for screening of cells harboring pBT_3567 was performed and result was shown in Fig 4.5 (B). All of clones with positive PCR result: clone 5, 6, 8, 14 and 17 were propagated in LB medium and then performed plasmid extraction. Plasmids were then transformed into Rosetta 2 (DE3). A small scale expression was then performed following of stevioside hydrolysis activity test. All of them possessed enzymatic activity on stevioside (data not shown).

To construct pBT_3567His, the *BT_3567* gene was firstly amplified from pBT_3567 using PCR with the primer pairs of T7 promoter and Bt3567EcoRIHisr: GTGGTGGTGGTGGTGGAATTCTTTTAGCGTGAATCGTGC (*EcoRI* site underlined). The reason of using pBT_3567 as a template for preparing of BT_3567 gene for construction of pBT_3567His is amplification of this gene from the gDNA template was hardly performed but it easily prepared from pBT_3567. Some of PCR product of *BT_3567* gene was monitored on agarose gel, as shown in Fig. 4.5 (C). The remaining PCR product and linearized pET28a vector were further co-transformed into *ccd*B cell. Since the cloning method in this experiment was performed by homologous recombination. It no need of sticky end provided by restriction enzyme digestion. However, PCR product of BT_3567 gene should be contained homologous arm for *in vivo* homologous recombination inside the *ccd*B cells. The homologous regions were designed as T7 promoter element and 6xHisTag element located on those two primers. After transformation, cells were grown on drug selective LB agar at 37 °C for 18 h. Three transformants can be observed on the agar plate (data not shown).

Colony PCR was subsequently performed using T7 promoter primer and Bt3567EcoRIHisr primer. As shown in Fig 4.5 (D), all of 3 clones exhibited positive results indicating presence of *BT_3567* gene. The version of *BT_3567* gene framed with *6xHis tag* on pEt28a expression vector from this experiment has no presence of *XhoI* cut site because it was deleted in the step of primer design for homologous cloning. Plasmid extraction was performed. Two plasmids were then digested with *NcoI*, *XhoI* digestion enzyme. In contrast with the old version of pBT_3567, it had two cut sites of *XhoI* and *NcoI.* pBT_3567 was cut into 2 pieces of DNA fragments and presented vector backbone (5.3 kBp) and BT_3567 gene (2.3 KBp) (Fig 4.5 (E), lane 1). While, newest version of pBT_3567His were linearized after cut with *NcoI-Xho I* (Fig 4.5 (E), lane 2, 3) and had no presence of two separated fragments as seen in the result of pBT-3567 digestion. pBT_3567His was subsequently subjected to perform DNA sequencing analysis. The result of nucleotide sequence was shown in Appendix B4.

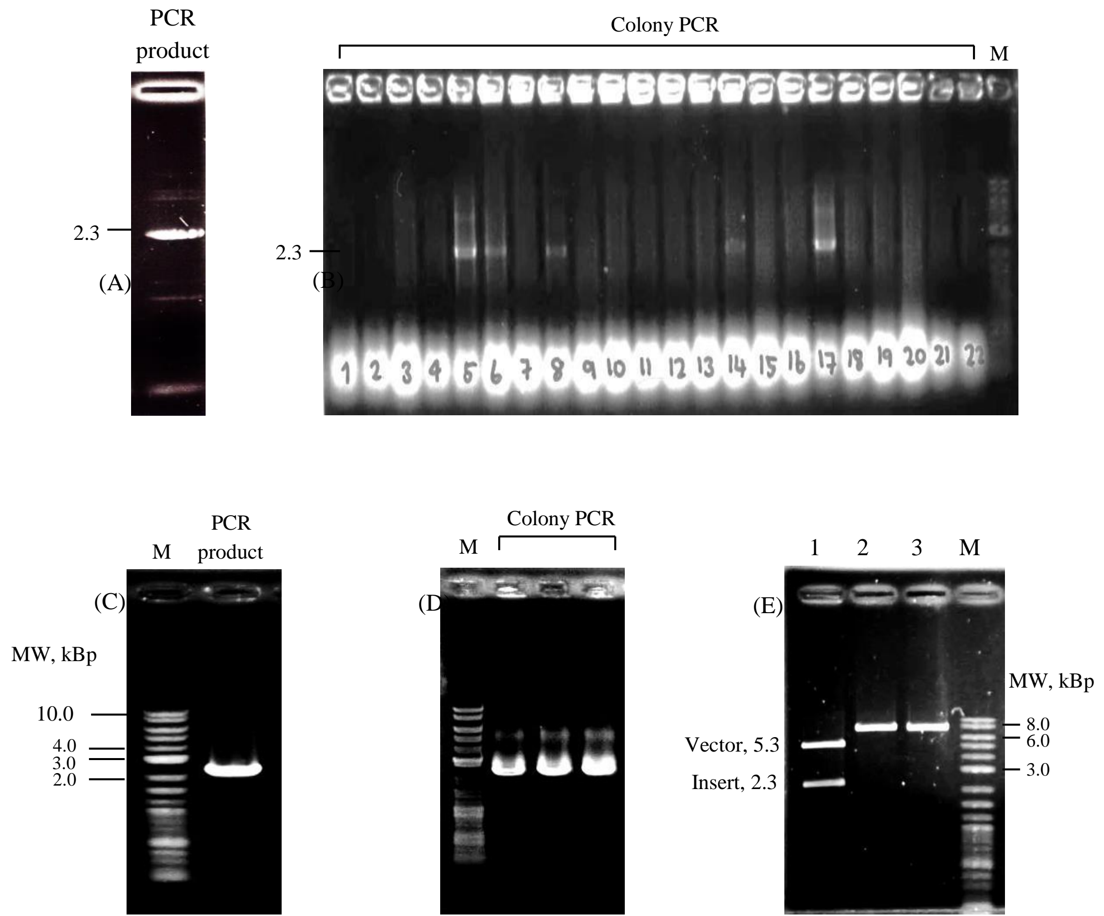


Figure 4.5 DNA agarose gel analysis. (A) BT_3567 gene product amplified from gDNA. (B) Colony PCR for screening of pBT_3567. (C) BT_3567 gene product amplified from pBT_3567. (D) Colony PCR for screening of pBT_3567His. (E) lane 1, cut pBT_3567; lane 2-3, cut pBT_3567His. M, 2-log DNA marker.

4.4 Recombinant BT_3567 gene and protein sequences analysis

Nucleotide sequence of *rBT_3567* gene was translated to amino acid sequence using the online translation program of ExPASy. The amino acid sequence of rBT_3567 was shown in Appendix B5.

Comparison of gene and protein identities of the reference strain, *B. thetaiotaomicron* VPI-5842, and the strain in this study, *B. thetaiotaomicron* HB-13, was performed by the BLAST program at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The results were shown that gene sequence identity of these two strains was 91% while amino acid sequence identity was 98%.

Basically, β-glucosidases are classified into the glycosyl hydrolase 1 (GH1) and 3 (GH3) families based on their substrate specificities and their amino acid sequence identity [45-47], as described in the CAZy database (http://www.cazy.org). To identify conserved domain presented in recombinant BT_3567 (rBT_3567), SMART server was performed. Revealed by analysis of amino acid sequence through the computer program, rBT_3567 composed of four domains Domain 1 (residues 1 to 22) is signal peptide. Domain 2 (residues 41 to 350) and 3 (residues 390 to 654) are conserved domains of glycosyl hydrolase GH 3. Domain 4 (residues 691 to 760) has a fibronectin type III fold with an unknown function.

According to Janbon *et. al.* 1995 [48] and Iwashita *et.al* 1999 [49], they have reported family 3 glycosylhydrolase contained H/E as putative proton donor while E/D was the active site nucleophile. To identify the expected acid/base nucleotide and catalytic site of the rBT_3567, the amino acid sequences of others six β-glucosidases from *B. thetaiotaomicron* and rBT_3567 were performed a multiple amino acid sequence alignment by ClustalW2. Central histidine (H333) in a conserved region of KHY/F (residue 333-335) was proposed to be proton donor while aspartate (D414) found in a conserved of SDC/Y/W or TDY (residue 413-415) was thought to be catalytic nucleophile, as shown in Fig. 4.6.

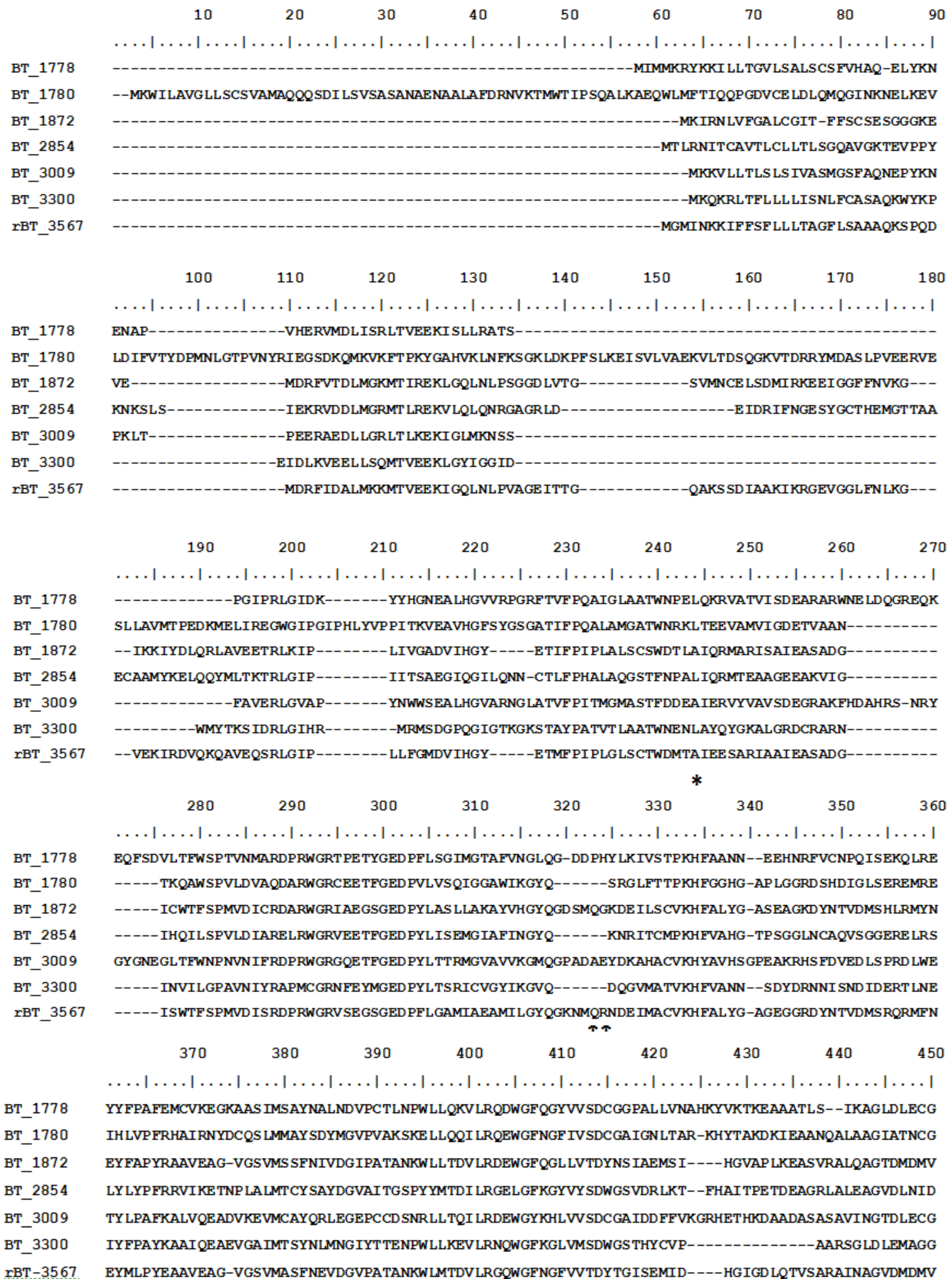


Figure 4.6 Multiple amino acid sequence alignment. Six β-glucosidase protein sequences obtained from *B. thetaiotaomicron* VPI-5842, BT_1778, BT_1780, BT_1872, BT_2854, BT_3009 and BT_3300 were aligned with protein sequence of rBT_3567. Residues 333-335 are the predicted as conserve motif for acid/base

catalysis and the central HIS333 (*) was proposed to be a proton donor. Residues 413-415 are predicted to be catalytic nucleophile motif and the Aspartate found in residue 414 (**) was thought to be a nucleophile residue.

4.5 Expression and purification of recombinant BT_3567

Expression of rBT_3567 was performed in the host of Rosetta 2 (DE3) *E. coli* strain. After transformation of pBT-3567His into the Rosetta 2 (DE3) cells, they were then cultured continuously until the density of cells when measured OD_{600} reach to 1.0. Cells were then induced with or without 200 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and then cultured at 25 °C for 16 h with shaking. Afterwards, protein samples from non-induced and induced cells were subsequently extracted and the soluble fraction was then migrated in SDS-PAGE. As shown in lane 2 of Fig. 4.7, the protein expression of non-induced cells were not shown prominent band as expected. While, the protein band of rBT_3567 of induced cells was observed at the position approximately 86 kDa (lane 3) indicated the expression of rBT_3567 was successfully performed in given condition of protein induction process. Later, rBT_3567 which was tagged with His_6 was purified from impurities using Ni-NTA chromatography. The protein was bound into the affinity bead while the unwanted proteins were eliminated by a stepwise elution with increasing of imidazole concentration. At the end, almost of impure proteins, unbound residue, were eluted with 100 mM imidazole and the purified rBT_3567, bound residue, was subsequently removed from the Nigel resin using imidazole at the final concentration of 150 mM. As shown in the lane 4, a single protein band of purified rBT_3567 was appeared.

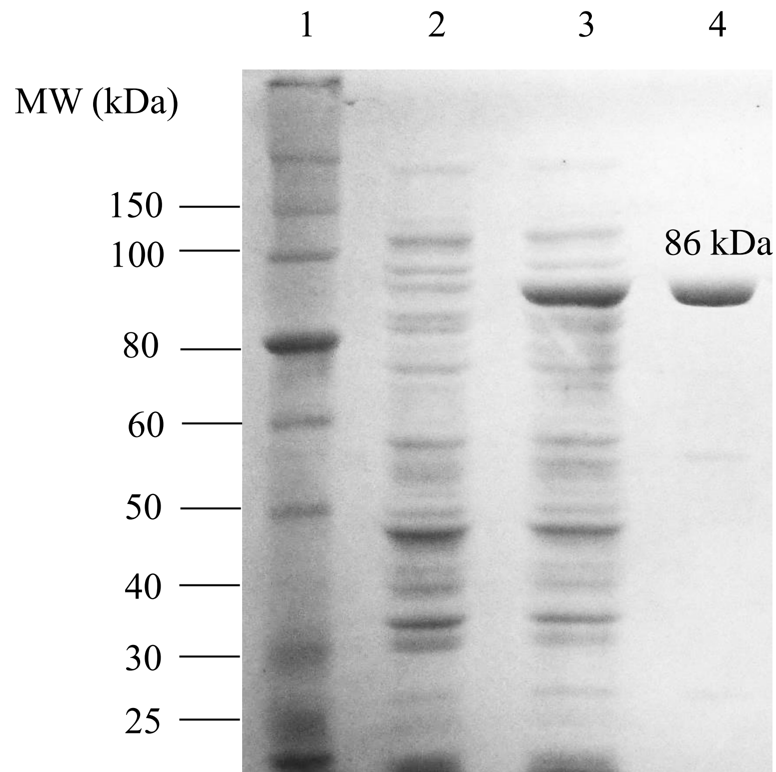


Figure 4.7 SDS-PAGE analysis of rBT_3567 expression. Lane 1, molecular weight standard; lane 2, crude extract of non-induced cells; lane 3, crude extract of the cells induced by IPTG; lane 4, purified rBT_3567

4.6 Internal peptide sequence analysis of recombinant BT_3567

To confirm identity of purified protein from previous result, the single protein band as shown earlier was excised and subsequently sent to perform LC-MALDI MS/MS. The 22 tryptic peptides sequence obtained from the result of mass analysis using Mascot software showed identical when comparison with the sequence of rBT_3567 that obtained from database (see Appendix C1). It had 32% coverage of matched peptide sequence and the peptide mapping was shown in Appendix C2. This result indicated that the purified protein from previous experiment was the rBT_3567 as targeted.

4.7 Hydrolyzed product analysis

To prepare hydrolytic product, enzyme-substrate reaction was performed by incubation of rBT_3567 with partially purified stevioside. At the end of hydrolysis reaction, stevioside was monitored to ensure that it was completely hydrolyzed by TLC and HPLC assay as shown in Figure 4.8 (A) and (B), respectively. Product was subsequently purified from impure substances using preparative TLC procedure. Purity of this product can be observed in the result of HPLC chromatogram as shown in Fig 4.8 (C). The result of mass spectrometry data showed stevioside-hydrolytic product displayed a signal at *m/z* corresponding to $[M^{+}Na]^{+}$, giving a molecular weight of 665.31 (see Appendix D1). The stevioside-hydrolytic product was here identified as rubusoside according to analysis of NMR spectra comparison with previous reports [42 and 50] as shown in Table 4.2.

In conclusion, rBT_3567 specifically cleaved β-1, 2-D-glucopyranosyl linkage of sophorose moiety at C-13 of stevioside then yield one mole of rubusoside and glucose.

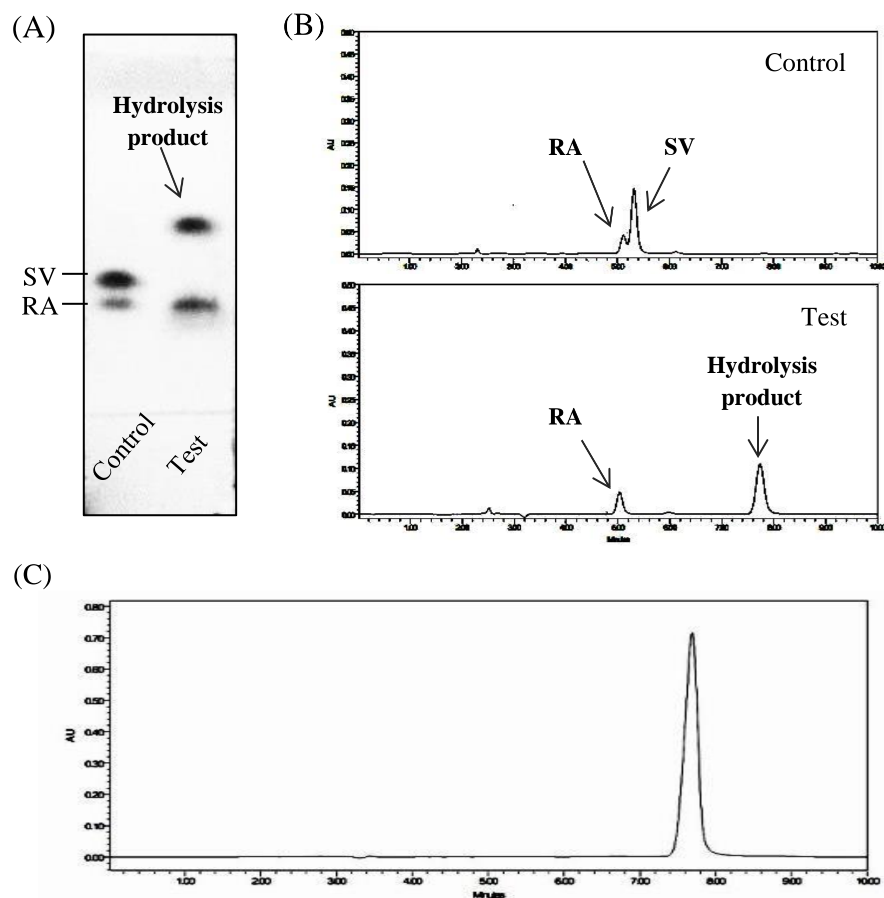


Figure 4.8 Result of enzymatic stevioside hydrolysis. Partially purified stevioside (10 mg/mL) was incubated with rBT_3567 in 50 mM sodium acetate buffer (pH 6.0) to perform hydrolysis reaction. At the end of assay, hydrolysis reaction was stopped by heating and then the reaction solution was monitored by TLC (A) and HPLC (B) assay. In this experiment, HPLC analysis was achieved using Acclaim 120 C18 column (Thermo Fisher Scientific, 4.6 mm×100 mm, 3 µm). The mobile phase was isocratic solvent of water/acetonitrile (65:35 v/v). Ten microliter of samples was injected with flow rate of 1 mL/min. The UV absorption was measured at 210 nm. (C) HPLC chromatogram of the purified hydrolysis product after performed preparative TLC. SV and RA was refered to stevioside and rebaudioside A, respectively.

Table 4.2 Comparative ^{13}C-NMR spectral data of the hydrolyzed product of stevioside.

^{13}C-NMR chemical shifts (ppm)

Residue	Carbon No.	RU[a]	RU[b]	Product[c]
Aglycone	C-1	40.8	40.2	40.3
	C-2	19.5	18.6	18.7
	C-3	38.4	37.4	37.5
	C-4	44.1	43.7	43.8
	C-5	57.4	56.7	56.9
	C-6	22.2	21.3	21.4
	C-7	41.7	40.8	40.9
	C-8	42.4	41.9	42.0
	C-9	54.0	53.2	53.3
	C-10	39.8	39.1	39.2
	C-11	20.7	20.2	20.3
	C-12	37.3	36.1	36.2
	C-13	85.9	86.5	86.6
	C-14	44.6	43.9	43.9
	C-15	47.8	46.9	47.0
	C-16	154.6	153.1	153.3
	C-17	104.4	104.5	104.6
	C-18	28.4	27.8	28.0
	C-19	177.0	178.8	178.8
	C-20	15.6	14.9	15.0

Table 4.2 Comparative ^{13}C-NMR spectral data of the hydrolyzed product of stevioside (continued).

^{13}C-NMR chemical shifts (ppm)

Residue	Carbon No.	RU[a]	RU[b]	Product[c]
C13-*O*-Glc	C-1	99.7	97.3	97.4
	C-2	75.2	73.2	73.3
	C-3	78.8	75.8	76.0
	C-4	71.1	69.7	69.9
	C-5	78.0	75.6	75.7
	C-6	62.1	60.7	60.9
C19-*O*-Glc	C-1	95.9	93.9	94.0
	C-2	74.0	71.8	72.0
	C-3	79.1	76.1	76.2
	C-4	72.3	69.1	69.3
	C-5	79.3	76.7	76.8
	C-6	63.0	60.5	60.6

[a]Ishikawa, H., et. al. 1990 [50]

[b]Wang, Z., et. al. 2015 [42]

[c]This study

4.8 Effects of pH on enzymatic activity and stability

To characterize biochemical properties of rBT_3567, effect of pH on rBT_3567 enzyme activity was examined. The assay of pH optimal was performed using both *p*NPG and stevioside as substrates. Enzyme-substrate reactions were performed in 50 mM Briston-Robinson Buffer in the pH ranges of pH 3-12 at 45 °C for 10 min and then the hydrolyzed products were measured. As shown in Fig. 4.9 (A), the optimum pH for hydrolysis activity of rBT_3567 on both substrates was the same at pH 6.0. To assay the pH stability, enzyme was incubated with different pH of buffer ranging of 3-12 before added of 5 volume buffer pH 6.0 to enhance enzymatic activity following with *p*NPG substrate to provide hydrolytic reaction. Remaining activity of the enzyme was then measured using the assay of *p*NP product. The result showed that rBT_3567 enzyme was maintained more than 80% of its maximal activity in the pH 6.0-8.0 as shown in Fig. 4.9 (B).

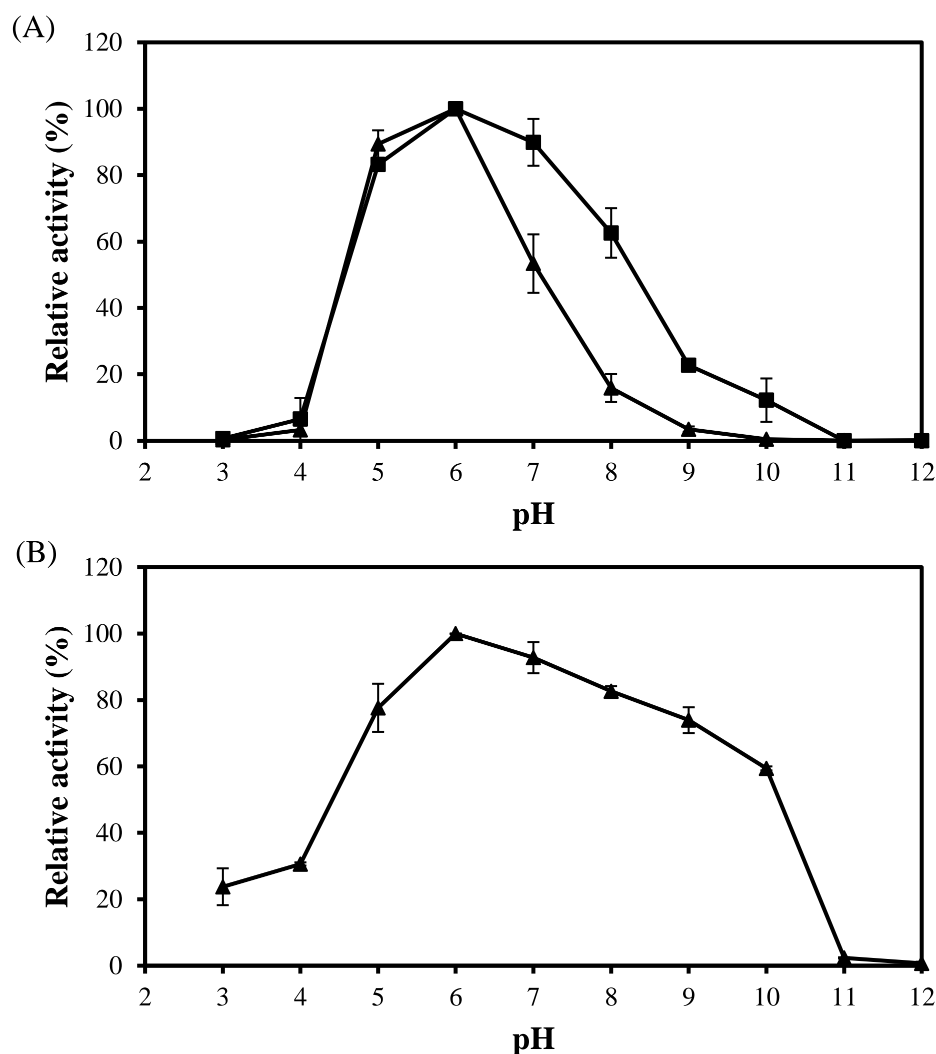


Figure 4.9 Effects of pH on activity and stability of recombinant BT_3567. Values are means calculating from triplicate determinations ±SD. Relative activity (%) was calculated by assuming that activities of rBT_3567 at pH 6.0 was 100%. (A) Effect of pH on the hydrolysis activity of 1 µg/mL rBT_3567 towards *p*NPG (▲) and stevioside (■). (B) The pH stability of rBT_3567 was examined by incubation of enzyme with 50 mM Britton−Robinson buffer at room temperature for 2 h. Afterwards the residual enzyme activity was measured using *p*NPG as substrate.

4.9 Effect of buffer type on enzymatic activity of recombinant BT_3567

Previous experiment, enzymatic activity was performed in Britton-Robinson buffer because it was universal buffer which was easily prepared for making of various pH values. To enhance hydrolysis activity of rBT_3567, buffer choice was thought. Enzymatic reaction of rBT_3567 and *p*NPG was performed in various buffers such as Britton-Robinson, Tris-HCl, sodium phosphate and sodium acetate with concentration of 50 mM and pH 6.0. Among them, the hydrolysis activity of rBT_3567 on *p*NPG has strongest in sodium acetate buffer as shown in the Fig. 4.10. Thus, sodium acetate buffer was considered to use for accomplishment enzymatic reaction of rBT_3567 in the next experiment.

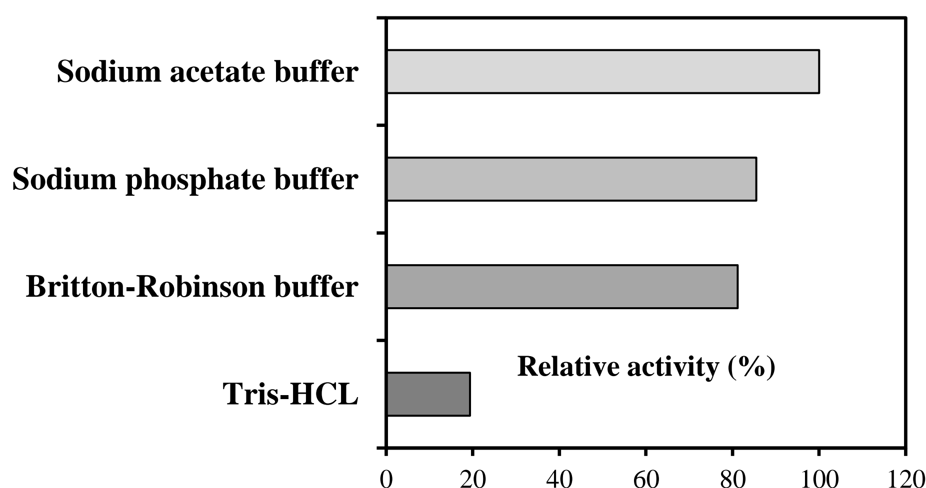


Figure 4.10 Comparison of buffer types on recombinant BT-3567 enzymatic activity. Relative activity (%) was calculated by assuming that activity of rBT_3567 in the presence of sodium acetate buffer was 100%. The enzyme was incubated with different buffers, Britton-Robinson, Tris-HCl, sodium phosphate and sodium acetate, at 45 °C for 10 min before measurement of the remaining activity using *p*NPG assay.

4.10 Effects of temperature on enzymatic activity and stability

Effect of temperature on rBT_3567 enzyme activity was examined using both *p*NPG and stevioside as substrates. As shown in Fig. 4.11 (A), the optimum temperature of rBT_3567 on *p*NPG- and stevioside-hydrolyzing activity was 45 °C. The effect of temperature on enzymatic stability was ascertained by pre-incubating the rBT_3567 in 50 mM sodium acetate buffer at the temperature 25- 65 °C for 0-160 min then remaining activity of the enzyme was examined at temperature of 45 °C in the presence of *p*NPG as a substrate. The result showed that thermal stability of enzyme was below the temperature of 25 °C. The recombinant enzyme was completely inactivated by heating at temperature of 65 °C for, at least, 5 min as shown in Fig. 4.11 (B).

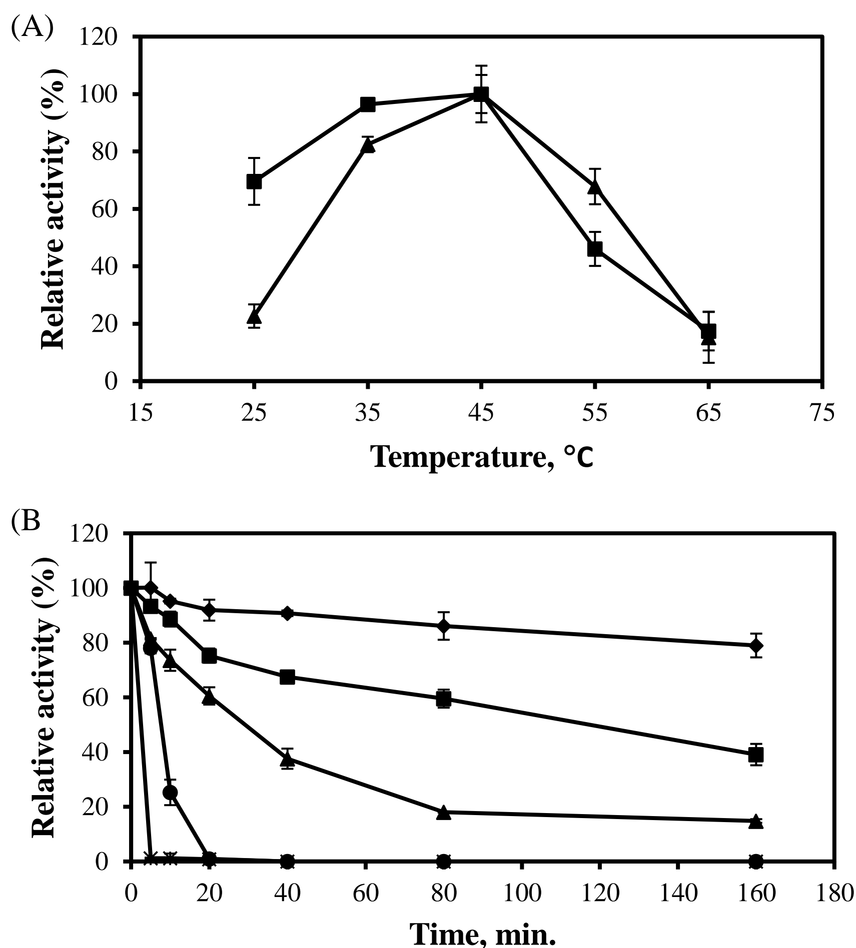


Figure 4.11 Effects of temperature on the activity and stability of recombinant BT_3567. Values are means calculating from triplicate determinations ±SD. Relative activity (%) was calculated by assuming that activities of rBT_3567 towards *p*NPG (▲) and stevioside (■) at temperature of 45 °C was 100%. (A) The optimum temperature was determined by pre-heating substrate solution for 2 min before incubating the enzyme at 25-65 °C for 10 min in 50 mM sodium acetate buffer (pH 6.0). (B) The thermal stability was ascertained by incubating the enzyme at 25 °C (♦), 35 °C (■), 45 °C (▲), 55 °C (●) and 65 °C (×) for 0-160 min in 50 mM sodium acetate buffer (pH 6.0). Afterwards, the residual enzyme activity was examined using *p*NPG as substrate.

4.11 Effects of metal ion on activity of recombinant BT_3567

The effects of metals were tested by incubation of the enzyme at room temperature for 30 min with various metal ions (final concentration of 1 mM). After that, the remaining activity of rBT_3567 on hydrolysis of *p*NPG was measured. As shown in Table 4.3, the strong inhibitor of rBT_3567 belongs to $HgCl_2$ and $CuCl_2$ which almost completely inhibited the activity of enzyme. In addition, $ZnCl_2$ also act as rBT_3567 inhibitor since it reduced the activity to approximately 40% comparison with control (without metal ions). Others metal ions as NaCl, $MnCl_2$, $MgCl_2$, KCl, $FeCl_3$, $CoCl_2$, $CaCl_2$, $BaCl_2$ did not inhibited the activity of rBT_3567. In addition, EDTA, a chelating agent, did not restrain an activity of rBT_3567.

Table 4.3 Effects of metal ions and chemical substance on activity of recombinant BT_3567.

Substance	Specific activity (U/mg)	% Residual activity*
Control	16.0±1.69	100
$BaCl_2$	17.0±1.35	106
$CaCl_2$	14.8±0.6	92.5
$CoCl_2$	15.6±0.4	97.5
$CuCl_2$	0.20±0.10	1.25
$FeCl_3$	15.8±0.70	98.0
KCl	16.8±1.29	105
$MgCl_2$	14.6±0.39	91.3
$MnCl_2$	16.2±1.35	101.3
NaCl	16.0±1.40	100
$ZnCl_2$	7.0±1.13	43.8
$HgCl_2$	0	0
EDTA	14.95±0.39	93.5

Values are means calculating from triplicate determinations ±SD.

*Relative activity (%) was calculated by assuming that activities of rBT_3567 on *p*NPG without pre-incubation with metal ion was 100%.

4.12 Substrate specificity of recombinant BT_3567

Actually, one enzyme may degrade several related substrates. To evaluate a specific hydrolysis activity of rBT_3567, some available substrates including rebaudioside A, aryl-, alkyl- and cyanogenic glycoside and natural saccharides were then examined. As shown in Table 4.4, rBT_3567 has a specific activity on stevioside and *p*NPG. Other substrates as *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, octyl-β-D glucopyranoside, amygdalin, lactose and rebaudioside A were weakly hydrolyzed by the enzyme. Recombinant BT_3567 had no hydrolysis activity on *p*NP-N-acetyl-β-D-glucosaminide, arbutin, cellobiose, sucrose, trehalose isomaltose.

Table 4.4 Substrate specificity test.

Substrates	**Linkage of glycosyl group**	**Relative activity (%)**
Steviol glycosides		
Stevioside	β-Glc	100±3.41
Rebaudioside A	β-Glc	1.74±0.16
Aryl glycosides		
*p*NP-β-D-glucopyranoside	β-Glc	57.11±4.46
*p*NP-α-D-glucopyranoside	α-Glc	0.11±0.012
*p*NP-β-D-galactopyranoside	β-Gal	0.66±0.12
*p*NP-N-acetyl-β-D-glucosaminide	β-Glc	0
Arbutin	β-Glc	0
Alkyl glycoside		
Octyl-β-D glucopyranoside	β-Glc	1.03±0.18
Cyanogenic glycoside		
Amygdalin	β-Glc	0.22±0.01
Saccharides		
Cellobiose	Glc β-(1→4) Glc	0
Lactose	Gal β-(1→4) Glc	0.14±0.10
Sucrose	Glc β-(1→2) Fru	0
Trehalose	Glc α-(1→1) Glc	0
Isomaltose	Glc α-(1→6) Glc	0

Data represent the means with standard deviation triplicates. The activity levels measured using stevioside as substrate were considered to be 100%.

4.13 Kinetic parameters of recombinant BT_3567 acting on *p*NPG and stevioside

Michaelis-Menten kinetic parameters of rBT_3567 were determined by fitting data to nonlinear regression analysis as shown in Fig. 4.10. The enzyme kinetic parameters of rBT_3567 for *p*NPG were as follows: *Km* 0.99±0.08 mM, *Vmax* 17.9±0.52 µmol min^{-1}, *kcat* 18.3±0.35 s^{-1}, and *kcat*/*Km* 18.4±1.25 s^{-1}•mM^{-1} (see Fig. 4.12 (A)). For stevioside were as follows: *Km* 1.12±0.21 mM, *Vmax* 39.6±2.32 µmol min^{-1}, *kcat* 38.8±2.74 s^{-1}, and *kcat*/*Km* 36.6±2.87 s^{-1}•mM^{-1} (see Fig. 4.12 (B)).

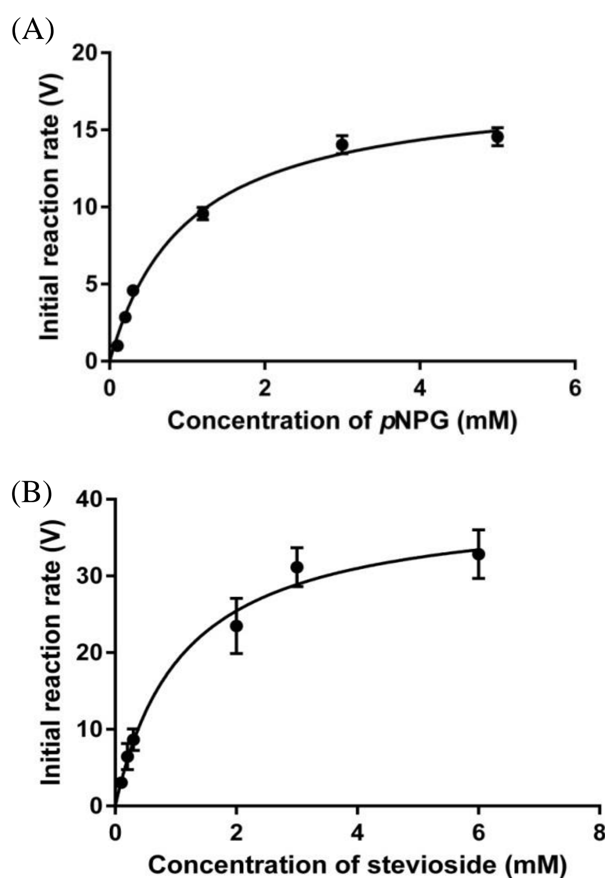


Figure 4.12 Non-linear regression curve of BT_3567. Enzymatic activity was tested on (A) *p*NPG and (B) stevioside.

4.14 Comparative method for preparing of crude *Stevia* extract

The procedures for preparation of crude *Stevia* extract were examined by varying solvent as well as time of incubation for extraction process. Water, 30% and 70% ethanol were used as solvent for extraction of SV from two random *Stevia* dried plants. As shown in Fig. 4.13 the result of stevioside content found in the extract of sample 1 and 2 using water extraction procedure for 10 min showed satisfactory yield. Therefore, water was considered to be used in the stevioside extraction process. In the next experiment, the sample of *Stevia* extract would be prepared by using water extraction with heat at 70 °C for 10 min.

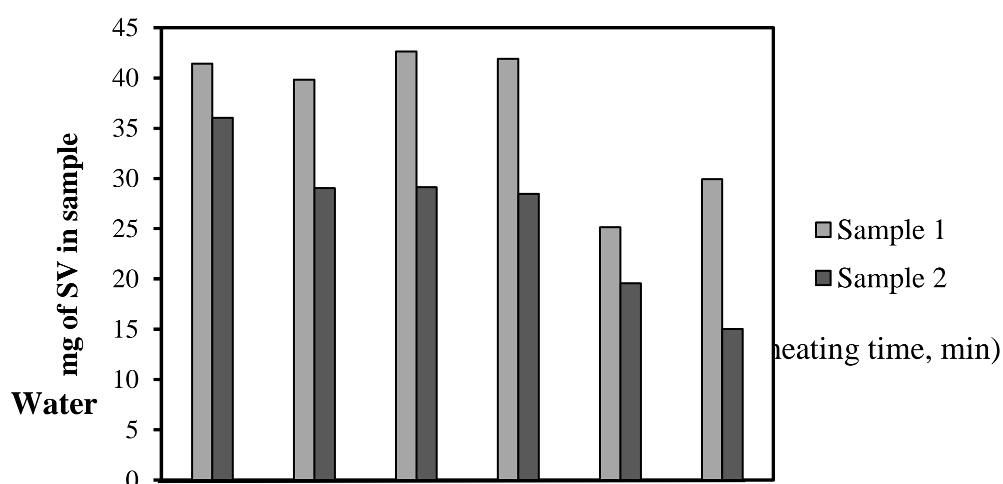


Figure 4.13 Effect of extraction method on the yield of stevioside. Ten mg/mL dried leaf of sample 1 and 2 were incubated with water or 30%, 70% ethanol and heating at 70 °C for 10 or 20 min. Amount of stevioside in the extracts was measured by HPLC.

4.15 Method optimization for stevioside assay in crude *Stevia* extract

In this experiment, 3 different co-substrates: ABTS, *o*-dianisidine and DAB, were prepared in 3 different pHs: 3.0, 4.0 and 6.0, of glucose oxidase and peroxidase reagent (GPO). Percentage of glucose recovery was examined to assume the potential of co-substrate and pH on the assay of glucose in crude *Stevia* extract sample. As shown in Table 4.5, glucose assay in the crude *Stevia* extract performed in GPO-DAB (pH 3.0) gave the best recovery percentage which was nearly 100%. Measurement glucose content should to perform in pH 3.0 and using of GPO-DAB system.

Table 4.5 Effect of pH on glucose assay in crude stevia extract with different co-substrates.

Co-substrates	pH	% Recovery*
ABTS	3	1.99±0.085
	4	2.45±0.087
	6	2.57±0.004
o-dianisidine	3	19.7±1.11
	4	23.7±0.26
	6	7.34±0.19
DAB	**3**	**100±1.39**
	4	85.8±0.97
	6	71.4±1.44

Data represent the means with standard deviation duplicates

*% Recovery = (measured value/ theoretical value)x100.

4.16 Effect of the plant matrix on glucose assay

To accomplish the limitation of crude extract concentration on assay of glucose, the crude *Stevia* extract was prepared with varying of the final concentration ranged from 0.5 to 2.5 mg/mL and the assay of glucose was then examined. As shown in Table 4.6, the final concentration at 0.5 mg/mL of crude *Stevia* extract gave the highest recovery percentage of glucose examination while in the crude sample which had concentration over than 0.5 mg/mL showed lower percentage of glucose recovery. Hence, the concentration of crude *Stevia* extract for enzymatic stevioside assay would be restricted within 0.5 mg/mL. On the other hand, the hydrolysis reaction can be performed at the final concentration of crude *Stevia* extract up to 50 mg/mL without inhibitory effect of *Stevia* plant matrix on rBT_3567 activity (data not shown).

Table 4.6 Effect of crude stevia extract concentration on glucose assay.

Stevia plant extract (mg of dried leaves/mL)	**% Recovery**
0.5	**100±1.39**
1.25	86.5±0.82
2.5	44.6±1.17

Data represent the means with standard deviation in triplicates

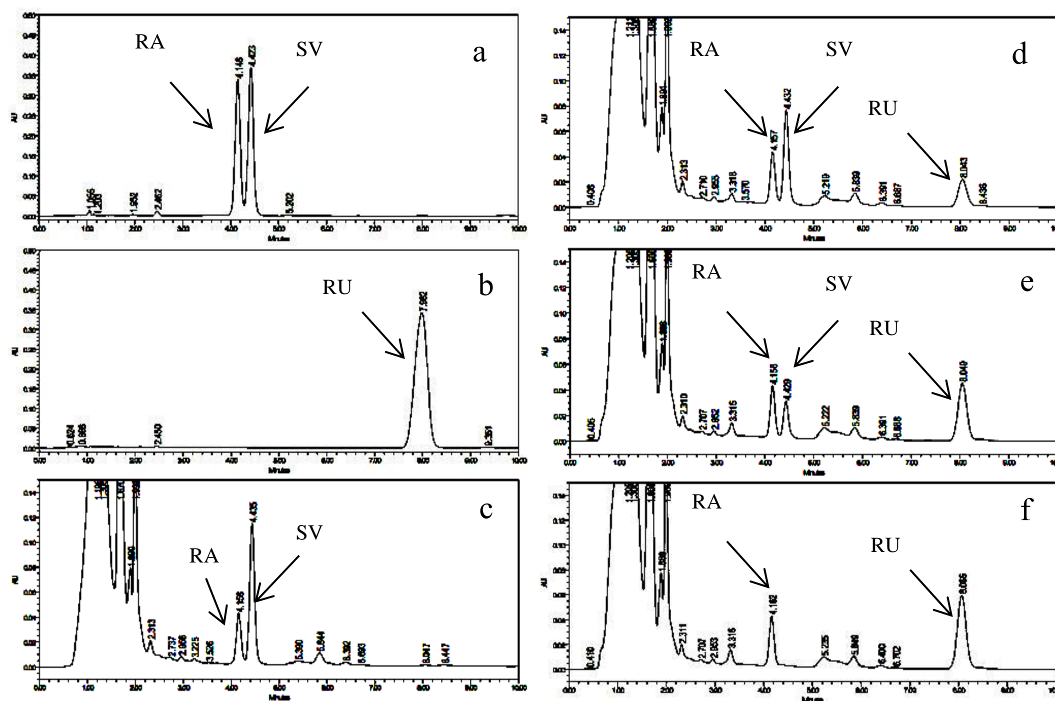
4.17 Time course of enzymatic analysis

To estimate time course of stevioside hydrolysis step, crude *Stevia* extract contained 150 µM of stevioside was incubated with rBT_3567 (0.05 U) and monitored amount of stevioside remaining in the extract using HPLC assay. Hydrolysis of stevioside in crude extract sample was completely hydrolyzed after 15 min as shown in Fig. 4.14 (A).

To monitor the time course of glucose assay, the colorimetric measurement of oxidized DAB was performed. Glucose standard sample (20 µM) and crude *Stevia* extract samples with and without stevioside hydrolysis were incubated with GPO-DAB. After 40 min of incubation time, the reaction was completely accomplished judged by unchanged A_{450} (see Fig 4.14 (B)).

Therefore, time course for enzymatic determination including stevioside hydrolysis and glucose assay processes in crude extract was totally estimated at 1 h.

(A)



(B)

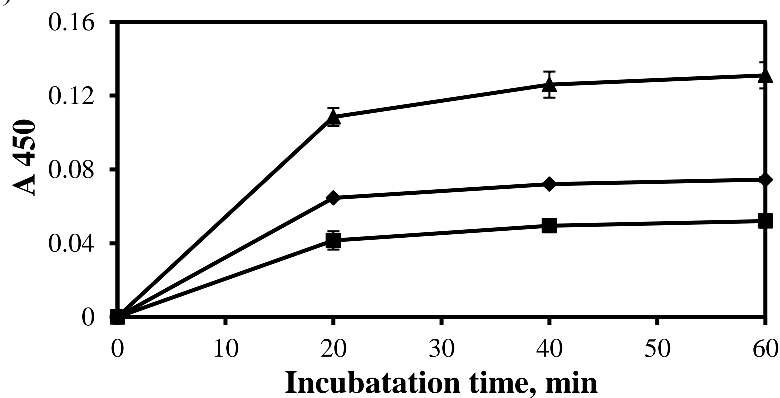


Figure 4.14 Time course analysis. (A) Time course of enzymatic hydrolysis of stevioside in crude *Stevia* extract sample using HPLC analysis. Fig. a and b, the HPLC profile of standard compound; rebaudioside A (RA), stevioside (SV) and rubusoside (RU). Fig. c, HPLC profile of crude *Stevia* extract sample (contained 150 µM of stevioside). Fig. d-f, HPLC profile of crude *Stevia* extract after incubation with rBT_3567 (0.01U) for 5, 10 and 15 min, respectively. (B) Time course of glucose assay. 20 µM glucose standard, (♦); non-hydrolyzed crude *Stevia* extract (contained 41 µM of stevioside), (■); hydrolyzed crude *Stevia* extract (▲).

4.18 Method validation

Selectivity of enzymatic stevioside determination

The selectivity of proposed enzymatic method was evaluated by analysis stevioside content in crude *Stevia* extract which were extracted from 16 different *Stevia* clones using enzymatic stevioside determination and comparing results with stevioside obtained from HPLC. The HPLC and enzymatic results of stevioside content from 16 crude *Stevia* extract samples were shown in Table 4.7. Results were processed by least-square regression analysis. Correlation plot and statistical parameters are shown in Figure 4.15.

Table 4.7 Comparison between HPLC and enzymatic assay of stevioside from 16 *Stevia* plant clones.

Sample No.	Amount of stevioside (µM)	
	HPLC method	Enzymatic method
1	20.94±1.44	22.45±0.63
2	24.28±1.20	24.14±1.23
3	24.84±1.02	23.10±3.12
4	25.24±0.98	23.41±2.66
5	28.03±1.07	28.49±0.51
6	28.36±1.87	30.57±3.34
7	29.95±1.43	31.47±1.96
8	29.46±1.11	31.81±2.92
9	31.97±1.66	32.31±3.09
10	32.05±0.96	33.34±0.58
11	33.38±2.01	33.97±3.21
12	37.01±1.58	39.37±1.11
13	38.37±2.71	37.18±2.21
14	38.43±1.9	40.54±4.96
15	41.72±1.48	43.40±3.94
16	54.91±1.52	52.70±0.88

Data represent the means with standard deviation duplicates

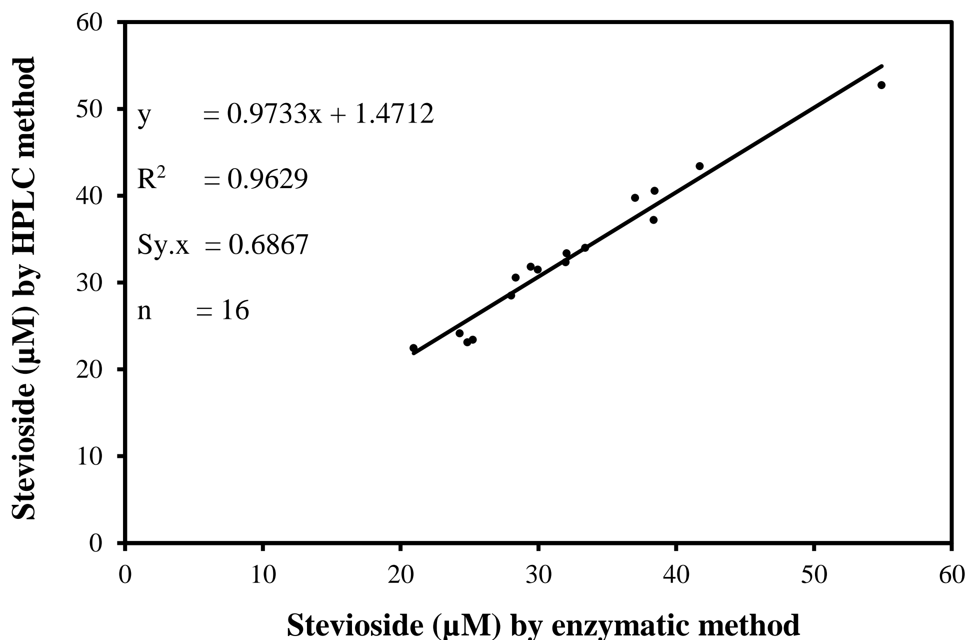


Figure 4.15 Correlation between HPLC and enzymatic method.

Precision and accuracy

As shown in Table 4.8, the variation co-efficient (CV) result of within-day precision was 1.86-3.21% in stevioside content ranging from 30-70 µM. The accuracy ranges for stevioside having concentration of 40, 50 and 70 µM were 98-105%, 97-105% and 98-104%, respectively. The CV result of between days precision was 1.80-3.28% in stevioside content ranging from 38-78 µM. The accuracy ranges for stevioside having concentration of 48, 58 and 78 µM were 96-100%, 98-105% and 95-103%, respectively (see Table 4.9).

Table 4.8 Analysis of within day and between days precision

Within day precision (n = 12)				
Stevioside (µM)			**% CV**[a]	**% Accuracy range**[b]
Original (mean±SD)	**Added**	**Detected (mean±SD)**		
29.37±1.00	10	40.21±1.10	2.74	98-105
	20	50.25±1.61	3.21	97-105
	40	70.43±1.31	1.86	98-104
Between days precision (n = 12)				
38.99±1.71	10	47.98±0.86	1.80	96-100
	20	58.67±1.34	2.29	98-105
	40	77.97±2.56	3.28	95-103

4.19 Analysis of stevioside content in *Stevia* product

Amount of stevioside in others *Stevia* samples were also tested to verify the usefulness and robustness of the enzymatic assay. Commercial *Stevia* products obtained from Sugavia Co., Ltd. such as steviol glycosides powder (SGP, n = 4) and syrup (SGS, n = 4) were examined stevioside content by both enzymatic assay and HPLC method. The result showed relative error were ranged in 0.15-4.97 % as shown in Table 4.9.

Table 4.9 Determination of stevioside content (%) in finished *Stevia* products.

Samples	Detected stevioside (%)[a]				% RE[b]
	HPLC method		Enzymatic method		
	Mean±SD	% CV	Mean±SD	% CV	
SGP (n = 4)	25.53±0.22	0.88	26.42±0.60	2.28	3.48
	22.26±0.70	3.17	22.80±0.22	0.98	2.33
	21.88±0.56	2.55	20.79±0.44	2.15	4.97
	33.30±0.35	1.07	34.65±0.44	1.29	4.04
SGS (n = 4)	29.11±0.72	2.53	29.93±0.67	2.27	2.79
	25.42±0.11	0.46	25.46±0.51	2.02	0.15
	27.86±0.16	0.60	28.33±0.74	2.64	1.68
	29.41±0.74	2.47	30.48±0.56	1.85	3.62

[a]Stevioside content (%) was presented in term of weight per weight (w/w)

[b]Relative error = |(enzymatic method value –HPLC method value) / HPLC method value| x100

CHAPTER V
DISCUSSION

The requirement of rapid and easily analytical method used for determination of steviol glycoside especially stevioside and rebaudioside A from stevia sample was gradually increased. Many chemical methods have been used to detect the compounds such as TLC, HPLC, NIR, mass spectrometry etc. Beyond these chemical analytic methods, we developed an alternative simple method based on enzymatic approach for measurement of steviol glycoside, in stevia plant extract and finished *Stevia* product. Enzymatic determination of steviol glycoside was performed by two main steps of enzymatic procedures including hydrolysis of glucose moiety from steviol glycoside-aglycone backbone and assay liberated glucose using glucose oxidase and peroxidase system. To develop the assay as described, relevant enzyme used in the first step was required. Since the specific steviol glycoside hydrolytic enzyme was not available. Therefore, screening of steviol glycoside hydrolytic activity from various microorganisms was firstly examined to select suitable enzyme to apply in the assay of steviol glycoside.

In order to screening of an active enzyme, microorganisms obtained from Tua-Nao were examined its ability on hydrolysis of steviol glycosides. Tua-Nao is a fermented soy bean food widely used in North-Thailand. *Bacillus subtilis* has been previously reported as the main microorganism of Tua-Nao. Amylase and pretease from the bacteria were reported that degrade insoluble sugar and proteins in raw soybean [51]. It might be degrade steviol glycosides from *Stevia* plant. Here, hydrolysis activity of microorganisms from Tua-Nao was evaluated. Result showed hydrolysis of stevioside after Tua-Nao microorganisms was cultured in medium plus steviol glycosides. Microorganisms were then isolated by colony streaking on medium agar. Two difference colonies were observed and named as TN1 and TN2. They were picked up from the agar and then tested hydrolysis activity on steviol glycosides again. TN1 and TN2, they did not exhibited degradation capability on stevioside or

rebaudioside A, after 3 days of incubation. The experiment was continuing performed till 1 month. TN1 exhibited hydrolysis activity on both stevioside and rebaudioside A. This result differed from the previous observation that microorganisms from Tua-Nao exhibited hydrolysis activity on stevioside but not for rebaudioside A. Contamination during performed the experiment might occur.

On the other hand, microorganisms from water extract of *Stevia* was examined their ability on steviol glycosides hydrolysis. They were thought that they might degrade steviol glycosides and used metabolized product as a nutrition source. After performed hydrolysis activity test similar with previous experiment, result showed that this contaminant (CM) can hydrolyze both of stevioside and rebaudioside A. CM microorganisms were isolated in individual. Four colonies were observed on medium agar. They were tested on steviol glycosides hydrolysis activity again. Result showed that both CM1 and CM4 hydrolyzed stevioside after microorganism were incubated with this compound for 3 days. Hydrolysis activity on rebaudioside A by CM1 occurred after incubation time was extended to 1 month. As mention in the result of hydrolysis activity assay of TN1, cultured medium of CM1 might contaminate by other microorganisms. Since after 3 days of incubation, degradation activity was not be observed from the same assay. CM1 and CM4 were identified using 16s rDNA molecular analysis. They were classified as *Enterococcus casseliflavus.* TN1, CM1 and CM4 were again examined their ability on steviol glycosides hydrolyzation. However, results took long time and could not be repeated. Since carbon source for growth of these bacteria were steviol glycosides which were not common as natural sugar. It might possible difference capacity of each strain on steviol glycosides hydrolysis when we performed the experiment on individual isolate. Therefore, hydrolysis activity from another microorganism was subsequently studied.

Since 1998, the list of microorganism and enzymes which had ability of steviol glycosides hydrolysis has been reported [34-43] as listed in Table 1.3. There are three reports of microorganisms which can hydrolyze both of reabaudioside A and stevioside included *Clavibacter michiganense* [34], *Flavobacterium johnsonae* [35] and a group of *Bacteroidaceae* [36]. Among them, *Bacteroides thetaiotaomicron* HB-13 was here designated to study. We was able to obtain the pure isolated strain *B. thetaiotaomicron* HB-13 and it had a hydrolysis activity on stevioside rapidly and

specifically. We therefore emphasis on study stevioside -hydrolytic enzyme from this microorganism and isolated genes to test their biochemical properties.

In general, one mole of stevioside consists of three moles of glucose. The positions of glucose moieties are located at C-13 and C-19 of steviol backbone. At the C-13 position, two molecule of glucose, a sopharose moiety, bond to steviol by ether linkage, while, the C-19 position one mole of glucose was linked with a backbone by ester linkage. With similarity of region-selectivity in hydrolysis of glucosidic linkages on stevioside, β-glucosidases were firstly served to be an enzymatic candidate. Since genome of our strain was not yet provided in GenBank database, primer design was performed using the genetic information of *B. thetaitaomicron* VPI-5482 as reference strain. There is a fact that different of strain may have some genetic variation. Thus, not all of seven genes can be successfully amplified and later then constructed into the expression vector, pET28a. Only three of seven β-glucosidases gene, *BT_1778, BT_1872* and *BT_3567* were cloned and then expressed in *E.coli* host strain of Rosetta 2 (DE3). The hydrolysis activity test proved rBT_3567 can degrade stevioside. After that, the version of his-tag recombinant BT_3567 was therefore performed. Finally, the expression vector of recombinant BT_3567 protein tagged His_{6}, namely pBT_3567His, was sent to perform the nucleotide sequencing. The gene identity of *BT_3567* compared with the reference strain was 91% while protein identity was 98%. Using SMART server domain analysis, recombinant BT_3567 was identified as a member of glycosylhydrolase family 3 (GH 3). According to Withers and Street, 1989 and Withers, 2001, the family 1 and 3 of glycosylhydrolase comprise retaining enzymes which hydrolyzed their substrate with net retention of anomeric configuration mechanism that occurs via a retaining mechanism [52, 53]. Therefore, our recombinant enzyme, BT_3567 was proposed that it hydrolyzed stevioside with retaining mechanism. Additionally, the catalytic sites, acid/base and nucleophile residue, were found in the position at residues of 209-211 (KHF) and residues of 287-289 (TDY), respectively (see Appendix B5). These conserved regions were predicted by amino acid sequence alignment analysis of others six annotated β-glucosidase sequences obtained from the reference strain and the sequence of rBT_3567 from our strain.

Recombinant BT_3567 tagged with 6xHis was therefore expressed in the pET28a/Rosetta 2 (DE3) system and purified by a step of purification using Ni-NTA chromatography. After perform protein purification, rBT_3567 exhibited a single band at 86 kDa as judged by SDS-PAGE indicating to homogeneously purification process. To confirm the identity of protein, this single band was then sent to perform molecular mass analysis. The result showed that amino acid sequence of BT_3567 from database was matched with the amino acid sequence analysis obtained from the experiment of mass spectrometry technique indicating that the visible band occurred in acrylamide gel was rBT_3567.

Since the stevioside hydrolyzed product was identified to rubusoside. Therefore, hydrolysis of one stevioside molecule is proportional to one mole of glucose. The optimum pH and temperature was 6.0 and 45 °C, respectively. Recombinant BT_3567 has a weak tolerant to temperature above 35 °C. Similary with previous studies, β-glucosidase are normally unstable at high temperature which was above 40 ˚C [54, 55]. The hydrolysis activity of rBtT_3567was significantly subsided upon presence of metal ions such as Hg^{2+}, Cu^{2+}, Zn^{2+}. These ions are common inhibitor for many enzymes. Hg^{2+} has been reported that it can inhibit the activity of the enzymes by forming coordinated covalent complex with sulfhydryl group of cysteine residue [56] indicating that rBT_3567 might present this group which required for the enzymatic activity. The rBT_3567 works well at the temperature of 25 or 37 ˚C. To prolong the activity of enzyme, the condition of enzymatic assay should be performed in this temperature range. The enzyme will loss its activity when temperature at or above 45 °C.

The result of substrate specific activity showed that this enzyme possessed a selective hydrolysis on stevioside. Apart from others β-glucosidases, which were previously reported as stevioside -hydrolytic enzyme [38-39, 42], rBT_3567 has a higher specific activity on stevioside than *p*NPG. The result indicated that rBT_3567 mostly prefered to hydrolyze stevioside. Moreover, result of kinetic study of rBT_3567 supported that rBT_3567 had more catalytic efficiency on hydrolysis of stevioside than *p*NPG as the value of *kcat*/*Km* for stevioside was approximately 2 times of *p*NPG.

The next part of this research is to assay liberated glucose in the sample. Although measurement of glucose content in the plant extract sample is complicated examination, it can be successfully performed in this study. To optimize the reaction condition used for determination of stevioside in crude extract, testing on the type of co-substrate used in the assay and optimum pH were performed. There are many types of co-substrate reported [57-63]. In this study, the co-substrates including *o*-dianisidine, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 3,3-diaminobenzidine (DAB) were examined. Method optimization was firstly performed with consideration of glucose recovery percentage as criteria of assay accuracy. In summary, results showed that assaying of stevioside by incubation of 0.05 U rBT_3567 with 0.25-0.5 mg/mL of crude *Stevia* extract (pH 6.0) for 20 min and following of assaying of liberated glucose using GPO-DAB (pH 3.0) and incubation for 45 min before stop reaction by adding of 80% (v/v) sulfuric acid gave the best procedure for stevioside determination.

Although, rBT_3567 has no hydrolysis activity on reabaudioside A, there are others member of steviol glycosides (e.g. dulcoside A, rebaudioside C, steviolbioside, rubusoside) as well as others natural compounds such as phenolic and flavonoid in *Stevia* plant that might be substrate of rBT_3567. However, as showed in the result of method comparison plot, the correlation between HPLC method and enzymatic method has demonstrated that others compounds were in small amount and did not interfere value of stevioside detection by rBT_3567. This can indicate selectivity of the assay. The method validation result also showed that enzymatic method for stevioside determination developed in this study can be repeated well judged by the percentage of acceptable co-efficient of variation (CV) and accuracy range according to international conference on harmonization (ICH) established [64].

Previously, in 1981, enzymatic determination of stevioside reported by Mizukami *et al*. They used crude hesperidinase to hydrolysis of stevioside and followed this enzyme with glucose oxidase and peroxidase for glucose assay. Comparison with this enzyme, it possessed non-specifically activity as well as lack of bioinformatics data of the enzyme. In contrast, rBT_3567 from our study was now established molecular information. It can be easily constructed by followed our experiment. Additionally, sample preparation in our study was also simpler than

Mizukami's method. Their method of them includes methanol extraction to prepare crude *Stevia* extract sample and following of PVP adding to inhibit the effect of interfere compounds existed in stevia extract sample while, our procedure is no need of methanol usage as well as PVP adding. All of these states can be designated that stevioside detection based on the application of rBT_3567 had more advantage than Mizukami's report.

Without of sample pre-treatment as well as requirement of general basic biochemical instrument such as spectrophotometer, enzymatic method exhibited simpler and more available assay than HPLC method for stevioside detection in crude *Stevia* extract. On the other hand, our method is eco-friendly due to no need of hazardous organic solvent use. In addition, by enzymatic procedure, high-throughput analysis can be achieved when using of 96-well microplate and microplate reader made it possible to reduce time-consuming of assay.

CHAPTER VI
CONCLUSION

In this study, we found that *B. thetaiotaomicron HB-13* carried a biocatalyst for degradation of stevioside. To capture gene encoded stevioside hydrolysis enzyme, BT_3567 β-glucosidase gene was cloned and performed a small scale protein expression following the test of hydrolysis activity on stevioside. Recombinant BT_3567, a member of β-glucosidase family 3, can hydrolyze stevioside yields glucose and rubusoside. Therefore, rBT-3567 was then applied in stevioside determination. Using of rBT-3567 in stevioside determination process, it should be performed under conditions of pH 6.0, temperature 25-37 °C and avoidance of Hg^{2+}, Cu^{2+} and Zn^{2+} contamination.

Glucose assay in crude stevia extract after stevioside hydrolysis performance should be examined in pH condition of 3.0 for the accuracy and precision of stevioside determination. Moreover, kind of co-substrate used in assay of glucose should to be tested first. Since the sensitivity of co-substrate for colored developing is generally different depend on its chemical properties. As seen in this study, among ABTS, *o*-dianisidine and DAB, the last one can be used to assay glucose in crude *Stevia* extract. It can be explained that glucose oxidase and peroxidase reagent used for stevioside determination should contain 0.1mg/mL DAB, 0.1 mg/mL peroxidase and 1 mg/mL glucose oxidase prepared in 50 mM sodium acetate buffer pH 3.0. In addition, the volume of stevioside hydrolysis reaction should restrict in volume of 110 µL while glucose oxidase and peroxidase reagent was added for 690 µL to completely recovered pH of the buffer for glucose assay.

Enzymatic method developed in this study has accuracy and precision when compared with HPLC method. Moreover, using of spectrophotometric-based approach, enzymatic assay of stevioside is easier than HPLC analysis due to less complicated operation for experimental performance. Moreover, enzymatic method for stevioside determination can shorten steps of the assay in which no need of pre-

treatment process. For example, using water extraction followed by centrifugation is sufficient for sample preparing step. Therefore enzymatic determination of stevioside is rapid and simple analytical methods and allowed a large throughput of samples. For such purpose, enzymatic stevioside determination is suitable for screening of high stevioside -produced breeding plant and for qualification of finished *Stevia* product during the production processing.

For further aspect, development of rebaudioside A measurement in crude *Stevia* extract should to start due to the need of fast and simple analysis of two major steviol glycosides, stevioside and rebaudioside A, in *Stevia rebaudiana*. It can be started from our preliminary results which were shown that CM microorganism can degrade rebaudioside A. There are interested sources of active microorganisms which might containof rebaudioside A bio-catalytic enzyme.

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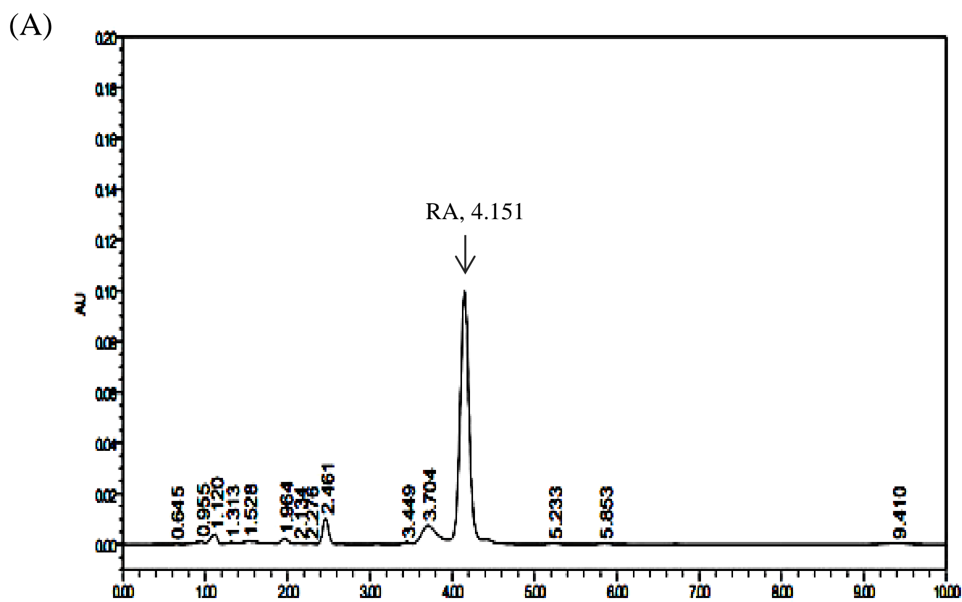
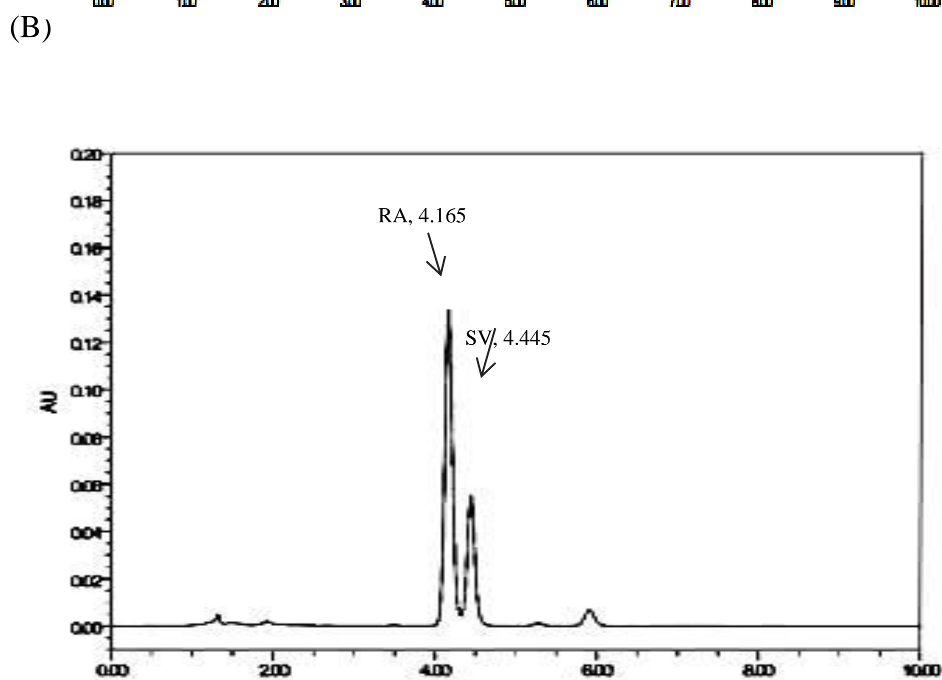
APPENDICES

APPENDIX A

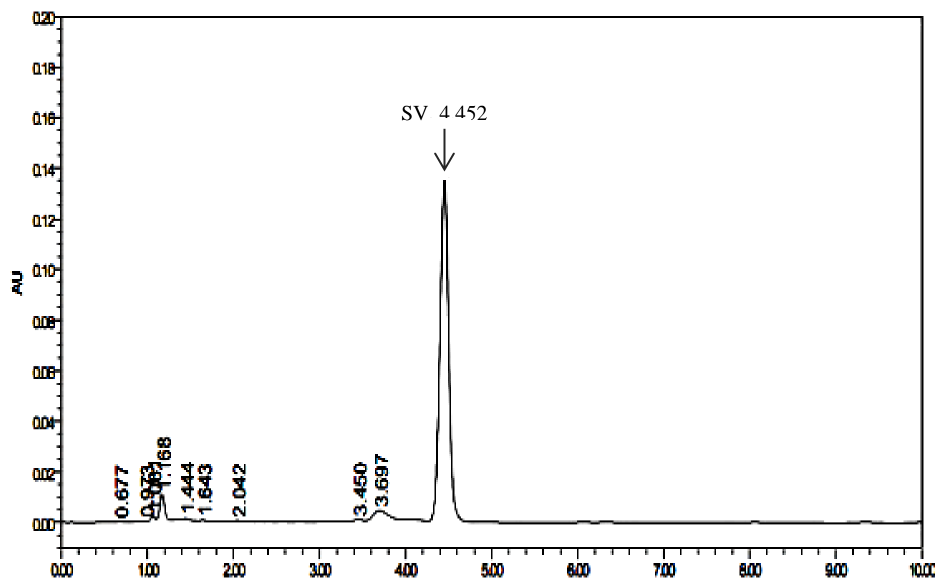
(A)



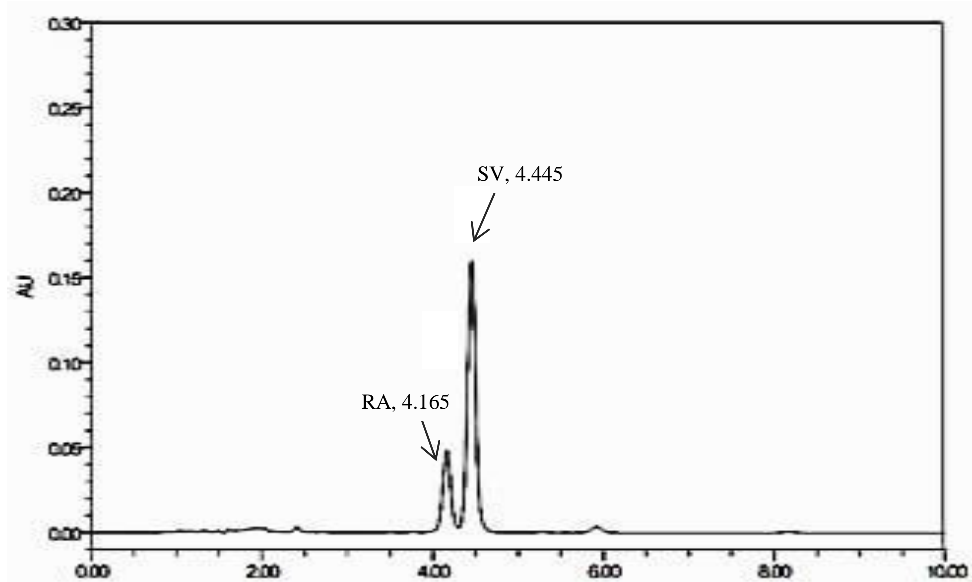
(B)



(C)



(D)



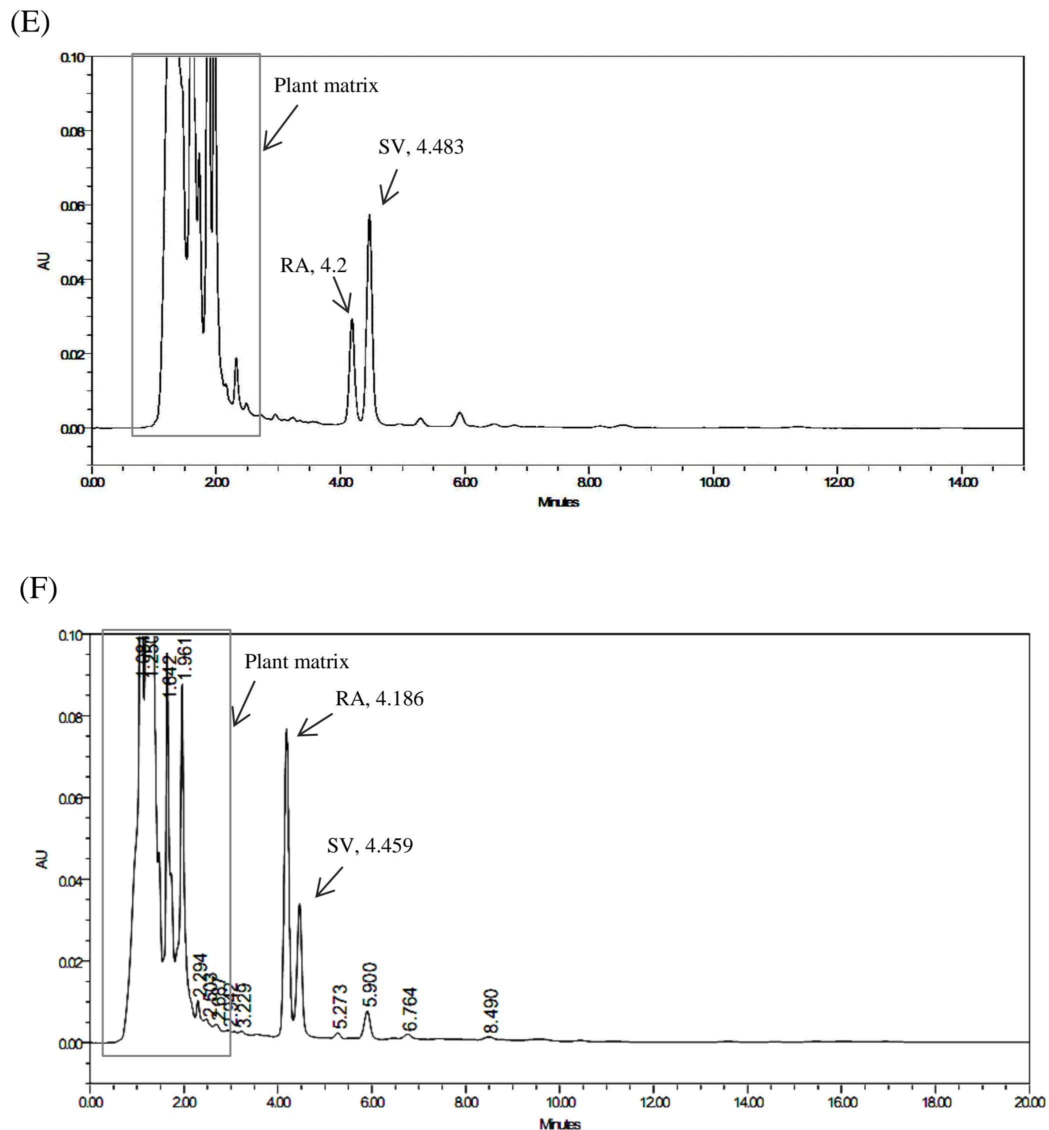


Figure A.1 The HPLC chromatogram of (A) standard RA, (B) ppRA and (C) standard SV, (D) ppSV. RA and SV appear at the retention time (tR) of 4.151 and 4.452 min. respectively. (E) HPLC chromatogram of RA and SV in crude extract of high SV-Stevia producing strain. (F) HPLC chromatogram of RA and SV in crude extract of high RA-Stevia producing strain.

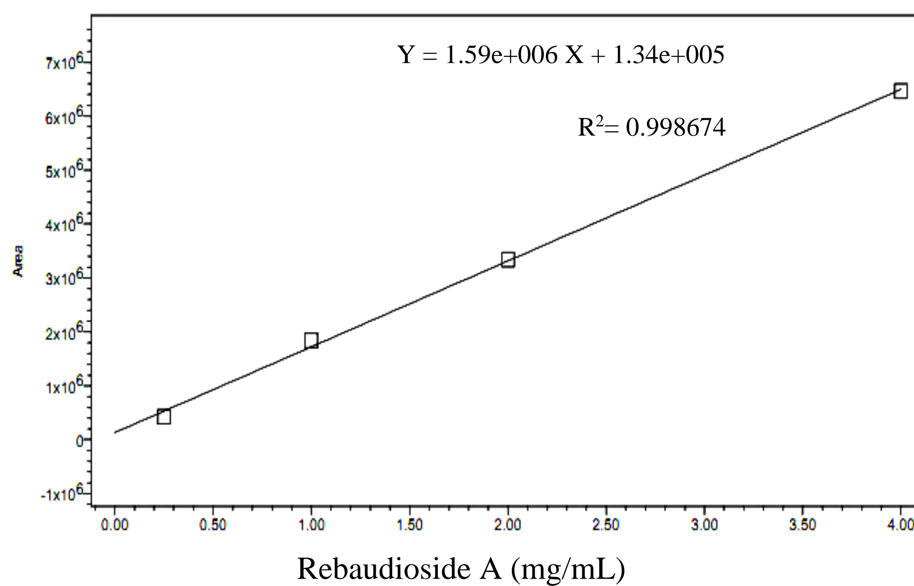


Figure A.2 Calibration curve of rebaudioside A standard for HPLC analysis.

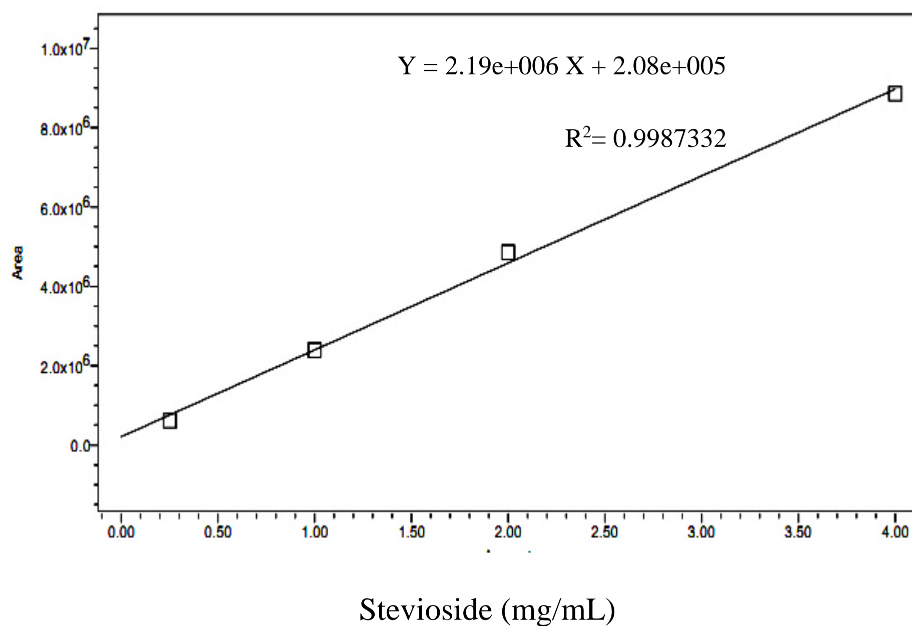


Figure A.3 Calibration curve of stevioside standard for HPLC analysis.

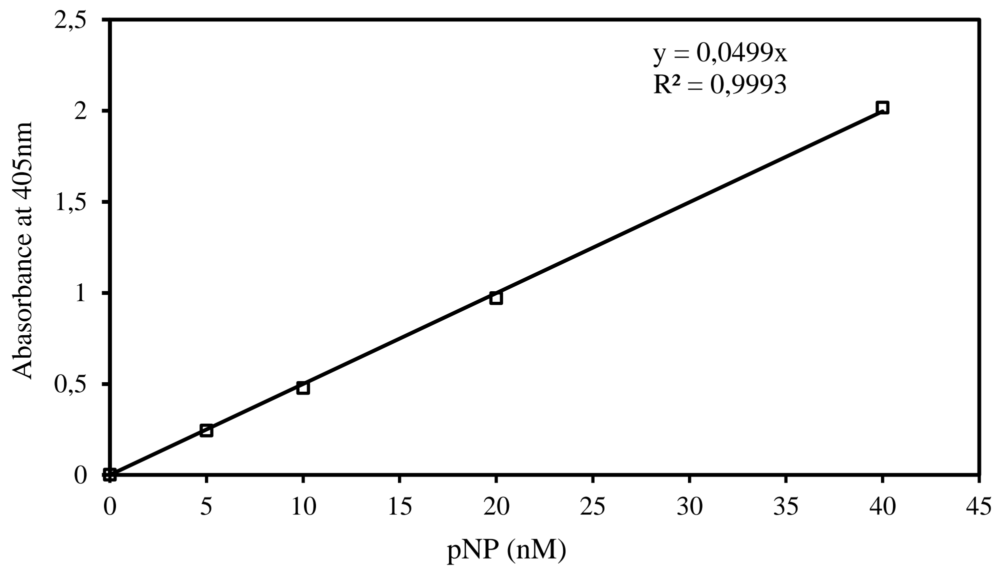


Figure A.4 Calibration curve of *p*NP for *p*NPG assay.

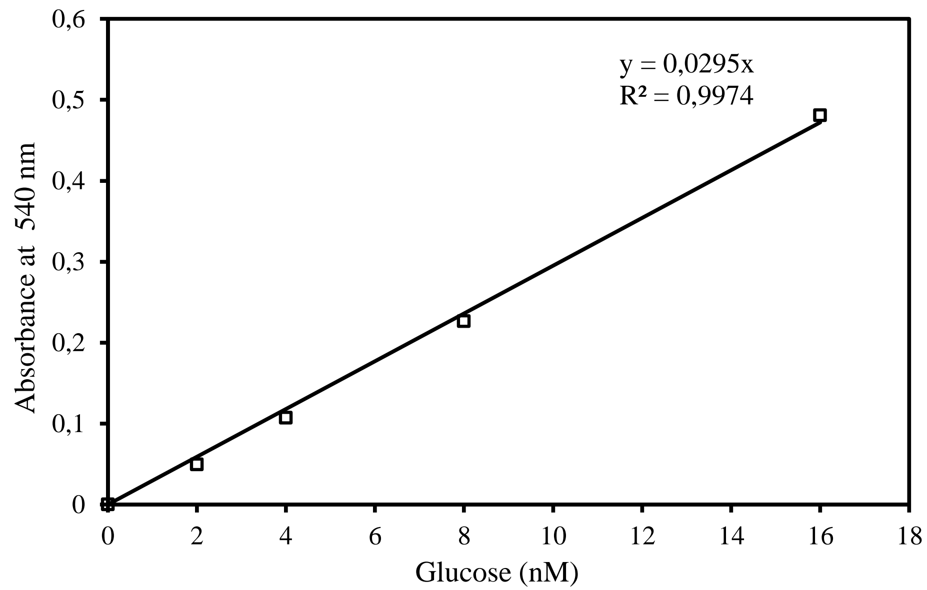


Figure A.5 Calibration curve of standard glucose using *o*-dianiside as a co-substrate.

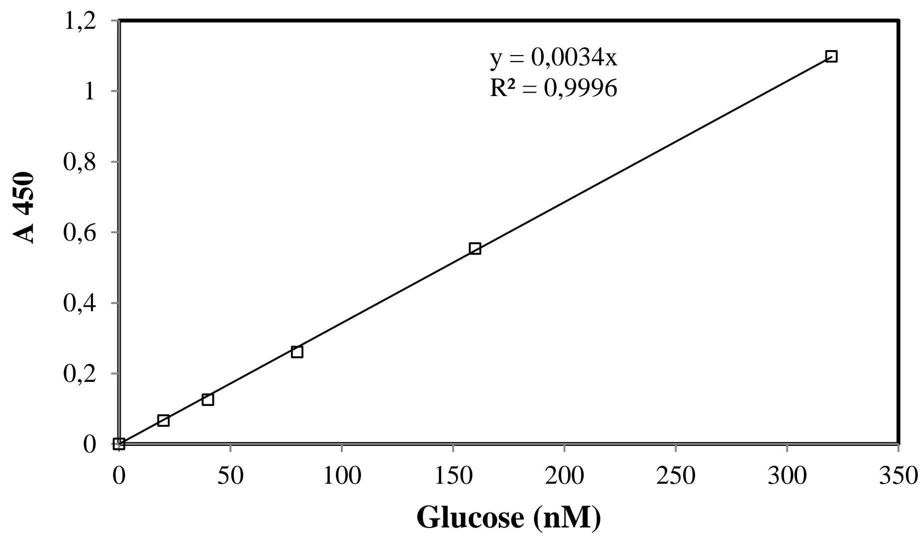


Figure A.6 Calibration curve of standard glucose using DAB as a co-substrate.

APPENDIX B

B1: Partial 16s rDNA nucleotide sequence of CM1. The percentage of query cover was 100% and gene identical was 99% comparison with *Enterococcus* sp.

A C G G C T C A C C A A G G C A A C G A T G C A T A G C C G A C C T G A
G A G G G T G A T C G G C C A C A C T G G G A C T G A G A C A C G G C C
C A G A C T C C T A C G G G A G G C A C A G C T A T G G A T A C T T C G G
C A A T G G A C G A A A G T C T G A C C G A G C A A C G C C G C G T G A
G T G A A G A A G G T T T T C G G A T C G T A A A A C T C T G T T G T T A
G A G A A G A A C A A G G A T G A G A G T A A A A T G T T C A T C C C T T
G A C G G T A T C T A A C C A G A A A G C C A C G G C T A A C T A C G T G
C C A G C A G C C G C G G T A A T A C G T A G G T G G C A A G C G T T G T
C C G G A T T T A T T G G G C G T A A A G C G A G C G C A G G C G G T T T
C T T A A G T C T G A T G T G A A A G C C C C C G G C T C A A C C G G G G
A G G G T C A T T G G A A A C T G G G A G A C T T G A G T G C A G A A G
A G G A G A G T G G A A T T C C A T G T G T A G C G G T G A A A T G C G T
A G A T A T A T G G A G G A A C A C C A G T G G C G A A G G C G G C T C
T C T G G T C T G T A A C

B2: Partial 16s rDNA nucleotide sequence of CM4. The percentage of query cover was 100% and gene identical was 95% comparison with *Enterococcus* sp.

T A A C G G C A C A C C T G G G C A A C C A T G C G C A G C C G A C C T G
A T A G G G A G A T C A G C C A C A C T G T T A C T G A G A C A C G T G A
C A C A C A C A T A C G C G A G G G A A C A G A C C T A C A T A C T T C G
G C A A T G G A C G A A A G T C T G A C C G A G C A A C G C C G C G T G
A G T G A A G A A G G T T T T C G G A T C G T A A A A C T C T G T T G T T

A G A G A A G A A C A A G G A T G A G A G T A A A A T G T T C A T C C C
T T G A C G G T A T C T A A C C A G A A A G C C A C G G C T A A C T A C G
T G C C A G C A G C C G C G G T A A T A C G T A G G T G G C A A G C G T T
G T C C G G A T T T A T T G G G C G T A A A G C G A G C G C A G G C G G T
T T C T T A A G T C T G A T G T G A A A G C C C C C G G C T C A A C C G G
G G A G G G T C A T T G G A A A C T G G G A G A C T T G A G T G C A G A
A G A G G A G A G T G G A A T T C C A T G T G T A G C G G T G A A A T G
C G T A G A T A T A T G G A G G A A C A C C A G T G G C G A A G G C G G
C T C T C T G G T C T G T A A C T G A C G C T

B3: Partial 16s rDNA nucleotide sequence of TN1. The percentage of query cover and gene identical were100% comparison with *Bacillus* sp.

G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C C A T A
A G A C T G G G A T A A C T C C G G G A A A C C G G G G C T A A T A C C
G G A T A A C A T T T T G A A C C G C A T G G T T C G A A A T T G A A A G
G C G G C T T C G G C T G T C A C T T A T G G A T G G A C C C G C G T C G
C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C A
A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G G C C A C
A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G
G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T
G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G C T T T C G
G G T C G T A A A A C T C T G T T G T T A G G G A A G A A C A A G T G C T
A G T T G A A T A A G C T G G C A C C T T G A C G G T A C C T A A C C A G
A A A G C C A C G G C T A A C T A C G T G C C A G C A G C C G C G G T A
A T A C G T A G G T G G C A A G C G T T A T C C G G A A T T A T T G G G C
G T A A A G C G C G C G C A G G T G G T T T C T T A A G T C T G A T G T G
A A A G C C C A C G G C T C A A C C G T G G A G G G T C A T T G G A A A
C T G G G A G A C T T

B4: DNA sequence of recombinant *BT_3567* gene presented in pBT_3567His.

```
         10        20        30        40        50        60
....|....|....|....|....|....|....|....|....|....|....|....|
ATGGGCATGATAAATAAGAAAATATTTTTTTCATTTCTACTGCTCACTGCCGGCTTCCTG

         70        80        90       100       110       120
....|....|....|....|....|....|....|....|....|....|....|....|
AGTGCGGCAGCGCAGAAATCGCCGCAGGACATGGACCGTTTCATCGACGCACTGATGAAG

        130       140       150       160       170       180
....|....|....|....|....|....|....|....|....|....|....|....|
AAGATGACTGTGGAAGAAAAGATAGGGCAACTGAACCTCCCCGTCGCAGGAGAGATCACT

        190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|
ACAGGACAAGCCAAAAGCAGTGACATCGCCGCCAAAATAAAACGGGGCGAAGTGGGCGGC

        250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|
CTGTTCAATCTGAAAGGAGTAGAGAAAATACGTGATGTACAGAAACAGGCAGTAGAACAA

        310       320       330       340       350       360
....|....|....|....|....|....|....|....|....|....|....|....|
TCCCGTCTGGGTATCCCCTTGCTGTTTGGTATGGATGTCATCCACGGATACGAAACAATG

        370       380       390       400       410       420
....|....|....|....|....|....|....|....|....|....|....|....|
TTCCCCATTCCGTTAGGACTGTCCTGTACCTGGGATATGACCGCCATTGAAGAATCGGCA

        430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|
CGCATTGCAGCCATAGAGGCCAGTGCCGACGGTATCTCATGGACATTCAGCCCGATGGTG

        490       500       510       520       530       540
....|....|....|....|....|....|....|....|....|....|....|....|
GACATCTCACGCGACCCACGTTGGGGACGTGTTTCCGAAGGCAGCGGTGAAGACCCGTTT
```

```
           550       560       570       580       590       600
....|....|....|....|....|....|....|....|....|....|....|....|
CTCGGAGCAATGATTGCCGAAGCAATGATACTCGGTTATCAGGGAAAGAATATGCAGCGG

           610       620       630       640       650       660
....|....|....|....|....|....|....|....|....|....|....|....|
AATGACGAAATCATGGCCTGCGTAAAACACTTTGCACTGTATGGCGCAGGGGAAGGCGGA

           670       680       690       700       710       720
....|....|....|....|....|....|....|....|....|....|....|....|
CGCGACTACAACACGGTAGACATGAGCCGCCAGCGGATGTTCAATGAATACATGCTGCCC

           730       740       750       760       770       780
....|....|....|....|....|....|....|....|....|....|....|....|
TACGAAGCTGCTGTTGAAGCCGGTGTAGGAAGTGTGATGGCATCGTTCAACGAAGTAGAC

           790       800       810       820       830       840
....|....|....|....|....|....|....|....|....|....|....|....|
GGTGTGCCTGCTACCGCCAACAAGTGGCTGATGACAGACGTACTGCGTGGTCAGTGGGGC

           850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|
TTCAACGGATTTGTAGTAACCGACTACACCGGAATCTCTGAAATGATCGACCACGGTATA

           910       920       930       940       950       960
....|....|....|....|....|....|....|....|....|....|....|....|
GGCGACCTGCAAACAGTTTCCGCACGTGCCATCAATGCCGGAGTAGACATGGATATGGTA

           970       980       990       1000      1010      1020
....|....|....|....|....|....|....|....|....|....|....|....|
AGCGAGGGCTTTGTCGGCACACTCAAAAAGTCTGTTCAGGAAGGCAAGGTTTCTATGGCA

           1030      1040      1050      1060      1070      1080
....|....|....|....|....|....|....|....|....|....|....|....|
ACACTGAATACCGCCTGCCGCCGTATTCTGGAAGCTAAATACAAACTGGGATTATTTGAC

           1090      1100      1110      1120      1130      1140
....|....|....|....|....|....|....|....|....|....|....|....|
AACCCCTACAAATATTGCGACCCGAAACGTCCTGCACGTGACATCTTTACGAAAGCGCAT
```

```
        1150      1160      1170      1180      1190      1200
....|....|....|....|....|....|....|....|....|....|....|....|
CGCGACGCTGCCCGCAGAATTGCCGCAGAAAGTTTTGTGCTTTTAAAGAACGACGACGTG

        1210      1220      1230      1240      1250      1260
....|....|....|....|....|....|....|....|....|....|....|....|
ACACTCATTCCGGGAACTCCTGCGGAACCTCTTCTTCCCTTCAATCCGAAAGGTAACATC

        1270      1280      1290      1300      1310      1320
....|....|....|....|....|....|....|....|....|....|....|....|
GCCGTGATAGGCCCGCTCGCCGACAGCCGGACAAACATGCCCGGTACATGGAGCGTAGCT

        1330      1340      1350      1360      1370      1380
....|....|....|....|....|....|....|....|....|....|....|....|
GCCGTACTCGACCGGTGTCCGTCGTTGGTAGAAGGACTGAAAGAAATGACTGCCGGAAAA

        1390      1400      1410      1420      1430      1440
....|....|....|....|....|....|....|....|....|....|....|....|
GCCAATATTCTTTATGCCAAAGGCAGCAATCTGATCAGTGACGCATCCTACGAAGAACGT

        1450      1460      1470      1480      1490      1500
....|....|....|....|....|....|....|....|....|....|....|....|
GCCACCATGTTCGGACGTTCGCTGAACCGTGATAACCGGACAGACCAGCAACTGCTGGAT

        1510      1520      1530      1540      1550      1560
....|....|....|....|....|....|....|....|....|....|....|....|
GAAGCATTGACAGTAGCCAACCAGTCGGATATCATCATCGCCGCCCTCGGAGAGTCATCC

        1570      1580      1590      1600      1610      1620
....|....|....|....|....|....|....|....|....|....|....|....|
GAAATGAGCGGAGAAAGCAGCAGCCGTACCGACCTGAACATTCCGGATGTACAGCAAAAC

        1630      1640      1650      1660      1670      1680
....|....|....|....|....|....|....|....|....|....|....|....|
CTGTTGAAGGAATTGCTGAAAACCGGGAAACCCGTTGTATTGGTATTATTTACAGGCCGT
```

```
      1690      1700      1710      1720      1730      1740
....|....|....|....|....|....|....|....|....|....|....|....|
CCGCTGACACTGACTTGGGAGCAGGAGCATGTACCCGCCATTCTGAATGTATGGTTCGGA

      1750      1760      1770      1780      1790      1800
....|....|....|....|....|....|....|....|....|....|....|....|
GGCAGCGAAGCTGCTTATGCCATTGGCGACGCGCTCTTCGGATATGTCAATCCGGGTGGA

      1810      1820      1830      1840      1850      1860
....|....|....|....|....|....|....|....|....|....|....|....|
AAACTGACCATGAGCTTCCCGAAGAATGTCGGTCAGATTCCGCTGTATTATGCGCATAAG

      1870      1880      1890      1900      1910      1920
....|....|....|....|....|....|....|....|....|....|....|....|
AATACAGGACGTCCGCTGGCACAAGGCAAATGGTTCGAAAAGTTCCGCAGCAATTATCTG

      1930      1940      1950      1960      1970      1980
....|....|....|....|....|....|....|....|....|....|....|....|
GATGTAGACAACGAACCGCTTTATCCATTCGGATACGGACTTTCTTACACGACCTTCTCC

      1990      2000      2010      2020      2030      2040
....|....|....|....|....|....|....|....|....|....|....|....|
TACGGGGATATCGACCTGAGCCGTTCTACCATCGATATGACGGGAGAGCTGACGGCAGCA

      2050      2060      2070      2080      2090      2100
....|....|....|....|....|....|....|....|....|....|....|....|
GTAATGGTAACCAATACCGGGACATGGCCGGGATCAGAAGTAGTGCAACTCTACATTCGG

      2110      2120      2130      2140      2150      2160
....|....|....|....|....|....|....|....|....|....|....|....|
GATCTTGTCGGCAGTACCACCCGTCCGGTGAAAGAACTGAAAGGCTTTCAGAAGATATTC

      2170      2180      2190      2200      2210      2220
....|....|....|....|....|....|....|....|....|....|....|....|
CTCGAACCGGGACAGTCAGAGATTGTACGGTTCAAGATTGCACCGGAAATGCTGAGGTAT
```

```
             2230      2240      2250      2260      2270      2280
....|....|....|....|....|....|....|....|....|....|....|....|
TACAACTACGACCTGCAACTGGTAGCAGAACCGGGTGAGTTTGAAGTAATGATCGGAACA

             2290      2300      2310      2320      2330      2340
....|....|....|....|....|....|....|....|....|....|....|....|....|.
AACAGCCGGGACGTGAAATCCGCACATTTCACGCTAAAAGAATTCCACCACCACCACCACTGA
```

B5: Amino acid sequence of recombinant BT_3567 presented in pBT_3567His. HIS210 (*) was proposed to be a proton donor in acid/base catalytic residues. Aspartate found in residue 288 (**) was thought to be a nucleophile residue.

```
        10        20        30        40        50        60        70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MGMINKKIFFSFLLLTAGFLSAAAQKSPQDMDRFIDALMKKMTVEEKIGQLNLPVAGEITTGQAKSSDIA

        80        90       100       110       120       130       140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AKIKRGEVGGLFNLKGVEKIRDVQKQAVEQSRLGIPLLFGMDVIHGYETMFPIPLGLSCTWDMTAIEESA
                                                                     *
       150       160       170       180       190       200       210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RIAAIEASADGISWTFSPMVDISRDPRWGRVSEGSGEDPFLGAMIAEAMILGYQGKNMQRNDEIMACVKH

       220       230       240       250       260       270       280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FALYGAGEGGRDYNTVDMSRQRMFNEYMLPYEAAVEAGVGSVMASFNEVDGVPATANKWLMTDVLRGQWG

       **
       290       300       310       320       330       340       350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FNGFVVTDYTGISEMIDHGIGDLQTVSARAINAGVDMDMVSEGFVGTLKKSVQEGKVSMATLNTACRRIL

       360       370       380       390       400       410       420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
EAKYKLGLFDNPYKYCDPKRPARDIFTKAHRDAARRIAAESFVLLKNDDVTLIPGTPAEPLLPFNPKGNI

       430       440       450       460       470       480       490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AVIGPLADSRTNMPGTWSVAAVLDRCPSLVEGLKEMTAGKANILYAKGSNLISDASYEERATMFGRSLNR

       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DNRTDQQLLDEALTVANQSDIIIAALGESSEMSGESSSRTDLNIPDVQQNLLKELLKTGKPVVLVLFTGR

       570       580       590       600       610       620       630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PLTLTWEQEHVPAILNVWFGGSEAAYAIGDALFGYVNPGGKLTMSFPKNVGQIPLYYAHKNTGRPLAQGK
```

```
         640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
WFEKFRSNYLDVDNEPLYPFGYGLSYTTFSYGDIDLSRSTIDMTGELTAAVMVTNTGTWPGSEVVQLYIR

         710       720       730       740       750       760       770
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DLVGSTTRPVKELKGFQKIFLEPGQSEIVRFKIAPEMLRYYNYDLQLVAEPGEFEVMIGTNSRDVKSAHF

         780
....|....|.
TLKEFHHHHHH
```

APPENDIX C

C1: Identification of internal peptides obtained from tryptic-digested recombinant BT_3567 by LC MALDI MS/MS.

Observed m/z	Mr (expt)	Mr (calc)	Ions score	Expect Peptide
491.2988	980.5830	980.5365	22	R.FIDALMKK.M
604.0398	1809.0975	1808.9996	71	K.IGQLNLPVAGEITTGQAK.S
595.3685	1188.7225	1188.6615	11	K.RGEVGGLFNLK.G
548.2718	1094.5291	1094.4736	30	R.NDEIMACVK.H
617.8327	1233.6508	1233.5891	28	K.HFALYGAGEGGR.D
558.7633	1115.5121	1115.4553	29	R.DYNTVDMSR.Q
525.3036	1048.5926	1048.5376	24	K.WLMTDVLR.G
738.7388	2213.1947	2213.0708	27	R.AINAGVDMDMVSEGFVGTLKK.S
620.3264	1238.6382	1238.5747	25	K.VSMATLNTACR.R
453.2671	1356.7796	1356.7078	40	K.YKLGLFDNPYK.Y
533.8106	1065.6067	1065.5495	35	K.LGLFDNPYK.Y
545.8567	1089.6989	1089.6434	13	R.IAAESFVLLK.N
750.1084	2247.3035	2247.1787	52	K.NDDVTLIPGTPAEPLLPFNPK.G
641.8939	1281.7732	1281.7041	52	K.GNIAVIGPLADSR.T
480.9173	1439.7302	1439.6528	66	K.GSNLISDASYEER.A
805.9859	1609.9572	1609.8	21	R.TDLNIPDVQQNLLK.E
420.2408	838.4	838.4259	46	K.LTMSFPK.N
468.2776	1401.8110	1401.7405	50	K.NVGQIPLYYAHK.N
586.8641	1171.7135	1171.6561	48	R.DLVGSTTRPVK.E
694.4201	1386.8257	1386.7507	87	K.IFLEPGQSEIVR.F
560.8401	1119.6656	1119.6110	25	R.FKIAPEMLR.Y
423.2518	844.4890	844.4476	34	K.IAPEMLR.Y

C2: Amino acid sequence of rBT_3567 and matched peptide analysis. Matched peptides shown in underline. The protein sequence coverage was 32%.

M G M I N K K I F F S F L L L T A G F L S A A A Q K S P Q D M D R F I D A L M K K M T
V E E K I G Q L N L P V A G E I T T G Q A K S S D I A A K I K R G E V G G L F N L K G V
E K I R D V Q K Q A V E Q S R L G I P L L F G M D V I H G Y E T M F P I P L G L S C T W
D M T A I E E S A R I A A I E A S A D G I S W T F S P M V D I S R D P R W G R V S E G S
G E D P F L G A M I A E A M I L G Y Q G K N M Q R N D E I M A C V K H F A L Y G A G
E G G R D Y N T V D M S R Q R M F N E Y M L P Y E A A V E A G V G S V M A S F N E V
D G V P A T A N K W L M T D V L R G Q W G F N G F V V T D Y T G I S E M I D H G I G
D L Q T V S A R A I N A G V D M D M V S E G F V G T L K K S V Q E G K V S M A T L N
T A C R R I L E A K Y K L G L F D N P Y K Y C D P K R P A R D I F T K A H R D A A R R I
A A E S F V L L K N D V T L I P G T P A E P L L P F N P K G N I A V I G P L A D S R T N
M P G T W S V A A V L D R C P S L V E G L K E M T A G K A N I L Y A K G S N L I S D A
S Y E E R A T M F G R S L N R D N R T D Q Q L L D E A L T V A N Q S D I I I A A L G E S
S E M S G E S S S R T D L N I P D V Q Q N L L K E L L K T G K P V V L V L F T G R P L T
L T W E Q E H V P A I L N V W F G G S E A A Y A I G D A L F G Y V N P G G K L T M S F
P K N V G Q I P L Y Y A H K N T G R P L A Q G K W F E K F R S N Y L D V D N E P L Y P
F G Y G L S Y T T F S Y G D I D L S R S T I D M T G E L T A A V M V T N T G T W P G S
E V V Q L Y I R D L V G S T T R P V K E L K G F Q K I F L E P G Q S E I V R F K I A P E
M L R Y Y N Y D L Q L V A E P G E F E V M I G T N S R D V K S A H F T L K E F H H H H
H H

APPENDIX D

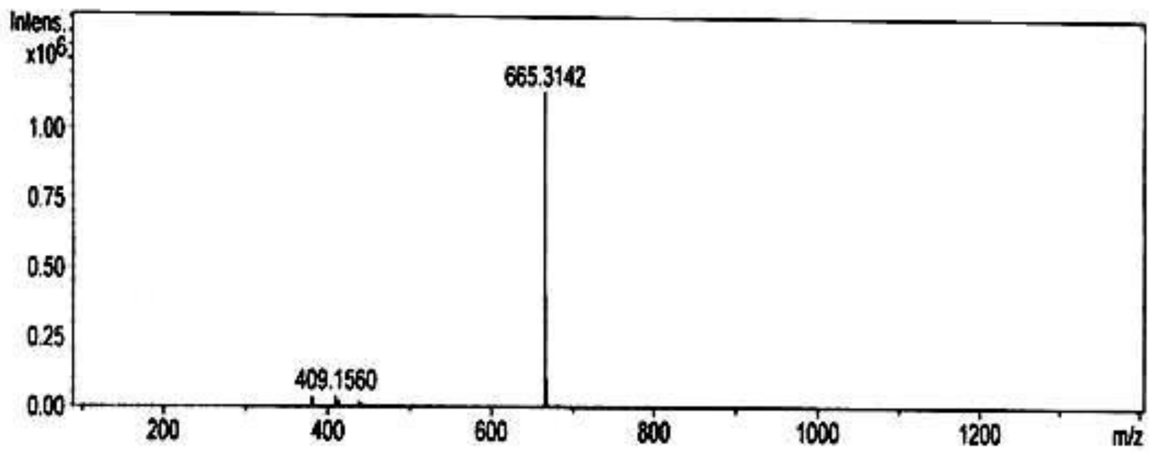


Figure D.1 Mass spectrum report of rubusoside.

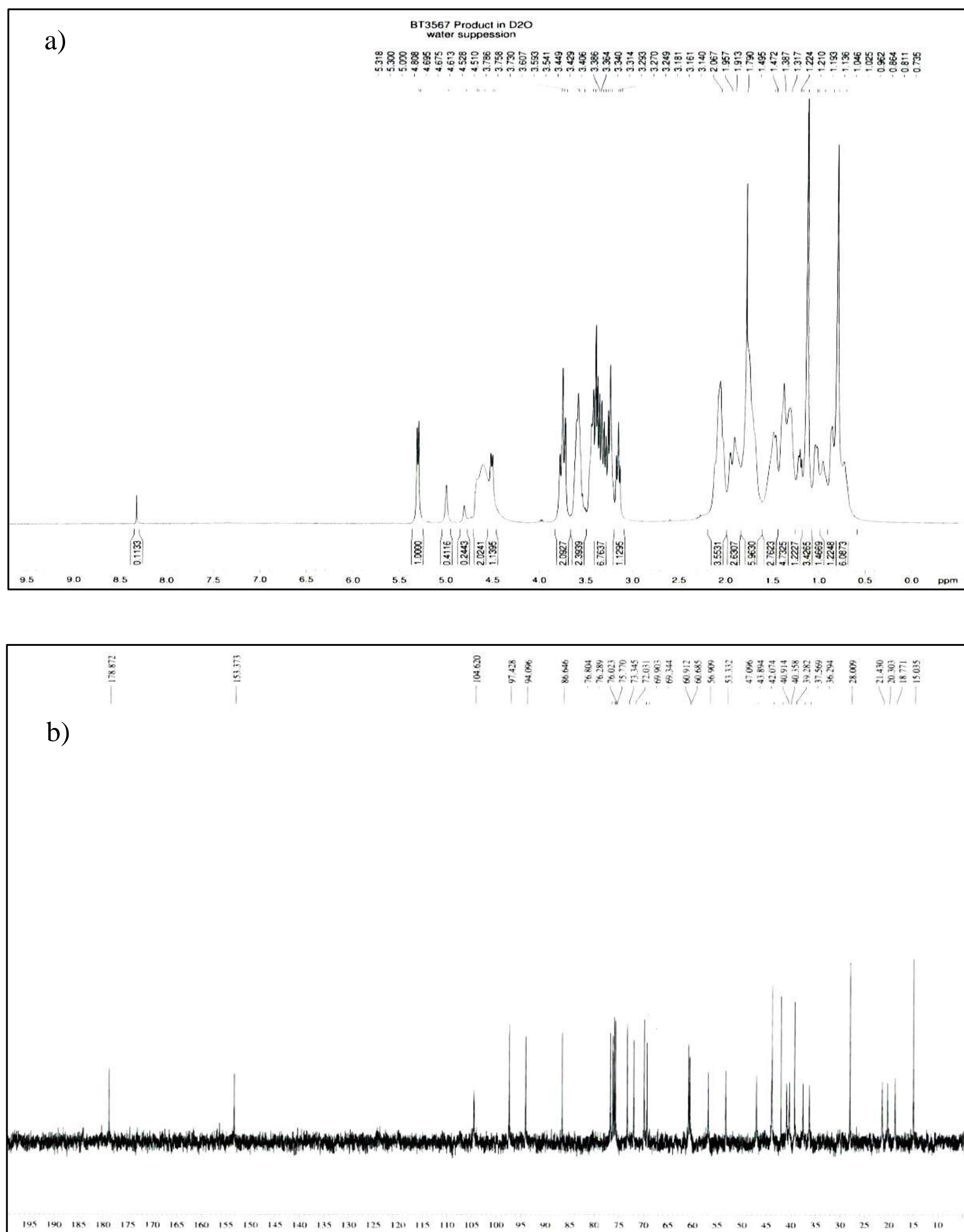


Figure D.2 The NMR spectrum of rubusoside. a) ^{1}H NMR spectrum. b) ^{13}C NMR spectrum.

BIOGRAPHY

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PUBLICATION	Somsiri Udompaisarn, Dumrongkiet Arthan and Jamorn Somana. Development and validation of an enzymatic method to determine stevioside content from *Stevia rebaudiana.* Journal of Agricultural Food Chemistry 2017, 65 (15), 3223-3229. DOI: 10.1021/acs.jafc.6b05793