ISOLATION AND CHARACTERIZATION OF BIOCATALYST DIGESTING STEVIOSIDE

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ABSTRACT

Enterococcus casseliflavus BO2, isolated from microbial contaminant of stevia leaf extract, was found to catalyze hydrolysis of steviol glycosides, sweet components of *Stevia rebaudiana* Bertoni (stevia) leaves. Previous studies, however, have shown that enterococci bacteria have no activity in the metabolism of steviol glycosides as well as rarely constructed recombinant steviol glycosides hydrolyzing enzyme. In this study, to ascertain *E. casseliflavus* BO2 responsible for this role, the β-glucosidase candidate genes were cloned and expressed in *Escherichia coli* with C-terminal His_6-tagged form. An EcBgl4 presented both *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and stevioside hydrolysis activity. The gene sequence analysis indicated that EcBgl 4 was a member of GH 3 glycosyl hydrolase β-glucosidase consisting of 721 amino acids, and corresponding to a molecular mass of 79.37 kDa. EcBgl 4 performed the highest functionally active form upon induction with 0.2 mM IPTG at 25°C and overnight. The protein was purified to homogeneity and the biochemical properties were characterized. Both crude extract and purified form of EcBgl 4 specifically hydrolyzed the glucose moiety of stevioside to produce rubusoside. The purified EcBgl 4 exhibited an optimum pH and temperature at 6.0 and 37°C, respectively against both *p*NPG and stevioside. Kinetic constants, k_{cat}/K_m for *p*NPG and k_{cat}/K_m for stevioside were calculated to be 8583 $mM^{-1}s^{-1}$ for and 95.41 $mM^{-1}s^{-1}$, respectively, indicating that EcBgl 4 hydrolyzed *p*NPG had more efficiency than stevioside. Importantly, EcBgl 4 was found to be the highest efficient enzyme that catalyzed both *p*NPG and stevioside compared to the stevioside hydrolyzing β-glycosidases previously reported. The enzyme showed substantial activity towards amygdalin and also hydrolyzed natural saccharides but no hydrolytic activity was observed on tested aryl-glycoside synthetic substrates. The results revealed that *E. casseliflavus* BO2 indeed possessed stevioside hydrolyzing activity via a novel β-glucosidase, which also served as a potential enzyme for future applications.

KEY WORDS: STEVIOL GLYCOSIDES / STEVIOSIDE / RUBUSOSIDE / β-GLUCOSIDASE / *ENTEROCOCCUS CASSELIFLAVUS*

90 pages

การจำแนกและศึกษาคุณลักษณะของสารย่อยสตีวิโอไซด์

ISOLATION AND CHARACTERIZATION OF BIOCATALYST DIGESTING STEVIOSIDE

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บทคัดย่อ

Enteroflavus cassiliflavus BO2 ได้จากการแยกเชื้อปนเปื้อนในน้ำใบหญ้าหวานสกัด สามารถย่อยสตีวิออลไกลโคไซด์ซึ่งเป็นสารหวานในใบหญ้าหวาน *Stevia rebaudiana* Bertoni แม้ว่าการศึกษาก่อนหน้าระบุว่าแบคทีเรียกลุ่มนี้ ไม่เกี่ยวข้องกับการเมทาบอลิซึมของสตีวิออลไกลโคไซด์ในลำไส้ และการผลิตรีคอมบิแนนต์เอนไซม์ที่ย่อยสารนี้มีน้อยมาก เพื่อทดสอบว่า *E. cassiliflavus* BO2 ย่อยสตีวิออลไกลโคไซด์ได้จริง การศึกษานี้ได้โคลนและเหนี่ยวนำการแสดงออกของยีนเบต้า-กลูโคซิเดส จาก *E. cassiliflavus* BO2 ในรูปแบบที่มี His_6-tagged ที่ปลาย C-terminus ใน *E. coli* พบว่าโปรตีน EcBgl 4 ที่แสดงออกสามารถย่อย *p*NPG และสตีวิโอไซด์ จากการวิเคราะห์ลำดับกรดอะมิโนพบว่า EcBgl 4 ประกอบด้วยกรดอะมิโน 716 ตัว มีขนาดโมเลกุล 79.37 กิโลดาลตัน และเป็นเอนไซม์ในกลุ่ม GH 3 ไกลโคซิลไฮโดรเลสเบต้ากลูโคซิเดส เอนไซม์นี้สร้างได้ดีที่สุดใน *E .coli* เมื่อถูกกระตุ้นการแสดงออกของยีนโดยใช้ IPTG 0.2 มิลลิโมลาร์ และเลี้ยงเซลล์ไว้หนึ่งคืนที่อุณหภูมิ 25 องศาเซลเซียส ทั้งเอนไซม์ที่ยังไม่บริสุทธิ์และบริสุทธิ์แล้วมีความจำเพาะในการย่อยกลูโคสจากสตีวิโอไซด์ได้เป็นรูบู-โซไซด์ เอนไซม์ที่บริสุทธิ์แล้วมีความสามารถย่อยสูงสุดต่อทั้ง *p*NPG และสตีวิโอไซด์ ที่ pH 6 และอุณหภูมิ 37 องศาเซลเซียส ผลการศึกษาจลศาสตร์ของเอนไซด์ต่อ *p*NPG และสตีวิโอไซด์ ได้ค่า k_{cal}/K_m เท่ากับ 8583 และ 95.41 มิลลิโมลาร์$^{-1}$ วินาที$^{-1}$ ตามลำดับ ซึ่งแสดงให้เห็นว่าเอนไซม์ย่อย *p*NPG ได้อย่างมีประสิทธิภาพมากกว่าย่อยสตีวิโอไซด์ และยังเป็นเอนไซม์ที่มีประสิทธิภาพสูงสุดในการย่อย *p*NPG และสตีวิโอไซด์เมื่อเทียบกับเอนไซม์อื่นที่ถูกรายงานก่อนหน้า นอกจากนี้ยังพบว่าเอนไซม์สามารถย่อย amygdalin และสารตั้งต้นตามธรรมชาติอื่น แต่ไม่ย่อยสารตั้งต้นสังเคราะห์ที่นำมาทดสอบได้ ดังนั้นผลการศึกษานี้จึงชี้ว่า *E. cassiliflavus* BO2 สามารถย่อยสตีวิโอไซด์ได้จริงโดยใช้เอนไซม์เบต้า-กลูโคซิเดส และเอนไซม์นี้ยังมีคุณสมบัติเป็นเอนไซม์เบต้า-กลูโคซิเดสตัวใหม่ที่น่าจะนำไปประยุกต์ใช้ได้เป็นอย่างดีในอนาคต

90 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

A	absorbance
α	alpha
bp	base pair
β	beta
BSA	Bovine serum albumin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
°C	degree Celsius
EcBgl 1	*Enterococcus casseliflavus* β-glucosidase 1
EcBgl 2	*Enterococcus casseliflavus* β-glucosidase 2
EcBgl 3	*Enterococcus casseliflavus* β-glucosidase 3
EcBgl 4	*Enterococcus casseliflavus* β-glucosidase 4
g	gravity acceleration
h	hour
HPLC	high pressure liquid chromatography
kb	kilobase pair
k_{cat}	catalytic efficiency
kDa	kiloDalton
K_m	Michaelis-Menten constant
L	liter (s)
LB	lysogenic broth
µg	microgram
µl	microliter
µM	micromolar
min	minute
MW	molecular weight
µg	microgram

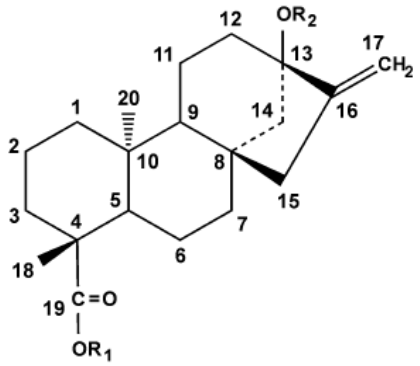
LIST OF ABBREVIATIONS (cont.)

Ni-NTA	Nickel-nitrilotriacetic acid
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
*p*NPG	*p*-nitrophenyl-β-D-glucopyranoside
*p*NP	*p*-nitrophenol
RA	rebaudioside A
rRNA	ribosomal ribonucleic acid
RU	rubusoside
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sp	species
SV	stevioside
TE	Tris-EDTA buffer
V/cm	Volt per centimeter
TLC	thin-layer chromatography
TSB	tryptic soy broth
U	unit (s)
v_0	initial velocity
V_{max}	maximum velocity
v/v	volume per volume
w/v	weight per volume

CHAPTER I
INTRODUCTION

1.1 Steviol glycosides

Steviol glycosides or steviosides are natural sweetener with high-potency non-calories presented in leaves tissue of *Stevia Rebaudiana* Bertoni (stevia). The steviol glycosides are a group of diterpene glycoside consist of the same aglycone steviol backbone (*ent*-13-hydroxykaur-16-en-19-oic acid) and differ in sugar residues. The backbone has two hydroxyl groups bound by glucose, rhamnose and/or, xylose, one attached to the C-19 of the C-4 carboxyl (R_1) and the other attached to the C-13 (R_2). Both of these positions can be further glycosylated at the 13-O-glucose of the C-2' and C-3' to produce the distinct steviol glycosides identified as stevioside, steviolbioside, rebaudioside A, B, C, D, F and dulcoside A common accumulation in the leaves (Brandle, Starratt, & Gijzen 1998; Swati et al., 2010). Structures of these compounds are given in Figure 1.1. However, occurred by occasionally hydrolyze might be artifacts from the extraction procedure caused steviolbioside and rebaudioside B but not naturally found as constituents of stevia (Kenelly, 2002; Geuns, 2003; Prakash, Dubois, Clos, Wilkens, & Fosdick, 2008). In addition, depending on growth conditions and mainly on stevia cultivar, their content varies between 4 and 20% of the dry leaf weight (Geuns, 2003). Details of steviol glycosides are listed in table 1.1. Typical proportion of glycosides found in the dry leaf is 4–13% w/w stevioside, followed by 2–4% w/w rebaudioside A, 1–2% w/w rebaudioside C, and 0.4–0.7% w/w dulcoside A (Makapugay, Nanayakkara, & Kinghorn, 1984). The minor constituents, steviolbioside, rebaudioside B, D, E and F are also presented in the leaf extracts (Geuns, 2003). On the other hand, high purity stevioside (>80%) or rebaudioside A (>90%) are obtained from purified of the leaf extracts as well as the commercially products (Gardana, Scaglianti, & Simonetti, 2010).



Structure name	R_1	R_2
Steviol	H	H
Steviolmonoside	H	*β*-Glc
Rubusoside	*β*-Glc	*β*-Glc
Steviolbioside	H	*β*-Glc- *β*-Glc(2→1)
Stevioside	*β*-Glc	*β*-Glc- *β*-Glc(2→1)
Rebaudioside A	*β*-Glc	*β*-Glc- *β*-Glc(2→1) | *β*-Glc(3→1)
Rebaudioside B	H	*β*-Glc- *β*-Glc(2→1) | *β*-Glc(3→1)
Rebaudioside C (Dulcoside B)	*β*-Glc	*β*-Glc-α-Rha(2→1) | *β*-Glc(3→1)
Rebaudioside D	*β*-Glc- *β*-Glc(2→1)	*β*-Glc- *β*-Glc(2→1) | *β*-Glc(3→1)
Rebaudioside F	*β*-Glc	*β*-Glc- *β*-Xyl(2→1) | *β*-Glc(3→1)
Dulcoside A	*β*-Glc	*β*-Glc-α-Rha(2→1)

Figure 1.1. Structures of steviol aglycone and the steviol glucosides. Glc, Xyl, and Rha represent, glucose, xylose, and rhamnose sugar moieties, respectively.

As a result of differential glucosylated counterparts as mentioned before, the steviol glycosides have distinctive properties, sweetness and taste quality (de Oliveira, Packer, Chimelli, & de Jesus, 2007). For example, rebaudioside A which contains one more glucose residue than stevioside is 350-450-fold, while, stevioside is 250-300-fold more sweeter than sucrose by weight equivalent as well as more soluble in water, which allows a variety of formulations. (Bridei & Lavieille, 1931; Kohda, Kasai, Yamasaki, Murakami, & Tanaka 1976; Catharino & Santos, 2012; Montoro et al., 2013). Indeed, a significant less bitter aftertaste was found in pure rebaudioside A than pure stevioside, hence, it is judged to have the most desirable sensory attributes of the steviol glycosides (Phillips 1987; Tanaka 1997). Extracts with higher proportions of rebaudioside A have a much improved flavor profile as significantly reduced bitter aftertaste. Therefore, in order to improve and apply this source of natural sweeter as industrial scale, new characteristics of stevia with higher levels of rebaudioside A is a challenge object to research groups and industries concerned (Mondaca, Antonio Vega-Ga´lvez, Bravo & Hen, 2012).

1.2 Applications of steviol glycosides

1.2.1 Stevioside and rebaudioside A

Besides innutritious of the synthetic sweeteners such as aspartame, neotame, saccharin, acesulfame-K, and sucralose, some of them are potentially carcinogenic and are involved in many other diseases as well (Priya, Gupta & Srikanth, 2011). For these reasons with regardless to human health concern, naturally sugar substituents with similar characteristics and properties to those artificial has been investigated and interested. One of the most prominent compounds is the extract from the leaves of stevia, steviol glycosides which have been used as a natural sweetener substitute for those synthetic sweeteners and also as an alternative to sucrose. Actually, stevia has been used for centuries by the indigenous Guarany natives as a traditional sweetener (for herbal, yerba maté and other beverages) (Puri, Sharma & Tiwari, 2011). The commercial exploitation of stevia has significantly increased since 1970s, when the procedure of extraction and refining sweetener

compounds, steviol glycosides, were developed by several Japanese researchers (Dacome et al., 2005). Among the steviol glycosides, the main constituents are stevioside and rebaudioside A, which represents 90 wt.% of all sweet glycosides in the leaves (Bergs, Burghoff, Joehnck, Martin & Schembecker, 2012), they are non-metabolizable (non-caloric) as well as high potency (~300 times sweeter than sucrose) makes them attractive as sugar substitutes. Moreover, the advantages of using steviol glycosides as a dietary supplement are manifold: high solubility in water, heat-stable up to 200 °C, acid-stable and not fermentable. For several decades, they have been commercially used as sweeten in a wide range of products including food stuffs and beverages in Japan, Korea, China, South-East Asia and South America (Koyama et al., 2003). Since December 2008 when the FDA stated that purified rebaudioside A can be considered GRAS (Generally Regarded As Safe) status, it has been used to sweeten beverages and some foods (FDA GRAS Notice GRN 000253 and GRN 000252) (Mondaca, Antonio Vega-Ga´lvez, Bravo & Hen, 2012). As a result, in a number of industrial foods, currently, these steviol glycosides are used as a sweetener additive, such as soft drinks or fruit drinks, desserts, breads, and biscuits. They also replace saccharose in many foods, for example, pickles, yoghurt, candies, soju, soy sauce and seafoods (Koyama et al., 2003; Tadhani & Subhash, 2006; Wallin, 2007; Goyal, Samsher, & Goyal, 2010). Another notable benefit is available for both diabetics and PKU (Phenylketouria) patients, as well as obese people attempt to lose weight by avoiding sugar in the diet (Huxtable, 2002; Geuns et al., 2006). It has been found that stevioside and rebaudioside A show anti-inflammatory and diuretic properties (Toyoda et al., 1997), prevent ulceration of the gastrointestinal tract (Wingard et al., 1980; Kochikyan, Markosyan, Abelyan, Balayan, & Abelyan, 2006) and perform anti-hyperglycemic (Hsieh et al., 2003; Gregersen Jeppesen, Holst, & Hermansen, 2004; Feri et al., 2006) anti-hypertensive,anti-tumor, anti-diarrheal and immunomodulatory activities (Chatsudthipong & Muanprasat, 2009). In addition, steviol glycosides play a role in the reduction of blood cholesterol (Ateh et al., 2008) and enhance cell regeneration and blood clotting (Wingard et al., 1980; Maki et al., 2008). Their further properties have also been presented, for example antibacterial, antiamnesic and antiviral activities. The positive therapeutic effects have been exhibited in the treatment of

neuralgia, anemia, lumbago, rheumatism, eczema and dermatitis (Puri, Sharma, Barrow, & Tiwari, 2011). Hence, the aforementioned beneficial effects of these steviol glycosides implying that they are not only used as natural sweeteners, but also as active pharmaceutical agents.

1.2.2 Rubusoside

In contrast to the main constituents, rubusoside a rare component in the leaves extract provides more valuable which is approximately 10-fold more expensive than stevioside (Ko et al., 2013). Besides it is used as a sweetener with 114- fold sweeter than sucrose and it also acts as an excellent solubilizing agent. Rubusoside with its amphipathic ability to be a potent natural solubilizer that enhances the solubility of various pour water soluble pharmaceutically important compounds, such as paclitaxel, curcumin, capsaicin, cyclosporine, nystatin, erythromycin (Zhijun, 2010), liquiritin, teniposide, and etoposide (Nguyen et al., 2014; Zhang et al., 2011; 2012). Furthermore, rubusoside has been found to be a bioactive compound performs antiangiogenic activity and antiallergic activity (Koh, Chou, & Liu, 2009; Liu et al., 2006). Actually, it is produced primarily by extraction from *Rubus suavissimus* S. Lee, which known in China as tiancha or Chinese sweet tea (Koh, Chou, & Liu, 2009; Prakash Chaturvedula & Prakash, 2011). This tea plant, the main source for rubusoside production grows only in southern China with variable yearly yield depending on local climate resulting in economically impractical for large-scale production (Ko et al., 2012; Chou et al., 2009). Besides an analog of rubusoside, stevioside exists in stevia leaf is more abundant and can be specifically hydrolyzed to produce rubusoside.

1.2.3 Steviol

Steviol is the aglycone and the precursor of steviol glucosides first isolated from stevia (Bridel & Lavielle, 1931). The study of metabolism of steviol glycosides demonstrates that they are degraded finally to steviol by human intestinal microflora. It has been used as a pharmaceutical compound for the improvement of cognitive functions such as learning, memory, alertness, and psychotic stability (Fowler et al. 2009). Suppression of synthesis of pro-inflammatory cytokines

stimulated by both cytokine (TNF-α) and bacterial cell wall part, lipopolysaccharides by steviol as well as stevioside are found (Boonkaewwan & Burodom, 2013). Moreover, by altering the morphology of intestinal absorptive cells of steviol, glucose absorption is inhibited (Toskulkao, Sutheerawatananon, Wanichanon, Saitongdee, & Suttagit, 1995). Besides therapeutics used, steviol with gibberellins like activity is indicated as a plant growth regulator and also an important starting material of synthetic bioactive chemicals (Oliveira, Stiirmer, Filho, & Ayub, 2008).

Table 1.1 Steviol glycosides represent in *Stevia rebaudiana* Bertoni. The contents are defined as concentration in leaf (% w/w) and the relative sweetness is compared to sucrose.

Steviol glycosides	Molecular formular	Relative sweetness	Content	Reference
Stevioside	$C_{38}H_{60}O_{18}$	250-300	4-14	Briedel & Lavieille, 1931
Steviolbioside	$C_{32}H_{50}O_{13}$	100-125	< 0.4	Kohda et al., 1976
Rebaudioside A	$C_{44}H_{70}O_{23}$	350-450	2.0-4.0	idem
Rebaudioside B	$C_{38}H_{60}O_{18}$	300-350	<< 1.0	idem
Rebaudioside C	$C_{44}H_{70}O_{22}$	50-120	1.0-2.0	Sakamoto et al., 1997
Rebaudioside D	$C_{50}H_{80}O_{28}$	200-300	<< 1.0	Sakamoto et al., 1997
Rebaudioside E	$C_{44}H_{70}O_{23}$	250-300	<< 1.0	idem
Rebaudioside F	$C_{43}H_{68}O_{22}$	nd	<< 1.0	Brandle et al., 2002
Dulcoside A	$C_{38}H_{60}O_{17}$	50-120	0.4-0.7	Kobayashi et al., 1977

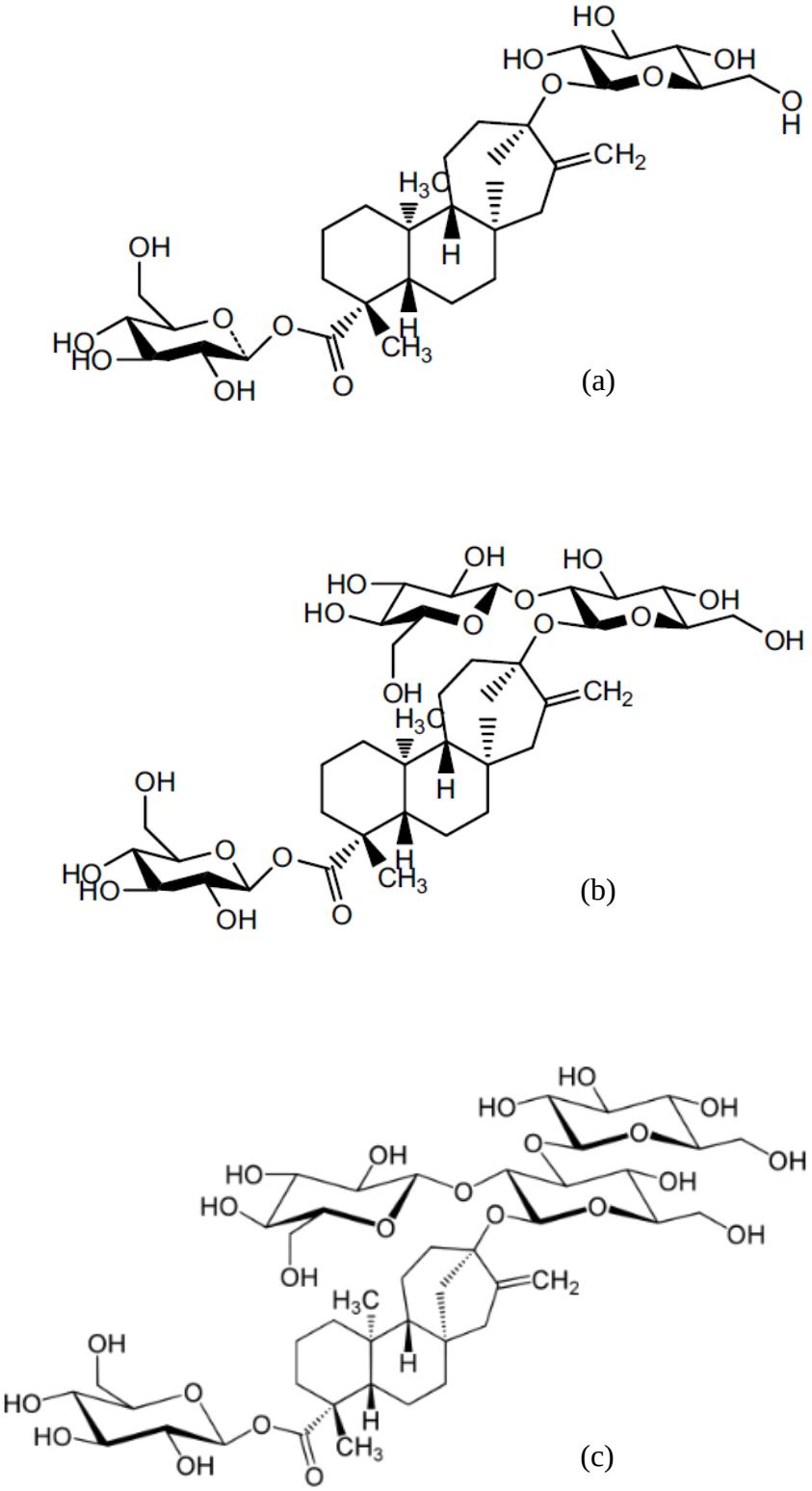


Figure 1.2 Steviosides used in this study, (a) rubusoside, (b) stevioside (c) rebaudioside A.

1.3 Steviol glycosides hydrolyzing enzymes

The demand for steviol glycosides has increased considerably as both health and application aspects, also they are recently known as the 'sweeteners of the future' (Esmat, Azza, & Ferial, 2010; Brahmachari, Mandal, Rajeev, Mondal, & Brahmachari, 2011; Mondaca, Antonio Vega-Ga´lvez, Bravo, & Hen, 2012; Rao Reddy, Ernala, Sridhar, & Ravikumar, 2012). Even the compound mainly obtained from the stevia plant, but it is limited in total area of cultivation as well as fluctuations in total productivity (Philippe et al., 2014). Hence, to reach the desirable quantity, many studies have tried to investigate the mass production of valuable steviol glycosides. With regard to the industrial used, some researchers have attempted to produce the rare component of steviol glycosides by chemical modification. For example, acid hydrolysis under extremely acidic conditions was used for produce steviol from stevioside (Kohoda, Kasai, Yamasaki, Murakami, & Tananka, 1976) together with alkaline hydrolysis of stevioside to steviol (Ogawa, Nozaki, & Matsui, 1980) and steviolbioside and rebaudioside B (Prakash Chaturvedula & Prakash, 2011). The chemical process requires a highly diluted system with a large excess of the expensive sodium periodate to overcome the useful yields lead to high cost investment and the environmental hazard. Consequently, it is not suitable for the large-scale production. Enzyme-based procedure, therefore, with considerable advantages over the former method, such as regioselectivity, time- and cost-effective, and eco-friendly was explored.

Structurally, stevioside comprises of three glycosidic bonds (an ester β-glucosidic linkage on the C-19 carboxyl group, *β*-linked sophorose, and *β*-1,2-D-glucopyranosyl on C-13) (Avent, Hansons, & Oliveira, 1990; Okamoto, Nakano, Yatake, Kiso, & Kitahata, 2000; Milagre, Martins, & Takahashi, 2009; Wehrli, 2011). In order to cleavage of an ester linkage at the C-19 esterases or lipases may be the first candidate, but till now the lipases containing this activity not yet reported (Chen et al., 2016) The next possible enzyme candidates may responsible for cleaving stevioside is glucosidase which involved in different regioselectivities in hydrolysis of glucosidic linkages. Steviol glycosides hydrolyzing enzymes have been achieved from different sources and proposed in detail including β-glucosidases, β-galactosidases, and lactase as listed in table 2. By mean of directly enzyme

purification several β-glucosidases hydrolyze steviol glycosides have been performed. β-glucosidases from *Clavibacter michiganense* (Nakano, Okamoto, Yatake, Kiso, & Kitahata, 1998) and *Flavobacterium johnsoniae* (Okamoto, Nakano, Yatake, Kiso, & Kitahata, 2000) hydrolyze the β-glucosidic linkage of the C-19 but not at the C-13 of rebaudioside A and stevioside. Interestingly, only these two enzymes able to hydrolyze rebaudioside A have been reported. Selective cleaving at the sophorosyl residue at C-13 of stevioside by β-glucosidase from *Chryseobacterium* sp (Jiang et al. 2011) and *Aspergillus aculeatus* (Ko et al. 2012) able to produce rubusoside. A purified β-glucosidase from *Penicillium decumbens* (Ko et al. 2013) converted stevioside to steviol by double cleave at the C-13 and C-19. Recently, the first β-glucosidase from *Streptocomyces* sp. GTX6 is successful cloned and expressed to produce rubusoside from stevioside (Wang et al. 2015). β-galactosidases also exhibit activity toward stevioside to produce rubusoside, steviol, and steviolbioside by *Aspergillus* sp. (Wan, Tao, Kim, & Xia, 2012), *Sulfolobus solfataricus* (Chen et al., 2014), and *Kluyveromyces lactis* (Chen et al., 2016) β-galactosidase, respectively. Unlike other stevioside hydrolyzing enzymes A b-galactosidase from *K. lactis* was found to specifically catalyze hydrolysis of the glycosyl ester linkage on the C-19 carboxyl group of stevioside to yield steviolbioside. All of these reported β-galactosidases however were purchased from commercially avialable enzymes. Moreover, recombinant lactase from *Thermus thermophilus* with the ability to converse stevioside to rubusoside was performed by synthesized the gene (Nguyen et al., 2014). Among those reported steviol glycosides hydrolyzing enzymes, not only β-glucosidases are the most extensively studied but yield a variety of products as well.

Table 1.2 Reported steviol glycosides hydrolyzing enzymes. S, steviol; SV, stevioside; RA, rebaudioside A; RU, rubusoside; SB, steviolbioside.

Enzymes	Organisms	Sources	Activities	References
β-glucosidase	*Clavibacter michiganense*	Purification	Cleave at C19 of steviosides	Nakano et al. ,1998
	Flavobacterium johnsoniae	Purification	Cleave at C19 of steviosides	Okamoto et al. 2000
	Chryseobacterium sp.	Purification	SV → RU	Jiang et al. 2011
	Aspergillus aculeatus	Purification	SV → RU	Ko et al. 2012
	Penicillium decumbens	Purification	SV → S	Ko et al. 2013
	Streptomyces sp. GXT6	Recombinant	SV → RU	Wang et al. 2015
β-galactosidase	*Aspergillus* sp.	Commercial	SV → RU	Wan et al. 2012
	Sulfolobus solfataricus	Commercial	SV → S	Chen et al. 2014
	Kluyveromyces lactis	Commercial	SV → SB	Chen et al. 2015
Lactase	*Thermus thermophilus*	Recombinant from synthetic gene	SV → RU	Nguyen et al. 2014

1.4 β-Glucosidases

β-glucosidases (β-D-glucoside glucohydrolase, E.C.3.2.1.21) catalyze the hydrolysis of β-glycosidic bonds formed between D-glucose and the hydroxyl group of disaccharides, oligosaccharides alkyl-, and aryl-β-glucosides to release nonreducing terminal glucosyl residues. The enzyme is ubiquitous and occurs in all the living taxa including bacteria, fungi, plants, and animals. A variety of the enzyme sources resulting in the distinct specificity towards aglycone moieties of the glycoside substrates, however they exhibit notable similarity in substrate specificity for glycone (glucose) and some non-physiological aglycones (e.g. nitrophenol). The enzyme play an important role in many crucial biological pathways, such as degradation of structural and storage oligosaccharides, lignification, cellular signaling, host–pathogen interactions, and also in a variety of biotechnological applications (Bhatia, Mishra, & Bisaria, 2002).

1.4.1 Classification of β-Glucosidases

Since the IUB Enzyme Nomenclature (1984) has defined the name and the corresponding E.C. number(s) based on the type of reaction that enzymes catalyse and on their substrate-specificity. The system, however, provides little about mechanism of action, structure, and relationship to other glycoside hydrolases, and one enzyme may catalyze hydrolysis of several related substrates (Ketudat Cairns & Esen, 2010). Development of an alternative classification system for glycoside hydrolases based on amino acid sequence and structural similarity was established by Henrissat 1991 and this is the currently accepted method for classification of these enzymes (Henrissat, 1991; Henrissat & Davies, 1997; Cantarel et asl., 2009). In this system, the enzymes are clustered into the same family contain sequence and folding similarities and 135 glycosyl hydrolase families are listed in enZYme (CAZY) Web site (http://www.cazy.org). β-glucosidases have been classified as GH 1 and GH 3 glycosyl hydrolase. GH 1 enzymes mostly consist of bacterial, archaeal, plant, and animal β-glucosidases whereas GH 3 enzymes are some bacterial, all yeast and fungi glucosidases (Marques, Coutinho, Videira, Fialho, & Correia, 2003).

β-Glucosidases have various structures, but the overall fold of the catalytic domain is similar in each GH family. GH1 have similar $(\beta/\alpha)_8$-barrel domains that contain the conserved glutamate residue in NEP and ENG motif acts as potential acid/base catalyst and active site nucleophile, respectively. Conversely, GH3 enzymes have two domains contributing to their active site, a $(\beta/\alpha)_8$-barrel with glutamate/aspartate active site nucleophile in conserved SDW motif followed by an $(\alpha/\beta)_6$ sandwich domain comprises histidine/aspartate proton donating residues in KHF motif (Bhatia et al., 2002)

1.4.2 Mechanism of β-Glucosidases

Most characterized β-glucosidases perform hydrolysis of the glycosidic linkages using the retaining catalytic mechanisms via overall retention of the anomeric configuration. The double displacement of two amino acid residues of the enzyme: a general acid (proton donor) and a nucleophile/base involve in two steps, glycosylation and deglycosylation (Withers, Street, Bird, & Dolphin, 1987; Withers, 1990; Withers, 2001). In glycosylation, the aglycone leaving group gain a proton from the catalytic acid while the catalytic nucleophile attacks from the opposite site to form an α-linked covalent enzyme-aglycone intermediate. Later, in deglycosylation step, the process is reversed, as a water molecule attacks with basic assistance from the catalytic base (the same caboxylate as the catalytic acid) to attack at the anomeric carbon and displace enzyme (Figure 1.3).

1.5 Rationale

Besides in our laboratory, the presenting of extract of the leave of stevia contaminated with microorganisms was observed, suggesting that they use the extract constituents as a reservoir source. The microorganisms were isolated and tested their ability to convert steviol glycosides using TLC. The preliminary result based on 16s rRNA conserved region, a bacterium with the detected reaction product was identified as *Enterococcus casseliflavus* BO2. This bacterium is a member in large genus of lactic acid bacteria of the phylum Firmicutes, but is a minor group of human intestinal microflora where bacteroides is a dominant group. Enterococci

are Gram-positive cocci that often represent in pairs (diplococci) or short chains. However, it sometimes is a rare opportunistic infection while two species are common commensal organisms in the intestines of humans: *E. faecalis* (90–95%) and *E. faecium* (5–10%).

In order to demonstrate that stevia extracts are safe, the metabolism of steviol glycosides in humans needs to be examined. The previous studies have been considered of interest to investigate the role of human fecal microflora in the hydrolysis of the steviol glycosides. Major groups of intestinal bacteria including enterococci, lactobacilli, clostridia, bacteroides and bifidobacteria were isolated, cultured and incubated with stevioside and rebaudioside A. The result showed that only the bacteroides via their β-glucosidase activity performed hydrolyze on both rebaudioside A and stevioside (Gardana, Simonetti, Canzi, Zanchi, & Pietta, 2003). However, steviol glycosides were not absorbed directly even though undergo hydrolysis to steviol (Koyama et al., 2003), fortunately, the human gut microflora have no degradation activity on steviol (Gardana, Simonetti, Canzi, Zanchi, & Pietta, 2003; Renwick & Tarka, 2008). β-glucosidase are produced by most of the major groups of intestinal organisms (Hawksworth & Hill, 1971) such as enterococci, lactobacilli, clostridia, bacteroides and bifidobacteria.

As mentioned above, according to the prelude result indicating that *Enterococcus casseliflavus* BO2 possesses steviol glycosides hydrolyzing activity and the gut intestinal β-glucosidase may perform this role. Together, β-glucosidases have been the most intensely investigated among the steviol glycosides hydrolyzing enzymes. Up to date, only a recombinant β-glucosidase from *Streptomyces* sp. GXT6 that hydrolyze the glucose moiety in stevioside has been proposed. Thus, this study aimed to investigate a novel steviol glycosides catalyzing enzyme from *E. casseliflavus*. Full-length of β-glucosidases was cloned and expressed in *Escherichia coli* before screening of steviol glycosides degradation activity. Indeed, the desirable β-glucosidase was purified and further characterized by monitoring the activity towards hydrolyze *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NPG) and steviol glycosides.

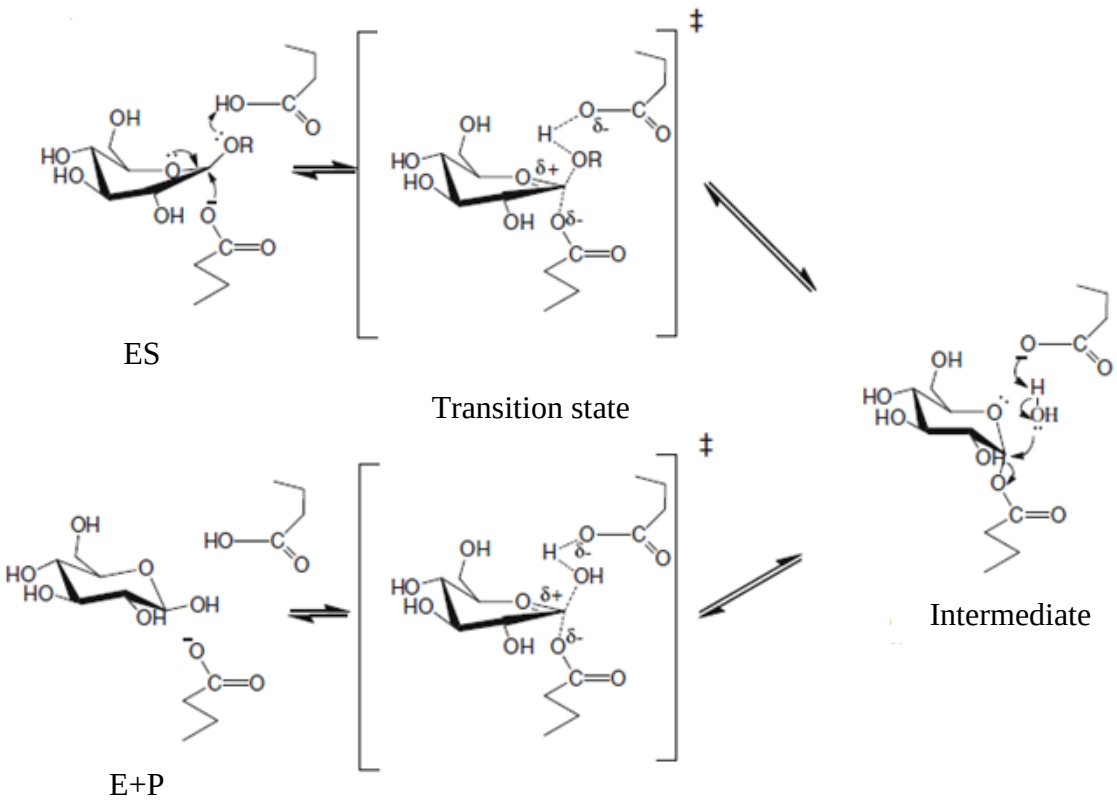


Figure 1.2 Retaining catalytic mechanism of GH 1 and GH 3 betaglucosidases (Ketudat Cairns and Esen, 2010).

CHAPTER II
OBJECTIVES

This study aims to identify and characterize the stevioside hydrolytic enzyme from *Enterrococcus casseliflavus* BO2.

CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used in this thesis were the highest purity and commercially available. Specific reagents and instruments in some assays, the distributors were provided when first mentioned either as shown in sentence or parenthesis.

3.2 Materials

3.2.1 Bacterial strains

Enterococcus casseliflavus BO2 was isolated and identified by using 16S rRNA gene sequence analysis from extract of the leave of stevia contaminated microorganisms by Somsiri Udompaisarn.

Escherichia coli strain *ccd*B was used as competent cells to perform an *in vivo* homologous recombination cloning.

E. coli BL21 (DE3) was used as expression host of β-glucosidase candidate genes.

E. coli DH5α was used to clone propagate and maintain plasmids.

3.2.2 Plasmids

A pET-28a (+) obtained from Novagen (Novagen, USA) was used as an expression vector.

3.3 General methods

3.3.1 Plasmid DNA and genomic DNA extraction and purification

3.3.1.1 Extraction of plasmid DNAs by rapid alkaline method (miniprep)

E. coli plasmid DNA was extracted by small-scale preparation of plasmid DNA using SDS and high pH to lyse the cells. Briefly, a single colony of *E. coli* was inoculated in 5 ml LB broth (1% peptone, 0.5% yeast extract, and 1% sodium chloride) containing 50 µg/ml kanamycin for overnight at 220 r.p.m. and 37°C. The cells were harvested in a 1.5 ml microcentifuge tube by centrifuging for 30s at 13,000 r.p.m. then re-suspended in 100 µl alkaline Miniprep lysis solution I (50 mM Tris, 10 mM EDTA, 100 ug/mL RNase A, and pH 8.0) Next, 200 µl Miniprep solution II (1% SDS, 0.2 M NaOH) was added to reaction followed by 150 µl of Miniprep solution III (3.0 M Potassium Acetate, pH 5.5). The suspension was centrifuged and taken to a new tube. 500 µl cold phenol:chloroform: isoamyl alcohol (25:24:1 v/v) was added. After the centrifugation of the mixture, clear supernatant was taken to a new tube. The precipitated plasmid DNA was carried out by adding of 1 ml absolute ethanol and centrifugation. The DNA pellet was dried before redissolved by adding of 50 µl TE buffer and stored at -20°C.

3.3.1.2 Extraction of plasmid DNAs by using plasmid extraction kit

TIANprep Rapid Mini Plasmid kit (Tiangen, China) was used for small-scale (3-5 ml cell) plasmid DNA extraction and purification according to the manufacturer instruction. The purified DNA was dissolved in 30-50 µl TE buffer or sterile water and stored at -20°C.

3.3.1.3 Extraction of *E. casseliflavus* genomic DNA

E. casseliflavus was grown in 5 ml LB medium for overnight at 220 r.p.m. and 37°C. Cells were harvested in a 1.5 ml microcentifuge tube by centrifuging for 30 s at 13,000 r.p.m. The cell pellet was re-suspended in 100 µl alkaline Miniprep lysis solution I and heated at 70 °C for 2 min to lyse cells. The lysate was allowed to cool for a few min before neutralized by adding 3 µl glacial acetic. Further steps of the extraction were same as the procedure of plasmid DNAs extraction by rapid alkaline method (miniprep) shown in section 3.3.1.1.

3.3.2 Agarose gel electrophoresis

DNA fragments were examined by agarose gel electrophoresis. 1% agarose gel in 0.5X TAE buffer was performed. The DNA samples were mixed 1X loading dye before loaded onto the gel. 2-log DNA ladder was used as standard marker to estimate the size of the DNA fragments in base pairs. By using the same buffer, the electric current was applied to the gel with the voltage of 10V/cm for 30 min. After electrophoresis, the gel was subjected to ethidium bromide staining for 1 min, and then destained with sterile water for 10 min. The DNA was visualized on a UV transilluminator and photographed.

3.3.3 Purification of DNA fragments and quantification

The desired DNA band of linearized vector or PCR product on agorose gel was transfer to a 1.5 ml microfuge tube. The purification of DNA was carried out by TIANGEN universal DNA purification kit (Tiangen, China), according to the manufacturer instruction. The quantification of purified DNA was performed at $OD_{260/280\ nm}$ by Nanodrop™ 2000/2000c spectrophotometers (Thermo Scientific). The purity of DNA was estimated from ratio of OD_{260}/OD_{280}. The ratio value more than 2.0 showed high purity of DNA but the ratio value less than 2.0 corresponding low purity of DNA with contaminated from phenol or protein.

3.3.4 Transformation of plasmid DNA into E. coli competent cells

3.3.4.1 Bacterial transformation by KCM transformation method

A linearized vector and a PCR product were directly co-transformed into *E. coli ccd*B competent cells by KCM transformation. The mixture was performed in a 50 µl final volume consist of an appropriate amount of linearized vector and PCR product, 10 µl 5X KCM solution (0.5 M KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) and adjusted to 50 µl by sterile distilled water. 50 µl competent cells were gently mixed with 50 µL of DNA mixture. The reaction was incubated on ice for 10 min before placed at 25°C for 5 min. After that, 500 µl LB broth was added for allow the cells to recover and incubated at 37°C with shaking for 45 min. Finally,

transformation culture was spread on a LB agar plate containing 50 µg/ml kanamycin and incubated overnight at 37°C.

3.3.4.2 Bacterial transformation by heat shock transformation method

The recombinant plasmids were transformed into *E. coli* BL21(DE3) competent cells for further protein expression. The plasmid DNA was mixed with 50 µl competent cells and incubated on ice for 20 min. The transformation mixture was heated at 42°C for 1 min and immediately chilled on ice for additional 5 min. Next, 500 µl LB broth was added and incubated at 37°C with shaking for 45 min. Then, transformation culture was spread on a LB agar plate containing 50 µg/ml kanamycin and incubated overnight at 37°C.

3.3.5 Characterization of recombinant clones

3.3.5.1 Screening of recombinant clones by colony cracking

The putative recombinant clone was screened from the bacterial colonies represented on the plate after *in vivo* homologous recombination cloning. The colonies with ~2 mm in size were selected to re-suspend in 10 µl TE buffer followed by adding of the equal volume of 2X cracking buffer (0.2 M NaOH, 0.5% SDS, 10% glycerol). The mixture was heated at 55°C for 5 min before mixed with 4 µl cracking loading dye (2M KCl, 0.1% bromphenol blue) and immediately chilled on ice for 5 min. The reaction was centrifuged at 13,000 r.p.m. for 1 min then aliquoted 20 µl of the sample to perform agarose gel electrophoresis.

3.3.5.2 Determination of recombinant clones by restriction analysis

In order to verify the present of plasmid harbouring the insert gene, restriction digestion analysis was performed by using the appropriate restriction enzymes. The digestion reaction, 1 µg plasmid, the recommended buffer diluted to 1X final concentration, and the enzyme was incubated at 37°C for 1 hr. Then, the mixture was subjected to agarose gel electrophoresis as described in section 3.3.2. The putative recombinant plasmid was further confirmed by DNA sequencing.

3.3.6 Analysis of sequences

The DNA sequences and deduced amino acid sequences were searched for similar nuclotide identification using BLAST at (National Center for Biotechnology Information) NCBI GenBank. The molecular masses of the proteins were calculated based on their amino acid sequences using http://www.bioinformatics.org/sms/prot_mw.html. Protein sequence alignments were carried out with the CLUSTAL W program.

3.3.7 Determination of protein concentration

The amount of protein was monitored by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Bradford reagent (Bio-Rad, USA) (200 µl) was mixed with 1 µl of protein sample in a 96-well microtiter plate. The reaction mixture was incubated at room temperature for 5 min prior to measurement of absorbance at 595 nm using a microplate reader. For performing SDS-PAGE, the quantification of protein was performed at $OD_{280\ nm}$ by Nanodrop™ 2000/2000c spectrophotometers (Thermo Scientific).

3.3.8 Analysis of protein by SDS-PAGE

Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) was performed with low-range molecular weight markers from Bio-Rad (Laemmli, 1970). *E.coli* cells were lysed by lysis buffer (1% SDS, 1 mM PMSF, 0.25 % glycerol, 16.8 mM Tris-HCl buffer (pH 7.5)). 20 µg protein of cell lysate suspension was mixed with loading dye. The mixture was boiled for 5 min before loading onto 10% SDS separating gel with 4% stacking gel. The gel was visualized by staining with Coomassie Brilliant Blue R250 followed by destained with destaining solution. The amount of β-glucosidase expressed as percent of total protein was determined by densitometric scanning using image scanner (Amersham Pharmacia Biotech, Germany).

3.4 Experimental methods

3.4.1 Determination of steviosides digesting activity of *E. casseliflavus* BO2

E. casseliflavus BO2 was grown on TSB rich medium (Scharlau, Spain) for two days at 220 r.p.m. and 37°C. The cultured was then changed to M9 minimal medium containing steviosides (1 mg/ml) and also incubated for two days at 220 r.p.m. and 37°C. Steviosides conversion activity was determined from the supernatant by thin layer chromatography (TLC).

3.4.2 Preparation of expression constructs

3.4.2.1 Primers design

Complete genome sequence of *Enterococcus casseliflavus* BO2 (GenBank accession number NC020995.1) obtained from NCBI nucleotide sequence database was used for design primer of the steviosides degraded candidate genes, *β-glucosidases.* Two strategies to find out the target genes, data mining by direct searching for the annotated *β-glucosidases*, and homology search using amino acid sequence of a *β-glucosidase* from *Bacteroides thetaiotaomicron*. The precursor protein was used since it was expressed and functioned as stevioside hydrolytic enzyme performed by Somsiri Udompaisarn. Five *β-glucosidases* were obtained from the first strategy and a *β-glucosidase* from the later. To amplify these target genes for *in vivo* homologous recombination cloning, it was necessary to generate a PCR amplicon wherein the gene of interest is flanked by homology sequence of expression vector. Gene-specific primers, forward primers with *Nco*I and reverse primers with His_6-tagged homology sequence of linearized pET28a expression vector were synthesized. The primers were ordered from Pacific Science Co., Ltd. Thailand. Primers used and their detail were listed in Table 3.1 (only four primer pairs provided the PCR products of the target genes were listed).

3.4.2.3 Amplification of *β-glucosidase* genes

Full-length of the four *β-glucosidase* genes were amplified by PCR using Q5® High-Fidelity DNA polymerase. The conditions of PCR reaction and primers used were shown in table 3.1. The PCR products were analyzed by agarose gel electrophoresis and subjected to purify using DNA purification kit according to the manufacturer's instructions (Tiangen, China).

Table 3.1 Primers used for *β-glucosidase* genes amplification. The homology sequence of the genes and linearized vector were underlined.

Primer pair	Sequence 5'-3'	Description	PCR condition
pET28NcoI00184f	AGA AGG AGA TAT ACC ATG CAG ACA GAA GGT GG	Forward primer for *EcBgl 1*	98°C 30 s 10 cycles: 98°C 10 s, 61°C 30 s,72°C 40 s 20 cycles: 98°C 10 s, 72°C 30 s, 72°C 40 s 72°C 2 min
pET28Histag00184r	GTG GTG GTG GTG CTC GAG TTC TGT ATA TGG GTT TGT ATA G	Reward primer for *EcBgl*	
pET28NcoIRS04970f	AGA AGG AGA TAT ACC ATG ACT AAC YTG YAT TTT CCT AAA G	Forward primer for *EcBgl 2*	98°C 30 s 10 cycles: 98°C 10 s, 62°C 30 s,72°C 40 s 20 cycles: 98°C 10 s, 71°C 30 s, 72°C 40 s 72°C 2 min
pET28HistagRS04970r	GTG GTG GTG GTG CTC GAG GTC GAC AAA GCC GTT TTG	Reward primer for *EcBgl 2*	
pET28NcoIRS11275f	AGA AGG AGA TAT ACC ATG GTA AAA ATA CCT GAA ACA TT	Forward primer for *EcBgl 3*	98°C 30 s 10 cycles: 98°C 10 s, 58°C 30 s,72°C 40 s 20 cycles: 98°C 10 s, 70°C 30 s, 72°C 40 s 72°C 2 min
pET28HistagRS11275r	GTG GTG GTG GTG CTC GAG CAG AAT CAA TGG TTT TTC TTC	Reward primer for *EcBgl 3*	
pET28NcoIRS016010f	AGA AGG AGA TAT ACC ATG GAA CAG CAG AAA TTA ACC	Forward primer for *EcBgl 4*	98°C 30 s 10 cycles: 98°C 10 s, 61°C 30 s,72°C 70 s 20 cycles: 98°C 10 s, 72°C 30 s, 72°C 70 s 72°C 2 min
pET28HistagRS016010r	GTG GTG GTG GTG GTG GTG CCT AAC TAA TTG CAG GGT TG	Reward primer for *EcBgl 4*	

3.4.3 Cloning of the *β-glucosidase* genes in *E. coli*

The PCR fragments were cloned into the expression vector pET-28a(+) that had been double-digested with *BamH*I and *Xho*I. *In vivo* homologous recombination cloning was performed in *E. coli ccd*B as shown the strategy in Figure 3.1. The recombinant plasmids were verified according to the procedure in section 3.3.5 and confirmed by sequencing. The positive recombinant plasmids were extracted and transformed into *E. coli* BL21(DE3) by heat shock transformation method as described in section 3.3.4.1.

3.4.4 Expression of β-glucosidase in *E. coli* BL21(DE3)

E. coli BL21(DE3) harboring the recombinant plasmids were grown in LB broth containing 50 μg/ml kanamycin and incubated at 220 r.p.m and 37°C until an $OD_{600\ nm}$ reached 0.5. Culture was induced by the addition of 0.2 mM IPTG at 25°C for 4 h and pelleted the cell by centrifugation at 3,500*g* for 10 min. *E. coli* BL21(DE3) culture transformed of an vector control (pET-28a(+) without insert) was used as a negative control.

3.4.5 Large-scale expression and purification of protein

To obtain the highest activity of the target protein, the expression condition was optimized before performing large-scale cell growth and purification. Initially, in order to identify the optimum IPTG concentration, the cells were grown at 37°C until an $OD_{600\ nm}$ reached 0.5, variation of IPTG was added to a final concentration of 0.05, 0.1, 0.2, 0.25, and 0.3 mM then cultured the cell at 25°C for additional 4 h. Next, the incubation temperatures were varied including 37°C, 30°C, and 25°C. Lastly, the incubation time optimization ranging from 3 h to overnight was carried out. The optimum condition was calculated in term of highest specific activity on the crude extract as following:

Specific activity = amount of activity/amount of protein

Here, amount of activity was *β*-glucosidase activity by using glucose assay, 1 U was the amount of enzyme required to release 1.0 μmol of glucose per min. Amount of *β*-glucosidase was determined by Bradford as mentioned in section 3.3.7. % intensity was defined as percent of total protein on the crude extract

electrophoretic profile was determined by densitometric scanning (Amersham Pharmacia Biotech, Germany) using ImageMaster TotalLab Software. The supernatant from uninduced cell lysate was used as a negative control.

To purify the target protein, initially, induced cultures were harvested and disrupted the cell pellets by lysis buffer (50 mM sodium acetate (pH 6.0), 1 mg/ml lysozyme, 1 mM PMSF, 200 ug/ml DNase, 1 mg/ml RNase) at 37°C for 30 min. The cell debris was removed by centrifugation at 15,000*g* and 4°C for 20 min. The resulting supernatant was applied to a 10 ml column of Ni-NTA resin equilibrated in the buffer containing 50 mM sodium acetate buffer (pH 6.0), 200 mM sodium chloride and 20 mM imidazole. The column was washed with 10 column volumes of this buffer then eluted the bound fusion protein by an over 10 column volumes linear gradient of 75 to 150 mM imidazole in the same buffer. Active fractions were pooled, concentrated, and the buffer was exchanged using an Amicon Ultra Centrifugal filter 30,000 MWCO (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The protein concentrations were determined by using Bradford against a BSA standard as described in section 3.3.6. The fractions containing the tagged protein were monitored by SDS-PAGE followed the procedure in section 3.3.7.

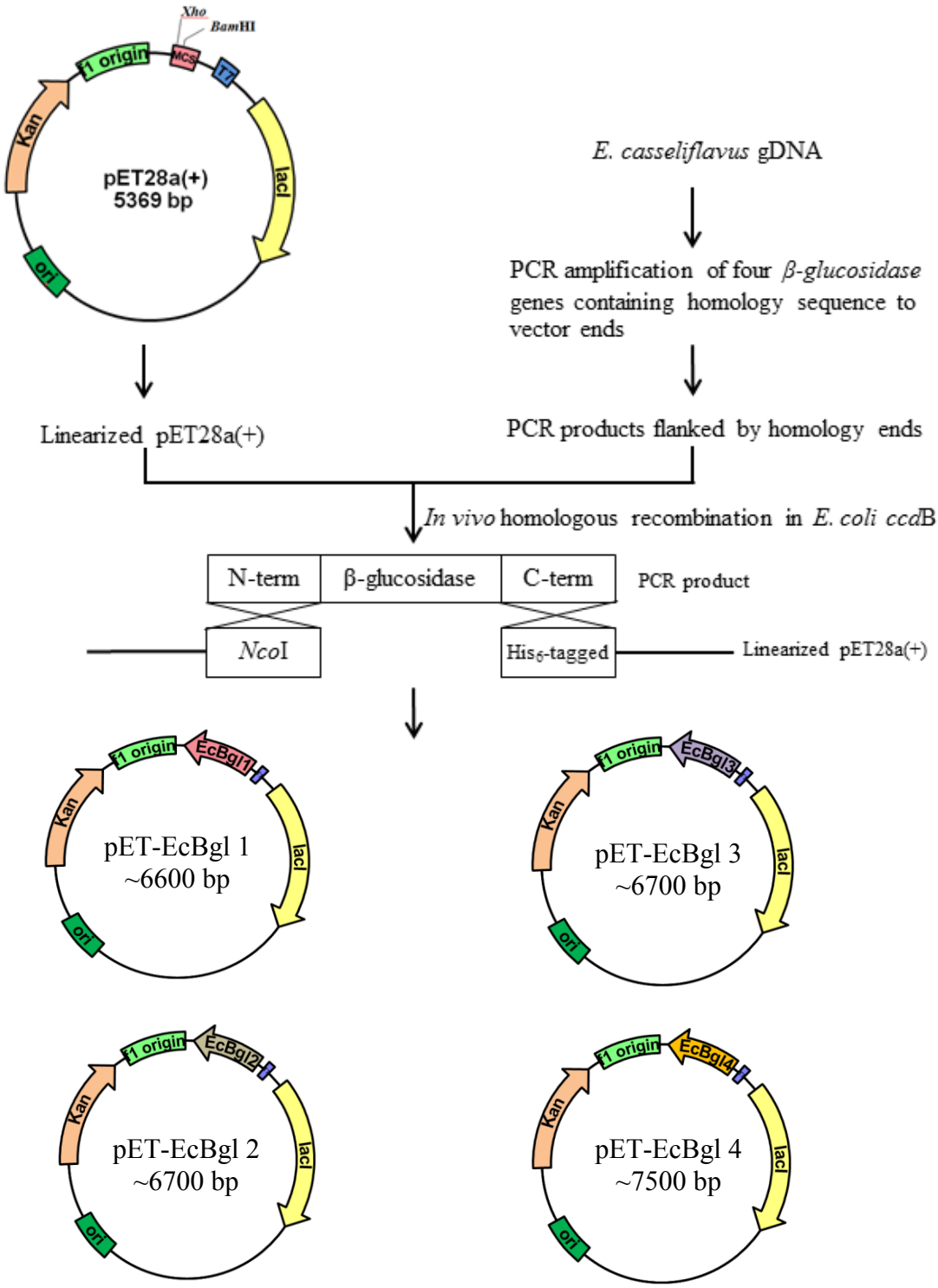


Figure 3.1 Schematic illustration of construction of *β-glucosidase* genes in pET-28a(+) expression vector

3.4.6 Determination of enzyme activity

3.4.6.1 *β*-glucosidase activity

3.4.6.1.1 Measurement of *p*NP released

The reaction was carried out photometrically using *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NPG) as a substrate. 1 mM of *p*NPG in 50 mM sodium acetate buffer pH 6.0 was prewarmed at 37°C for 2 min. The reaction was started by adding an appropriate amount of enzyme and then incubated for 10 min. and then stopped by adding of 0.1 M Na_2CO_3. The blank containing no enzyme was performed with the same manner as the samples. The release of *p*-nitrophenol (*p*NP) was determined by measuring the absorbance at 405 nm. A standard curve of *p*NP was used to quantify the product. One unit of *β*-glucosidase activity was defined as the amount of enzyme required to release 1.0 μmol of *p*NP per min.

3.4.6.1.2 Measurement of glucose released

The concentrations of glucose released from the reaction mixtures were determined by couple enzyme glucose assay against a glucose standard. The reaction mixture was heated at 65°C to inactivate the enzyme. After that, the mixture was incubated with 100 μl of glucose assay solution consists of 1 mg/ml glucose oxidase, 0.1 mg/ml peroxidase, and 0.06 mg/ml *o*-dianisidine at 37°C for 15 min. The reaction was stopped by adding of 80% H_2SO_4 and the absorbance was measured at 540 nm. One unit of the enzyme activity (U) was defined as the amount of enzyme that produced 1 μmol of glucose per minute

3.4.6.2 Steviosides hydrolysis activity

3.4.6.2.1 Determination of the reaction product by TLC

The reaction product of mixture of enzyme and 1 mg/ml steviosides was monitored by thin layer chromatography (TLC) using silica gel 60 $F_{254,}$ aluminium sheet (Merck, Germany). 1 μl of the reaction mix was spotted on the TLC plate and chromatographed vertically using isopropanol/ethyl acetate/acetone/water (4.5:7.95:0.3:2.25) as a mobile phase for approximately 11 min. Seviosides standard was run under the same condition. The reaction products were visualized by soaked rapidly TLC plate into 10% H_2SO_4 and heated at 100°C for 3 min.

3.4.6.2.2 Determination of the reaction product by HPLC

In order to identify the reaction products, 1 ml of reaction samples containing the enzyme and 1 mg/ml stevioside in 50 mM sodium acetate (pH 6) was incubated over night at 37°C. The mixtures were subjected to HPLC using a waters HPLC system on a e2695 separation module system, 2487 dual wavelength detector (Waters Corporation, Milford, USA) and a reverse phase Acclaim 120 C18 column (Thermo Fisher Scientific, 4.6 mm×100 mm, 3 µm) with UV detection at 210 nm. The temperature of the column was controlled at 60 ^{0}C. The samples were injected with an isocratic mobile phase of 68% water and 32% acetonitrile at a flow rate of 0.5 ml/min and run time 10 min. Rubusoside standard was run along with the sample.

3.4.7 Characterization of enzyme

All enzyme assays were determined against the product standard curves and performed in three independent experiments.

3.4.7.1 pH optimum and stability

To determine the optimum pH of *β*-glucosidase activity towards *p*NPG, the activity was measured over a pH range of 3.0–10.0 by using 50 mM Britton-Robinson universal buffer. 700 µl of 1 mM *p*NPG in the defined pH was pre-incubated at 37°C for 2 min before adding of 100 µl of the appropriately diluted of purified enzyme. The reaction was incubated for 10 min and then 200 µl of 0.5 M Na_2CO_3 was added to stop the reaction. The amount of released *p*NP was determined by an absorption increase at 405 nm on an automated microplate reader (PekinsElmer, USA). The highest enzyme activity on the substrate was expressed as 100%. A reaction mixture without enzyme was used as a blank control.

For the optimum pH of *β*-glucosidase activity on stevioside, the assay also performed in the same defied pH buffer. The enzyme (10 µl) was incubated with 90 µl of 1 mM stevioside at 37°C for 20 min. The reaction was stopped by heating at 65°C for 5 min. 60 µl of the reaction mixture was taken to mix with 140 µl of glucose assay solution in a microtiter plate and incubated at 37°C for

15 min. The reaction was stopped by adding 50 µl of 80% H_2SO_4. The absorbance was measured at 540 nm to monitor the liberated glucose using a microplate reader.

3.4.6.2 pH stability

To elucidate the pH stability, *β*-glucosidase pH stability was performed by using *p*NPG as a substrate. 100 ul of purified enzyme was incubated for 1 h at 25 °C in the absence of substrate in the same buffer as pH optimum experiment. The pH value of enzyme was readjusted to pH 6.0 by adding 700 ul 1 mM *p*NPG pH 6.0. Then, the residual activity was measured under the same assay conditions above.

3.4.6.3 Temperature optimum

Enzymatic activity towards *p*NPG was determined within a temperature range of 25°C–65 °C for 10 min. The reaction containing 700 µl of 1 mM *p*NPG in sodium acetate buffer (pH 6.0) was pre-incubated at the desired temperature for 2 min. Next, 100 µl of enzyme was added to initiate the reaction. After 10 min, the reaction was stopped by adding 200 µl of 0.5 M Na_2CO_3 and measured the absorbance at 405 nm. A blank of each temperature condition was run in parallel by replacing enzyme with the buffer. The highest residual activity was defined as 100%.

Using stevioside as a substrate, the reaction mixture containing 50 µl of 1 mM stevioside and 50 µl of enzyme was incubated at the defined temperature. After 20 min, the mixture was heated at 65 °C for 5 min to terminate the reaction. Then 100 µl of reaction was mixed with 100 µl of glucose assay solution. Glucose released from the reaction mixture was monitored at 540 nm using a microplate reader.

3.4.6.4 Temperature stability

Thermostability data was obtained by analyzing residual activity under the same assay conditions at temperatures ranging from 30 to 50 °C within 1 h. Every 10 min of incubation, the enzyme was allowed to cool at room temperature for 2 min before pre-incubating at 37°C for 2 min. The residual activities were measured under the same assay conditions as the temperature optimum activity on *p*NPG. The result was expressed as the relative activity with the initial activity as 100%.

3.4.6.2 Effect of metal ions and other reagents

Enzyme was incubated with 2 mM of cobalt chloride, mercury chloride, zinc chloride, potassium chloride, copper chloride, iron (III) chloride, calcium chloride, sodium chloride, barium chloride, magnesium chloride, manganese chloride, and silver nitrate. Effect of ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 1% sodium dodecyl sulfate (SDS) were also tested. The reaction containing 75 µl of enzyme and 75 µl of 2 mM ion solution in 50 mM sodium acetate (pH 6.0) were incubated at 25°C for 30 min. After an adequate incubation period, the mixture was pre-warmed at 37°C for 2 min, then 25 µl of 8 mM *p*NPG was added with continue incubated for 10 min. 25 µl of 0.8 M Na_2CO_3 was added to stop the reaction. Blank was run in parallel by replacing enzyme with 75 µl of the buffer. The activity of untreated reaction which no metal ion added was defined as 100%.

3.4.6.5 Substrate specificity

The assay of substrate specificity was performed by determining the enzyme activity on various substrates with 2 mM final concentration including aryl-glycoside chromogenic substrates and saccharides. Aryl-glycoside synthetic substrates used were *p*-Nitrophenyl β-D-glucopyranoside, *p*-Nitrophenyl α-D-glucopyranoside, *p*-Nitrophenyl β-D-galactopyranoside, *p*-Nitrophenyl-N-acetyl-β- and D-glucosaminide. For natural saccharides the substrates were tested including, cyanogenic β-glucoside (amygdalin), aryl-glucoside (arbutin), glucobiose (cellobiose), disaccharides (lactose and sucrose), trehalose, isomaltose. Two steviol glycosides, stevioside and rebaudioside A were also assayed. The enzyme activities towards the aryl-glycoside synthetic substrates were assayed by incubating the appropriately diluted of enzyme with the substrate in 50 mM sodium acetate buffer (pH 6.0) at 37°C for 10 min with the same manner standard of *p*NP and glucose released monitoring method as described in section 3.4.5.1. The reaction without enzyme was used as a blank performed along with each substrate. One unit of the enzyme activity (U) was defined as the amount of enzyme that produced 1 µmol of *p*-nitrophenol or glucose per min for aryl-glycoside synthetic substrates and sacharides, respectively. The result was performed in term of % relative activity and the enzyme activity against *p*NPG and stevioside was defined as 100% for aryl-glycoside synthetic substrates and sacharides, respectively.

3.4.6.6 Determination of kinetic parameters

The initial velocity (v_0) of the reaction was determined with enzyme concentrations and times that gave linear rates. The enzyme in 50 mM sodium acetate buffer (pH 6.0) was incubated with either *p*NPG (0.05 to 6 mM) or stevioside (0.5 to 30 mM) at 37°C. Kinetic parameters, K_m and V_{max} were calculated from (v_0) via the Michaeilis-Menten equation using a curve-fitting function of Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The apparent k_{cat} values were calculated by dividing the V_{max} by total enzyme concentration.

CHAPTER VI
RESULTS

4.1 Steviol glycosides conversion activity of *E. casseliflavus* BO2

Initially, in order to confirm the ability to convert steviol glycosides of *E. casseliflavus* BO2, the isolated cells were grown in a M9 minimal medium containing stevioside and rebaudioside A. *E. casseliflavus* BO2 showed the obvious reaction product compare to the mixture control performed by TLC (Figure 4.1), indicating it contained the hydrolytic activity on steviol glycosides.

4.2 Amplification of *β-glucosidase* genes

Full-length of *β-glucosidase* genes was amplified from genomic DNA of *E. casseliflavus*. The PCR amplicons of expected sizes were specifically amplified by using their primer pairs as aforementioned in table 3.1. Three *β-glucosidase* genes from data mining could be amplified, *EcBgl 1*, *EcBgl 2*, and *EcBgl 3*, which were similar in size corresponding to 1344 bp, 1392 bp, and 1416 bp, respectively. While one *β-glucosidase* genes from homology search, *EcBgl 4* was the largest size with 2151 bp (Figure 4.2).

4.3 Construction of the plasmid for expression of *β-glucosidase* genes

The PCR products from 4.2 were co-transformed with linearized pET-28a(+) into *E. coli ccd*B to allow *in vivo* homologous recombination cloning. The recombinant plasmids were verified by using method as described in section 3.3.5. The resulting recombinant plasmids containing the inserted genes then were named pET-EcBgl 1, pET-EcBgl 2, pET-EcBgl 3, and pET-EcBgl 4 (data not shown).

Figure 4.1 TLC chromatogram of stevioside hydrolysis by *E. casseliflavus* BO2.

E. casseliflavus BO2 was grown in M9 medium containing 1 mg/ml of steviosides, rebaudioside A (RA) and stevioside (SV). One microliter of the cell supernatant was loaded onto silica gel TLC with sulfuric acid staining. Lane 1, M9 medium control incubated with RA and SV; lane 2 *E. casseliflavus* BO2 incubated with RA and SV.

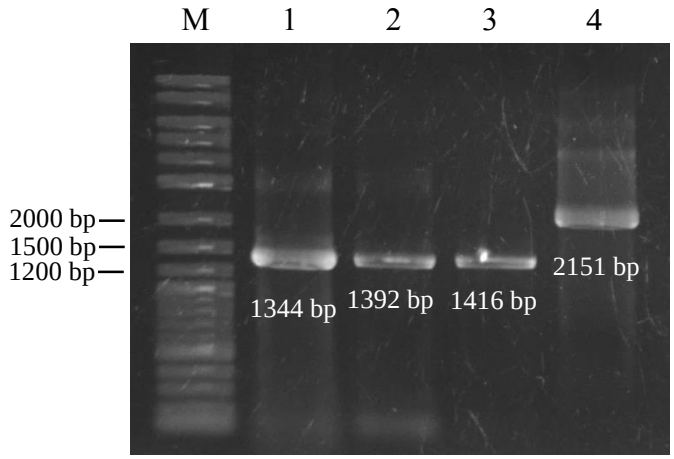


Figure 4.2 Agarose gel electrophoresis of *ß-glucosidase* genes of *E. casseliflavus* BO2 from PCR amplification.

Full-length of four *β-glucosidase* genes were amplified from genomic DNA of *E. casseliflavus*. Lane M, 2-log DNA marker; lane 1, *EcBgl 1* (1344 bp); *EcBgl 2* (1392 bp); lane 3 *EcBgl 3* (1416 bp); lane 4, EcBgl 4 (2151 bp).

4.4 Sequences analysis

The full length of β-glucosidase inserts in plasmids pET-EcBgl 1, pET-EcBgl 2, pET-EcBgl 3, and pET-EcBgl 4 were subjected to sequences analysis to confirm that the nucleotide sequences of the putative clones were correct. All plasmids showed the complete insert DNA of *β-glucosidases* with His_6-tagged sequences containing the open reading frame encoded a protein of 455 amino acids, 471 amino acids, 479 amino acids, and 721 amino acids for EcBgl 1, EcBgl 2, EcBgl 3, and EcBgl 4, respectively. The nucleotide sequence along with the amino acid sequence of each inserted gene was shown in figure 4.3 for EcBgl 4, and appendix B1, B2, and B3 for EcBgl 1, EcBgl 2, and EcBgl 3, respectively.

The deduced amino acid sequences were searched for protein homology identification by using BLASTP according to the procedure in section 3.3.5. The result revealed that EcBgl 1, EcBgl 2, and EcBgl 3 were GH 1 glycosyl hydrolase similar to β-glucosidase in contrast to EcBgl 4 was GH 3 glycosyl hydrolase similar to in contrast to β-glucosidase. EcBgl 1, EcBgl 2, and EcBgl 3 were aligned with the previous stevioside conversion GH 1 β-glucosidases from *Streptomyces* sp. GXT6. Since GH 3 β-glucosidase with steviol glycosides hydrolysing activity has not been annotated, EcBgl4 was aligned with GH 3 β-glucosidase precursor hydrolyze stevioside from *Bacteroides thetaiotaomicron* (BtBgl) (unpublished data). The deduced amino acid alignment represented GH 1 glycosyl hydrolases contained glutamate residues in NEP and ENG conserved motifs, which act as both acid/base catalyst and active site nucleophile (Figure 4.4). Based on the sequences of GH 3 of glycosyl hydrolases, the β-glucosidases consist of histidine proton donor in the KHF putative catalytic motif and the putative active site aspartate residue in the SDW conserved motif (Figure 4.5). These sequence results demonstrated that all plasmid constructed indeed containing *β-glucosidase* genes.

```
   1 ATGGAACAGC AGAAATTAAC CGAACTACTT TCAGAAATGA CCTTAGATGA
     M  E  Q  Q   K  L  T   E  L  L   S  E  M  T   L  D  E
  51 AAAAATCGAT CAGTTGCTAC AACTGGCAGC GGCTTTTTAT TCAGATAAAG
      K  I  D   Q  L  L  Q   L  A  A   A  F  Y   S  D  K  A
 101 CAGAAGAGAA AACAGGTCCG ATGGGCGACT TAGGACTGAC ACAAGAAAAC
       E  E  K   T  G  P   M  G  D  L   G  L  T   Q  E  N
 151 ATCAACAATG CGGGAACAAC GCTAGGTGTT TCTGGTGCAA AAGAAGCGAT
     I  N  N  A   G  T  T   L  G  V   S  G  A  K   E  A  I
 201 CCGGGTCCAA AAAGAGTATA TCGCCAATAA CCGCTTGAAT ATCCCGACGA
      R  V  Q   K  E  Y  I   A  N  N   R  L  N   I  P  T  I
 251 TATTGATGGC GGACATCATT CACGGCTTTC GGACGATTTT CCCGATTCCA
       L  M  A   D  I  I   H  G  F  R   T  I  F   P  I  P
 301 TTAGGATTAG GTAGTTCATG GGATTTGGCA GCAGCGGAGA AAATGGCGGA
     L  G  L  G   S  S  W   D  L  A   A  A  E  K   M  A  E
 351 AGTATCTGCC AAAGAAGCAG CTGTTTCTGG CTTGCATGTG ACCTTTTCAC
      V  S  A   K  E  A  A   V  S  G   L  H  V   T  F  S  P
 401 CGATGGTGGA CTTAGTAAGA GACCCACGCT GGGGCCGTGT CATGGAATCG
       M  V  D   L  V  R   D  P  R  W   G  R  V   M  E  S
 451 ACGGGGGAAG ATCCTTACTT GAACAGTCGC TTCGCTGAAG CCTTCGTCAA
     T  G  E  D   P  Y  L   N  S  R   F  A  E  A   F  V  K
 501 AGGCTATCAA GGGGATGATC TGCGAACGGA TTTCAACCGC GTGGCTGCTT
      G  Y  Q   G  D  D  L   R  T  D   F  N  R   V  A  A  C
 551 GCGTCAAACA TTTTGCGGCT TACGGTGCGG CTATCGGTGG TCGCGATTAC
       V  K  H   F  A  A   Y  G  A  A   I  G  G   R  D  Y
 601 AACACGGTCA ATATGTCAGA ACGCCAACTG CGAGAAAGTT ATTTGCCAGG
     N  T  V  N   M  S  E   R  Q  L   R  E  S  Y   L  P  G
 651 CTATAAAGCA GCCCTTGATG CTGGTGCCAA GCTGGTGATG ACCTCCTTTA
      Y  K  A   A  L  D  A   G  A  K   L  V  M   T  S  F  N
 701 ATACGGTAGA CGGCATTCCA GCCACGGCCA ATCGCTGGCT TTTCCGCGAT
       T  V  D   G  I  P   A  T  A  N   R  W  L   F  R  D
 751 GTTTTGCGAG AAGAATTCGG GTTTGAAGGC GTTGTGATCT CTGACTGGGC
     V  L  R  E   E  F  G   F  E  G   V  V  I  S   D  W  A
 801 AGCAATCAAA GAAGTGATCG CTCATGGCGC AGCGGAGGAT GAAAAACATG
      A  I  K   E  V  I  A   H  G  A   A  E  D   E  K  H  A
 851 CCGCTGAACT AGCCATCAAA GCTGGGGTCG ATATCGAGAT GATGACGACT
       A  E  L   A  I  K   A  G  V  D   I  E  M   M  T  T
 901 TGCTACACCG ATAACTTGAA AGAGTTGATC GCAGAAGGCA CCGTTGAGGA
     C  Y  T  D   N  L  K   E  L  I   A  E  G  T   V  E  E
 951 AGCCTTAGTC GATGAAGCGG TGCTAAGAAT CTTGACCTTA AAAAATGAGC
      A  L  V   D  E  A  V   L  R  I   L  T  L   K  N  E  L
1001 TGGGGCTATT TGAAAATCCA TACCGTGGCG CTGATGAAGC CGCTGAAGCA
       G  L  F   E  N  P   Y  R  G  A   D  E  A   A  E  A
1051 GCCACTGTTT TGTCTCAAGA ACACCGAGAG ATCGCCAGCG ATATCGCAAA
     A  T  V  L   S  Q  E   H  R  E   I  A  S  D   I  A  K
1101 GAAATCAATG GTGTTGTTGA AAAATGAAGG TATCCTGCCG TTGCAGAAAA
      K  S  M   V  L  L  K   N  E  G   I  L  P   L  Q  K  T
```

Figure 4.3 Nucleotide sequence and the deduced amino acid sequence of *EcBgl 4* gene.

Nucleotide sequence and the deduced amino acid sequence including His_6-tagged sequence of *EcBgl 4* gene showed an open reading frame of 2172 bp encoding 721 amino acids.

```
1151 CCGAAAAAGT CGCGATCGTG GGCCCAGGTG CTCACTCCCG TGATCTATTA
       E  K  V   A  I  V   G  P  G  A   H  S  R   D  L  L
1201 GGTGCTTGGT CTTGGCAAGG GAAACAAGAA GAAGTAGTGA CTTTAGTGGA
     G  A  W  S   W  Q  G   K  Q  E   E  V  V  T   L  V  E
1251 GGGTGCCCAA GCCTTGGGTG CGGATCTGTT GATCGGTCAA GAGCCCTTTG
      G  A  Q   A  L  G  A   D  L  L   I  G  Q   E  P  F  D
1301 ATTATTTTGC ACCGTCTGAA ACGGCGATCC AAGAAGCAAT CGAATTAGTG
       Y  F  A   P  S  E   T  A  I  Q   E  A  I   E  L  V
1351 AAGGCAGCCG ATAAAGTGGT CTTAGCACTA GGAGAGCAGG AATGGATGAG
     K  A  A  D   K  V  V   L  A  L   G  E  Q  E   W  M  S
1401 CGGTGAAGCT GCCAGCCGCA GCGACATTCG TTTGCCGCAA GCCCAATTGT
      G  E  A   A  S  R  S   D  I  R   L  P  Q   A  Q  L  S
1451 CCTTGGTTGA AACCTTAAAA GAATACAACG AGCAATTGAT CGTAACTCTT
       L  V  E   T  L  K   E  Y  N  E   Q  L  I   V  T  L
1501 TATAACGGTC GTCCTCTTGA TTTGCAAGGT GTGGATGCAG CCAAAGCGAT
     Y  N  G  R   P  L  D   L  Q  G   V  D  A  A   K  A  I
1551 CGTCGAGGCT TGGTTCCCTG GAACCGAAGG CGGAAACGCA CTTGCTCAGA
      V  E  A   W  F  P  G   T  E  G   G  N  A   L  A  Q  I
1601 TCCTGTGGGG AGAATACAAT CCAAGCGGTC GCTTGAGCAT GTCATTCCCA
       L  W  G   E  Y  N   P  S  G  R   L  S  M   S  F  P
1651 GAAACCGTTG GACAAGTTCC TGTGTATTAC AACGTTGACA ATACCGGTCG
     E  T  V  G   Q  V  P   V  Y  Y   N  V  D  N   T  G  R
1701 TCCTTATGAA AGTGCACCGG ATGAAAAATA TGTCTCGAAA TATTTGGATG
      P  Y  E   S  A  P  D   E  K  Y   V  S  K   Y  L  D  V
1751 TCTCCAATTA TGCCAAATAT CCATTTGGGT TTGGCTTGAG TTATAGCCCA
       S  N  Y   A  K  Y   P  F  G  F   G  L  S   Y  S  P
1801 GTTGCGTATT CTGCGGTGAC GTTGGATCAG CCGACTATGA CCAAAGATCA
     V  A  Y  S   A  V  T   L  D  Q   P  T  M  T   K  D  Q
1851 AACGGTCACG GCTTCCATCA CTGTTACGAA CCAAGGAACA GCAGCGGTCT
      T  V  T   A  S  I  T   V  T  N   Q  G  T   A  A  V  W
1901 GGGAAACGGT CCAATGCTAC ATTCGCGATT TAGTCGGTGA AGTGGTTCGC
       E  T  V   Q  C  Y   I  R  D  L   V  G  E   V  V  R
1951 CCAGTGAAAG AACTGAAAGG CTTTAAGAAA ATTTGGCTAG AGGCTGGCGA
     P  V  K  E   L  K  G   F  K  K   I  W  L  E   A  G  E
2001 ATCAGCAACC GTTCAATTTG AGATCACGGA AGAACTGCTG CGCTATGTCC
      S  A  T   V  Q  F  E   I  T  E   E  L  L   R  Y  V  H
2051 ACAGCAACCA ACAAGTAAGC AGTGATCCAG GGAAATTCCA CATCATGATT
       S  N  Q   Q  V  S   S  D  P  G   K  F  H   I  M  I
2101 GGTGGGAACA GCCGAGACAC CCAACAAACA ACCCTGCAAT TAGTTAGGCT
     G  G  N  S   R  D  T   Q  Q  T   T  L  Q  L   V  R  L
2151 CGAGCACCAC CACCACCACC AC
      E  H  H   H  H  H  H
```

Figure 4.3 (continue) Nucleotide sequence and the deduced amino acid sequence of *EcBgl 4* gene.

Nucleotide sequence and the deduced amino acid sequence including His_6-tagged sequence of *EcBgl 4* gene showed an open reading frame of 2172 bp encoding 721 amino acids.

Figure 4.4 Multiple sequence alignment of the deduced amino acids of GH 1 β-glucosidase.

Deduced amino acid sequence of GH 1 β-glucosidases (EcBgl1, EcBgl2, EcBgl3) in the present study were aligned with a putative β-glucosidase hydrolyse the glucose moiety in stevioside previously reported *Streptomyces* sp. GXT6 (StBgl 1) (AIN35077). Identical residues shown by shaded characters. Catalytic residues, Glu; catalytic acid/base and active site nucleophile shown by rectangles.

Figure 4.5 Multiple sequence alignment of the deduced amino acids of GH 3 β-glucosidase.

Deduced amino acid sequence of GH 3 β-glucosidases EcBgl 4 in the present study was aligned with a putative β-glucosidase hydrolyse stevioside from *Bacteroides thetaiotaomicron* (BtBgl) (NP 812479, 41% identity). Identical residues shown by shaded characters. Catalytic residues, Asp; active site nucleophile and His; putative proton donor shown by rectangles.

4.5 Expression of recombinant β-glucosidases in *E. coli*

To investigate the function of the four *β-glucosidase* genes of *E. casseliflavus*, the genes were expressed as an C-terminal His_6-tagged fusion protein using pET-28a(+) expression system in *E. coli* BL21(DE3). SDS-PAGE (10%) revealed that molecular masses with His_6-tagged about ~53 kDa of EcBgl 1, ~55 kDa of EcBgl 2 and EcBgl 3, and ~80 kDa of EcBgl 4 as shown in figure 4.6. These results were corresponding to the total calculated molecular mass of the predicted amino acid sequence with a C-terminal His_6-tagged sequence of 53.06 kDa, 54.58 kDa, 56.78 kDa, and 79.37 kDa of EcBgl 1, EcBgl 2, EcBgl 3, and EcBgl 4 respectively. This result was also in agreement with the aforementioned result of the derived nucleotide sequence lengths from the recombinant clones in section 4.2. Indeed, all β-glucosidases were expressed in soluble form in comparable quantities.

4.6 Screening of β-glucosidase candidates by enzyme activity assay

To test whether recombinant enzymes from the four candidate β-glucosidases performed their function, the activity toward *p*NPG and steviosides were determined by using enzyme from the crude extracts. β-glucosidase activity was assayed using *p*NPG as a substrate by incubation the extract with 2 mM *p*NPG at 37°C for 10 min. Among the four assayed β-glucosidases, only EcBgl 4 provided the activity against *p*NPG (data not shown). To examine the steviosides hydrolyzing activity of the recombinant β-glucosidases, the crude enzymes were incubated with 1 mg/ml steviosides (rebaudioside A and stevioside) at 37°C for 1 h and the reaction mixture was analyzed by TLC. The obvious reaction product of steviosides degradation was presented by using EcBgl 4 as shown in figure 4.7. These results demonstrared that only EcBgl 4 contained both β-glucosidase and steviosides degradation activities. Hence, EcBgl 4 was chosen for the subsequent experiments.

In order to determine the enzyme substrate and the reaction product, EcBgl 4 was incubated with either1 mg/ml stevioside or rebaudioside A for 24 h and the reaction mixture was monitored by TLC. The result showed that in the reaction of EcBgl 4 incubated with stevioside, a reaction product was detected along with stevioside was disappeared (Figure 4.8). Following the TLC analysis, the reaction

samples were subjected to HPLC to ensure the stevioside hydrolysis activity performed by EcBgl 4 and also to identify the products (Figure 4.9). Complete disappearance of stevioside was accompanied by the accumulation of the prominently new peak which had a retention time corresponding to rubusoside standard, implying that rubusoside was the product. These results were supported by the NMR structural elucidation of rubusoside, the hydrolysis product of stevioside hydrolyzing β-glucosidase from *Bacteroides thetaiotaomicron* performed by Somsiri Udompaisarn as two enzymes produced the same retention time of the product demonstrated by HPLC. To confirm the stevioside cleaved by the enzyme, glucose assay was performed to determine glucose released from the reaction. Glucose product was detected, indicating that stevioside was hydrolyzed by indeed β-glucosidase activity of EcBgl 4. In addition, the purified EcBgl 4 also showed the hydrolysis activity in the same manner as the enzyme from crude extract and had no detectable product as well as no decrease in rebaudioside A was observed (Figure 4.14). Thus, EcBgl 4 specifically catalysed the conversion of stevioside into rubusoside but not rebaudioside A.

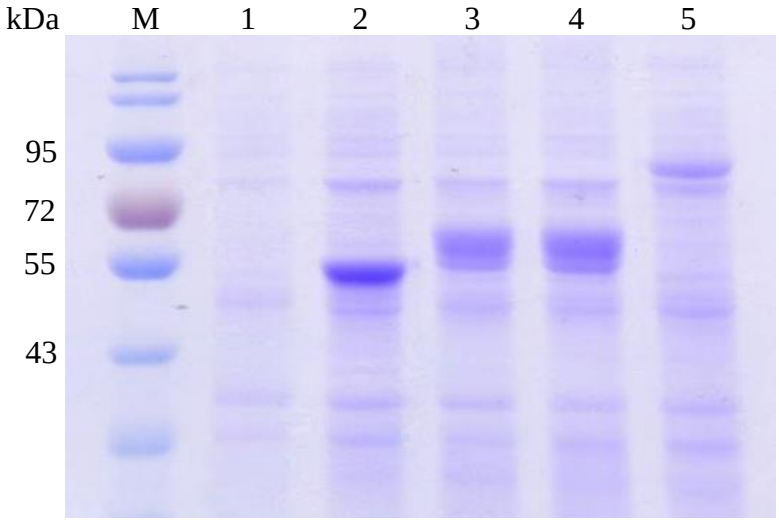


Figure 4.6 SDS- PAGE (10%) analysis of expressed β-glucosidases

E. coli BL21(DE3) harboring His_6-tagged β-glucosidases were induced by 0.2 mM IPTG at 25 °C for 4 hours. Soluble fractions of crude extracts were run on SDS-PAGE and the gel was visualized by staining with Coomassie Brilliant Blue R250. Lane M, broad range protein marker; lane 1, vector control whole cell lysate; lane 2, EcBgl 1; lane 3, EcBgl 2; lane 4, EcBgl 3; and lane 5, EcBgl 4.

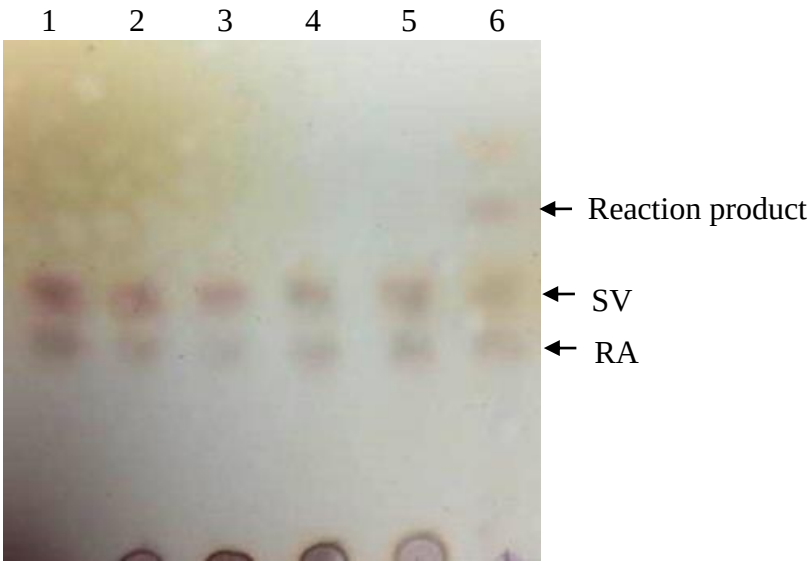


Figure 4.7 TLC chromatogram of stevioside hydrolysis by using recombinant β-glucosidases.

Crude extract of expressed β-glucosidase cells were screened for the hydrolytic activity towards steviosides by incubating with 1 mg/ml steviosides, rebaudioside A (RA) and stevioside (SV) at 37°C for 1 h. One microliter of the reaction mixture was loaded onto silica gel TLC with sulfuric acid staining. Lane 1, standard RA and SV; lane 2; crude extract vector control; lane 3, EcBgl 1; lane 4, EcBgl 2; lane 5, EcBgl 3; lane 6, EcBgl 4.

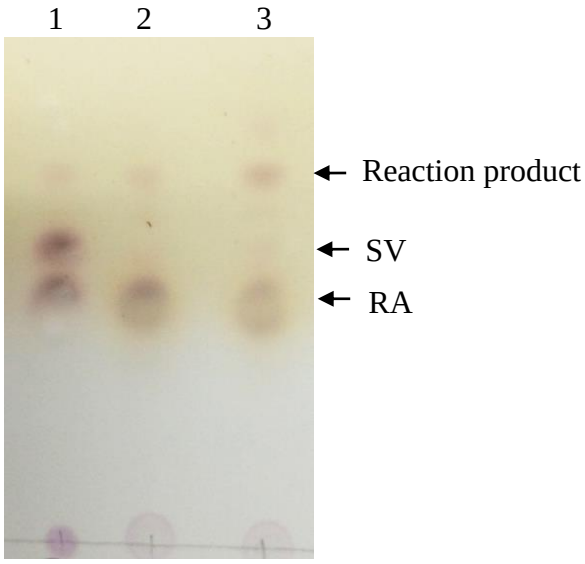


Figure 4.8 TLC chromatogram of the reaction product of hydrolysis of stevioside using EcBgl 4.

Enzyme crude extract EcBgl 4 was incubated with 1 mg/ml of either stevioside or rebaudioside A at 37°C for 24 h and the reaction mixture was monitored by TLC. Lane 1, crude extract vector control incubated with RA and SV; lane 2, EcBgl 4 incubated with RA; lane 3, EcBgl 4 incubated with SV.

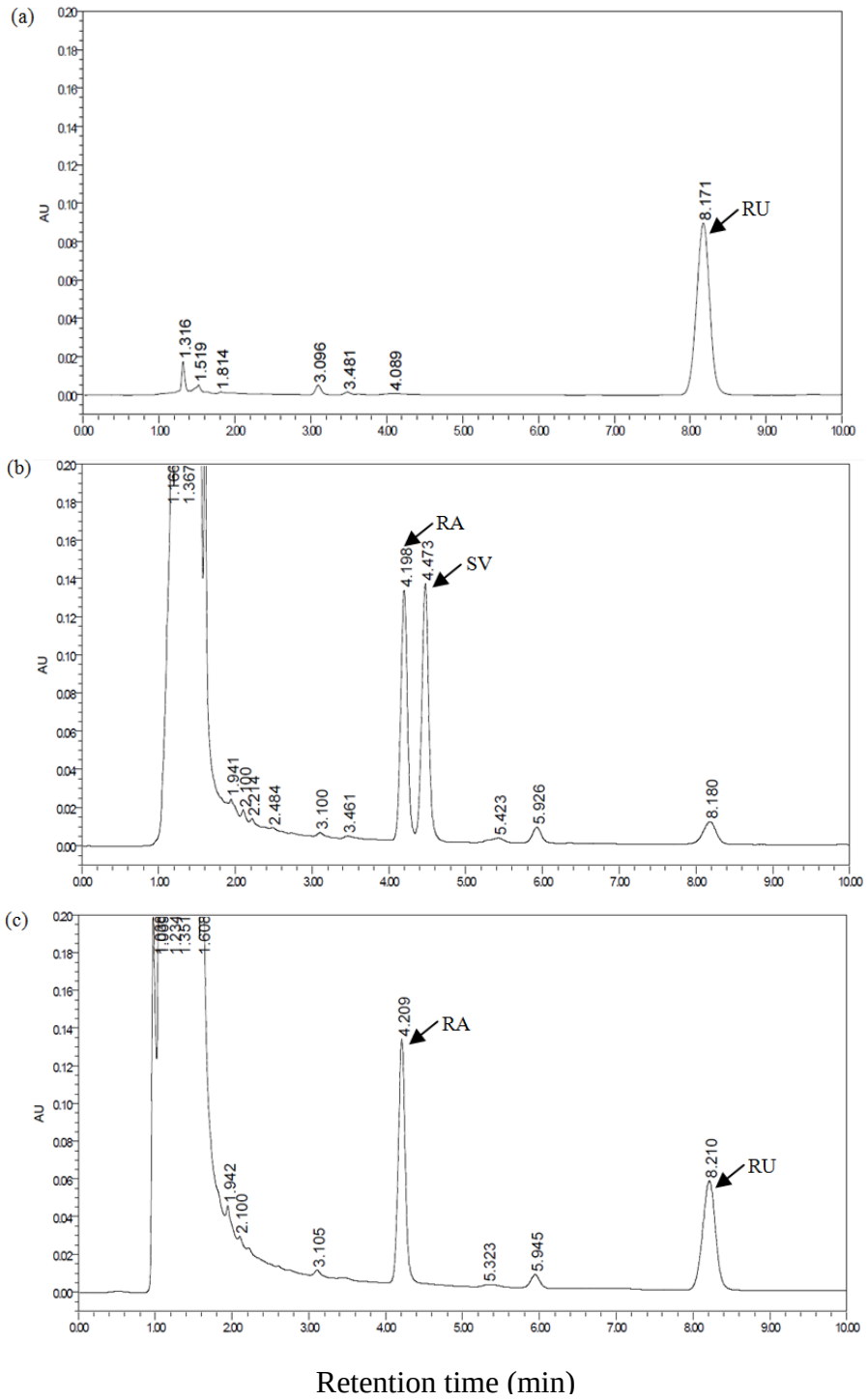


Figure 4.9 HPLC chromatogram of conversion stevioside to rubusoside by crude extract EcBgl 4.

(a) standard rubusoside; RU. (b) vector control+rebaudioside A; RA and stevioside; SV. (c) EcBgl4+RA and SV.

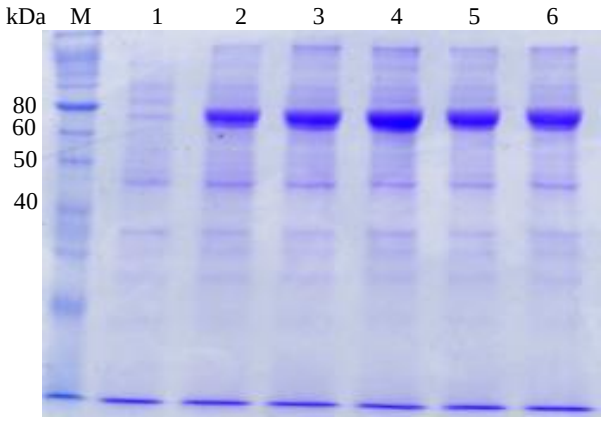
4.7 EcBgl 4 expression and purification

To achieve the highest performing activity, EcBgl 4 expression condition was optimized by variation of IPTG concentration along with incubation temperature and time. The enzyme activity was determined as the relative specific activity of the crude extract towards *p*NPG as described in section 3.4.6.1.1. Whole cell lysates were run along with the uninduced fraction which had no target protein represented, indicating that there were no constitutive expression in the absence of the inducer (i.e., IPTG). Within the same condition of variation, no significant difference in the EcBgl 4 expression level was monitored by densitometric analysis after SDS-PAGE. Similarly increased in the specific activity was observed by induction with 0.2, 0.25, and 0.3 mM IPTG and maximum activity was obtained by using 0.2 mM IPTG (Figure 4.10). In order to determine the optimum culture temperature, the cells were induced by 0.2 mM IPTG for 4 h at 37°C, 30°C, and 25°C. The specific activity was obviously increased in 25°C optimized condition (Figure 4.11). By inducing the cell culture with 0.2 mM IPTG at 25°C within the defined incubation time, overnight expression showed the highest activity (Figure 4.12).

After the optimum expression condition of EcBgl 4 was obtained, EcBgl 4 was subjected to large-scale cell growth and protein expression. The expressed protein was purified to homogeneity from the cellular extracts using a Ni-NTA column. SDS-PAGE analysis of EcBgl 4 expression profile and the purification steps were shown in figure 4.13. The molecular mass of the expressed EcBgl 4 and His_6-tagged was 79 kDa, which corresponded well with the predicted molecular mass for from the the amino acid sequences (79.37 kDa).

In addition, purified EcBgl 4 was tested the activity to hydrolyze stevioside. Similar to the enzyme crude extract, the purified form able to converse stevioside to rubusoside and also had no activity towards rebaudioside A as shown by HPLC result in figure 4.14. Rubusoside product was observed in the reaction of the enzyme incubated with impure rebaudioside A, which containing stevioside, so the product was produced by hydrolysis of stevioside.

(a)



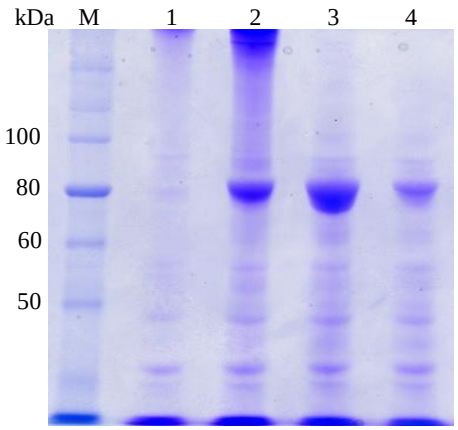
(b)

IPTG concentration (mM)	Amount of EcBgl 4 (% intensity)	Total activity (U)	Specific activity (U/mg)
0.050	67	1.0×10^{-3}	5.9×10^{-3}
0.10	72	2.0×10^{-3}	12×10^{-3}
0.20	70	3.9×10^{-3}	22×10^{-3}
0.25	66	3.3×10^{-3}	19×10^{-3}
0.30	67	3.0×10^{-3}	17×10^{-3}

Figure 4.10 Optimization of EcBgl 4 expression by variation of IPTG.

The cells were grown at 37°C until an $OD_{600\ nm}$ reached 0.5, defined concentration of IPTG was added and cultured at 25°C for 4 h. (a) SDS- PAGE (10%) analysis of whole cell lysates; lane M, broad range protein marker; lane 1, uninduced; lane 2, 0.05 mM; lane 3, 0.1 mM; lane 4, 0.2 mM; lane 5, 0.25 mM; lane 6, 0.3 mM. (b) Amount of EcBgl 4 on whole cell lysates determined by densitometric scanning. Total activity (U) was defined as the amount of enzyme required to release 1.0 μmol of glucose per min.

(a)



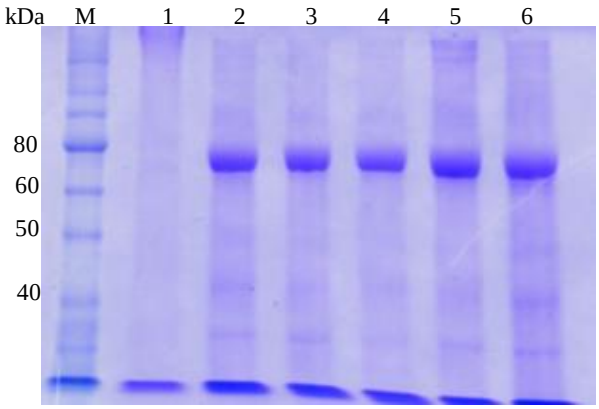
(b)

Induction temperature (°C)	Amount of EcBgl 4 (% intensity)	Total activity (U)	Specific activity (U/mg)
37	80	1.4×10^{-3}	17×10^{-3}
30	74	1.7×10^{-3}	24×10^{-3}
25	65	3.2×10^{-3}	79×10^{-3}

Figure 4.11 Optimization of EcBgl 4 expression by variation of temperature.

The cells were grown at 37°C until an $OD_{600\ nm}$ reached 0.5, 0.2 IPTG was added and cultured at defined temperature for 4 h. (a) SDS- PAGE (10%) analysis of whole cell lysates; lane M, broad range protein marker; lane 1, uninduced; lane 2, 37°C; lane 3, 30°C; lane 4, 25°C; (b) Amount of EcBgl 4 on whole cell lysates determined by densitometric scanning. Total activity (U) was defined as the amount of enzyme required to release 1.0 μmol of glucose per min.

(a)



(b)

Induction time (h)	Amount of EcBgl 4 (% intensity)	Total activity (U)	Specific activity (U/mg)
3.0	78	1.2×10^{-3}	39×10^{-3}
4.0	88	1.1×10^{-3}	67×10^{-3}
5.0	86	1.4×10^{-3}	49×10^{-3}
6.0	84	1.4×10^{-3}	38×10^{-3}
Over night	82	2.7×10^{-3}	73×10^{-3}

Figure 4.12 Optimization of EcBgl 4 expression by variation of induction time.

The cells were grown at 37°C until an $OD_{600\ nm}$ reached 0.5, 0.2 IPTG was added and cultured at 25°C for defined additional time. (a) SDS- PAGE (10%) analysis of whole cell lysates; lane M, broad range protein marker; lane 1, uninduced; lane 2, 3 h; lane 3, 4 h; lane 4, 5 h; lane 5, 6 h; lane 6, overnight. (b) Amount of EcBgl 4 on whole cell lysates determined by densitometric scanning. Total activity (U) was defined as the amount of enzyme required to release 1.0 μmol of glucose per min.

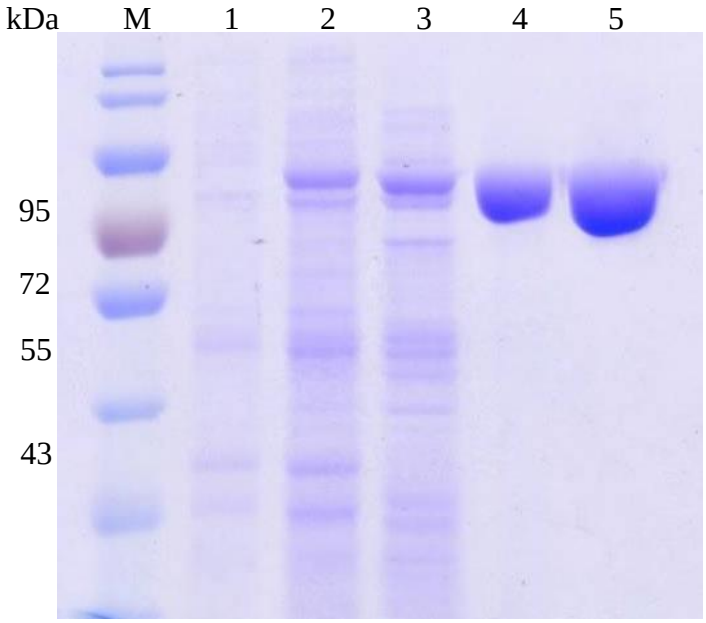


Figure 4.13 SDS- PAGE (10%) analysis of purification of His_6-tagged EcBgl 4.

Expressed EcBgl 4 was lysed and the supernatant was loaded onto Ni-NTA column. Lane M, broad range protein marker; lane 1, vector control whole cell lysate; Lane 2, crude extract; lane 3, flow through fraction in 20 mM imidazole; lane 4, fraction eluted with 75 mM imidazole; lane 5, fraction eluted with 150 mM imidazole.

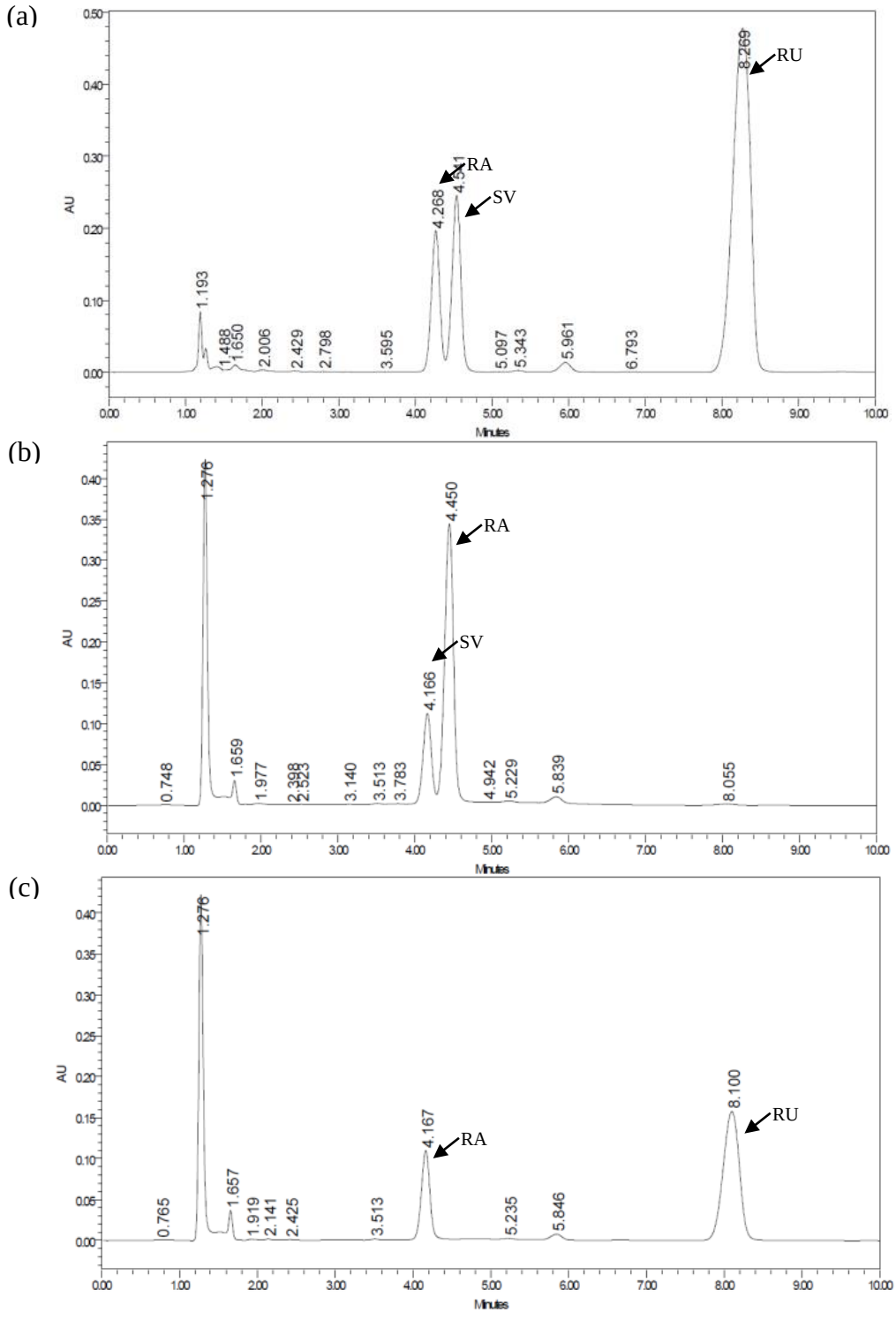


Figure 4.14 HPLC chromatogram of conversion stevioside to rubusoside by purified EcBgl 4.

(a) standard rebaudioside A, stevioside; SV, and rubusoside; RU. (b) control+ SV (c) EcBgl 4+SV.

4.8 Characterization of EcBgl 4

4.8.1 pH optimum

The effect of pH on purified EcBgl 4 activity against *p*NPG and stevioside was examined at 37°C in various pH of Britton-Robinson buffer ranging from pH 3.0 to 10.0. The activity on *p*NPG was retained more than 50% within a pH range of 5.0 to 7.0 (Figure 4.14). The maximum of EcBgl 4 towards both substrates was observed at pH 6.0. By using stevioside as a substrate, the enzymatic activity at pH 6.0 and pH 7.0 were similar but the activity was dropped rapidly at pH lower than 6.0.

4.8.2 pH stability

To elucidate the effect of pH on the enzyme stability, the purified enzyme was equilibrated in different pH of Britton-Robinson buffer ranging from pH 3.0 to 10.0 at 25 °C. After an hour of incubation, the enzyme solution was then readjusted to pH 6.0 with 1 mM *p*NPG in sodium acetate buffer pH 6.0. The enzyme activity was stabilized by retaining over 70% of its initial activity between pH 6.0 and pH 10.0 and provided the highest activity at pH 7.0. The enzyme had no stability in acidic pH below 6.0 (Figure 4.15).

4.8.3 Temperature optimum

The effect of various temperatures on enzyme activity was elucidated by incubating the enzyme at 25°C–65 °C for 10 min in 50 mM sodium acetate buffer (pH 6.0). The optimal temperature of recombinant EcBgl 4 activity on both *p*NPG and stevioside was 37 °C. The enzyme activity against *p*NPG retained over 50 % of its activity within the temperature range from 30–40 °C and less than 20% of the activity was observed at the temperature between 35-65°C (Figure 4.16).

4.8.4 Thermal stability

Enzyme stability was ascertained over the range of 30 to 50 °C within 1 h. Every 10 min of incubation, the residual activity against *p*NPG was monitored. The result in Figure 4.17 demonstrated that within 1 h of incubation the enzyme was

stable at 30°C and lost about 50% of its activity when incubated at 37°C. However, the enzyme showed a poor thermostability since it was unstable at 40°C by rapidly decreased in the activity after 20 min, and also completely lost its activity at 45°C and 50°C after a half time of incubation.

4.8.5 Effect of metal ions and substances

The effect of metal ions on EcBgl 4 activity was assayed by using *p*NPG as a substrate and the result depicted in Table 4.1. At 1 mM, Na^{+}, K^{+}, Mg^{2+}, and Co^{2+} provided no significant reductions in enzyme activity. The enzymatic activity showed partially inhibited in the reaction containing of Ba^{2+}, Fe^{3+}, and Zn^{2+}. Whereas, Hg^{2+}, Cu^{2+}, Ag^{2+} completely inactivated the enzyme activity and the presence of Mn^{2}+ mostly inhibited the activity. EDTA had on effect no enzyme activity while PMSF was found to enhance the activity. Moreover, 1% SDS strongly reduced enzyme activity.

4.8.6 Substrate specificity

The relative rates of hydrolysis of two types of the substrates were compared separately, aryl-glycoside synthetic substrates and sacharides with *p*NPG and steviosides, respectively as summarized in Table 4.2 and Table 4.3. Among the synthetic substrates, the enzyme had no activity towards *p*-Nitrophenyl-N-acetyl-β-D-glucosaminide, *p*-Nitrophenyl α-D-glucopyranoside or *p*-Nitrophenyl β-D-galactopyranoside. When the sacharides were used as substrates, interestingly, the enzyme showed substantial hydrolytic activity against a cyanogenic β-glucoside, amygdalin. Sucrose was hydrolyzed at the high rate (89%) and trehalose relative rate was twice as rebaudioside A. Less than 3% of the enzyme activity on cellobiose, lactose, and isomaltose were detected whereas arbutin was not hydrolyzed.

4.8.7 Kinetic study of EcBgl 4

The kinetic data was obtained by incubating purified EcBgl 4 with either *p*NPG (0.05 to 6 mM) or stevioside (0.5 to 40 mM) at 37°C and Michaelis-Menten curves were plotted using Graphpad Prism 5.0 software as shown in Figure 4.18 and Figure 4.19. The calculated Michaelis-Menten constants (K_m), maximal velocities

(V_{max}), and k_{cat} of *p*NPG and stevioside were summarized in Table 4.4. The kinetic parameters for *p*NPG were determined as follows: K_m 0.37±0.11 mM, V_{max} 1007±91.50 pmol/min, k_{cat} 3176 s^{-1}, and k_{cat}/K_m 8583 $mM^{-1}s^{-1}$, while for stevioside: K_m 1.820±0.5100 mM, V_{max} 2753±221.8 pmol/min, k_{cat} 173.6 s^{-1}, and k_{cat}/K_m 95.41 $mM^{-1}s^{-1}$. The K_m value for *p*NPG was lower than stevioside, implying that EcBgl 4 had higher affinity to *p*NPG than stevioside. In addition, since the resulting V_{max} and k_{cat} for *p*NPG were higher than stevioside, EcBgl 4 sinificantly higher hydrolyzed *p*NPG than stevioside with 90-fold catalytic efficiency. These results indicated that *p*NPG was better substrate than stevioside.

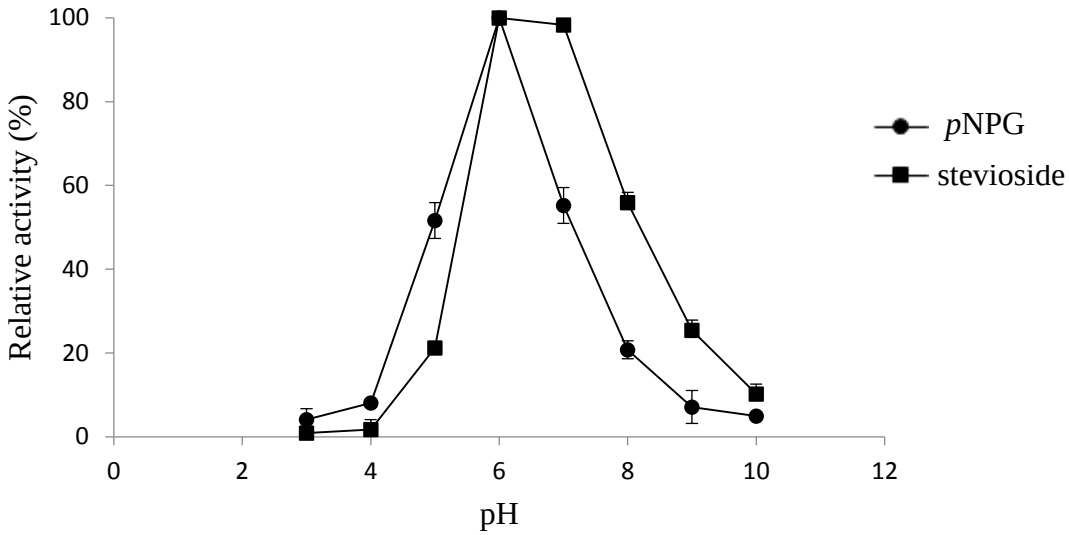


Figure 4.15 Optimum pH on enzyme activity.

The purified EcBgl 4 was assayed for enzyme activity at different pH ranging from 3.0 to 10.0 at 37°C using Britton-Robinson universal buffer. The residual activity on both *p*NPG and stevioside was expressed as a percentage of that at pH 6.0.

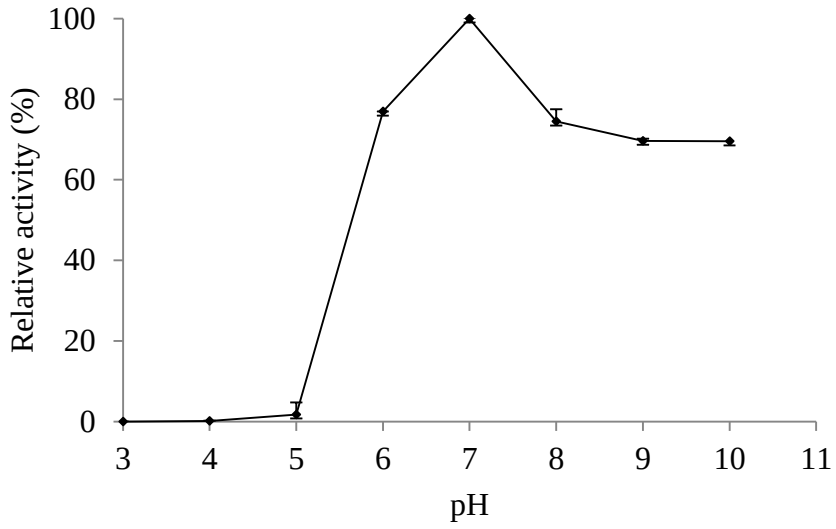


Figure 4.16 pH stability of EcBgl 4.

The purified EcBgl 4 was preincubated in Britton-Robinson universal buffer over a pH range of 3.0–10.0 at 25°C for an hour. The enzyme mixture was readjusted to pH 6.0 before monitoring the activity towards *p*NPG. The enzyme activity was expressed as a percentage of that at pH 7.0.

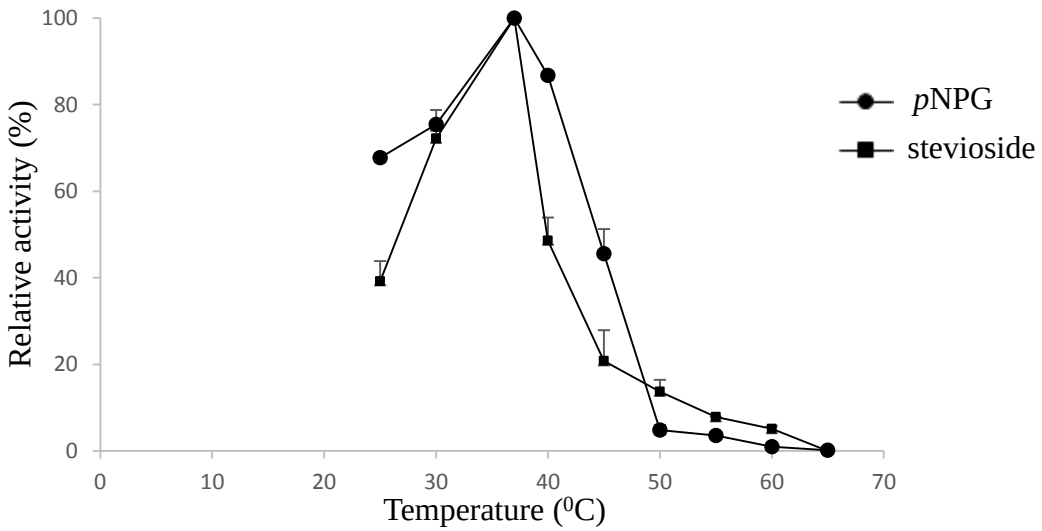


Figure 4.17 Optimum temperature on enzyme activity.

The purified EcBgl 4 was assayed at various temperatures as indicated (25°C–65 °C). The residual activity on both *p*NPG and stevioside was expressed as a percentage of that at pH 6.0 and 37°C.

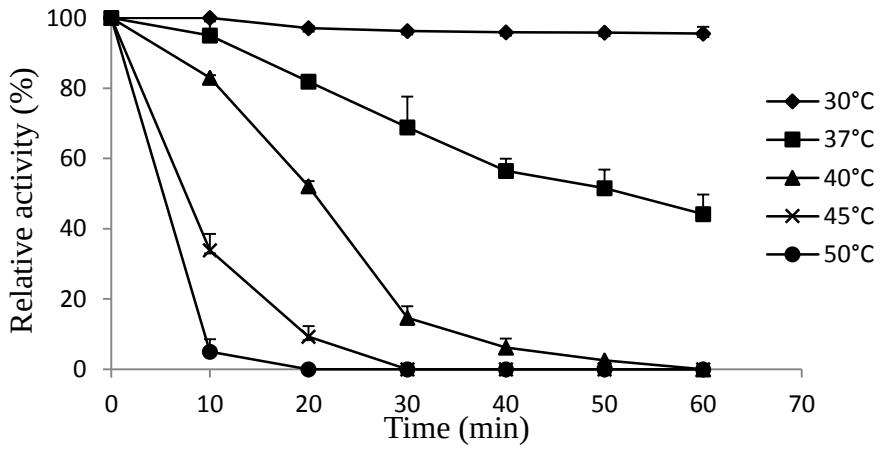


Figure 4.18 Thermal stability of EcBgl 4.

The purified EcBgl 4 was preincubated at various temperatures ranging from 30°C–50 °C for an hour. Every 10 min, the enzyme was aliquoted to measure the remaining activity on *p*NPG at pH 6.0 and 37°C.

Table 4.1 Effect of metal ions on EcBgl 4 activity.

Purified EcBgl 4 was incubated with various metal ions at final concentration of 1 mM at 25°C for 30 min. The remaining activity on *p*NPG was measured as a percentage of the activity assayed without metal ions added (control). The experiment was performed in three independent experiments.

Substance	Relative activity (%)
Control	100
NaCl	89.9±1.79
$MgCl_2$	89.7±0.90
KCl	89.6±2.58
$CoCl_2$	87.7±3.76
$CaCl_2$	84.7±0.0500
$BaCl_2$	82.1±4.38
$FeCl_3$	81.6±11.9
$ZnCl_2$	81.2±1.32
$MnCl_2$	9.90±1.78
$AgNO_3$	0
$CuCl_2$	0
$HgCl_2$	0
PMSF	120±8.80
EDTA	97.4±1.36
1%SDS	0.63±0.21

Table 4.2 Substrate specificity of EcBgl 4 on aryl-glycoside synthetic substrates.

The purified EcBgl 4 was incubated with 2 mM aryl-glycoside synthetic substrates in 50 mM sodium acetate buffer (pH 6.0) at 37°C for 10 min. The relative activity on different substrates was determined as relative values of the percentage activity obtained with *p*NPG.

Substrate	Linkage of glycosyl group	Relative activity (%)
p-Nitrophenyl β-D-glucopyranoside	β-Glc	100
p-Nitrophenyl α-D-glucopyranoside	α-Glc	0
p-Nitrophenyl β-D-galactopyranoside	β-Gal	0
p-Nitrophenyl-N-acetyl-β-D-glucosaminide	β-Glc	0.01±0.00

100% was corresponding to 4655.1±246.18 μmol/min/mg when *p*-Nitrophenyl β-D-glucopyranoside was used as substrate.

Table 4.3 Substrate specificity of EcBgl 4 on saccharides.

The purified EcBgl 4 was incubated with 2 mM saccharides in 50 mM sodium acetate buffer (pH 6.0) at 37°C for 10 min. The relative activity on different substrates was determined as relative values of the percentage activity obtained with stevioside.

Substrate	Linkage of glycosyl group	Relative activity (%)
stevioside	Glc β-(1→2) Glc	100
rebaudioside A	Glc β-(1→2) Glc β-(1→3) Glc	12.5±0.060
amygdalin	β-Glc	200 or 100±6.64*
arbutin	α-Glc	0
cellobiose	Glc β-(1→4) Glc	0.815±0.380
lactose	Gal β-(1→4) Glc	2.52±0.190
sucrose	Glc α-(1→2) Fru	88.9±18.4
trehalose	Glc α-(1→1) Glc	24.4±4.48
isomaltose	Glc α-(1→6) Glc	1.54±0.490

100% was corresponding to 16.2±1.23 μmol/min/mg when stevioside was used as substrate.

* Amygdalin contained two possible linkages that EcBgl 4 able to cleave. Cleavage of Glc β-(1→1) Glc provided a glucose molecule, relative activity should be 200%, whereas double cleavage of Glc β-(1→2) Glc and Glc β-(1→1) benzene glucose molecules were released, relative activity should be 100±6.64%.

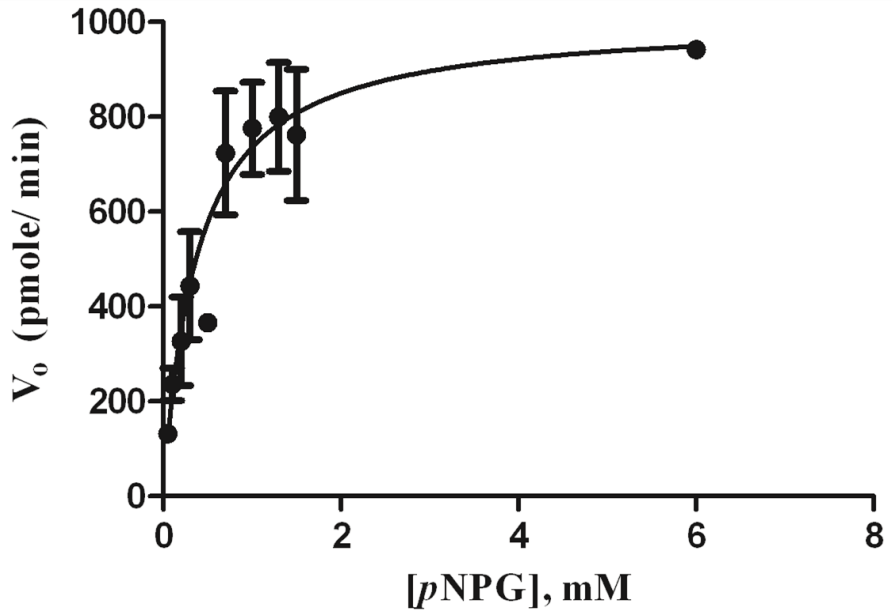


Figure 4.19 Michaelis-Menten plots of EcBgl 4 on *p*NPG.

The enzyme was incubated with *p*NPG concentrations ranging from 0.05 to 6 mM in 50 mM sodium acetate buffer (pH 6.0). The Michaelis–Menten curve was plotted using initial velocity (v_0) versus *p*NPG concentrations by a curve-fitting function of Graphpad Prism 5.0 software.

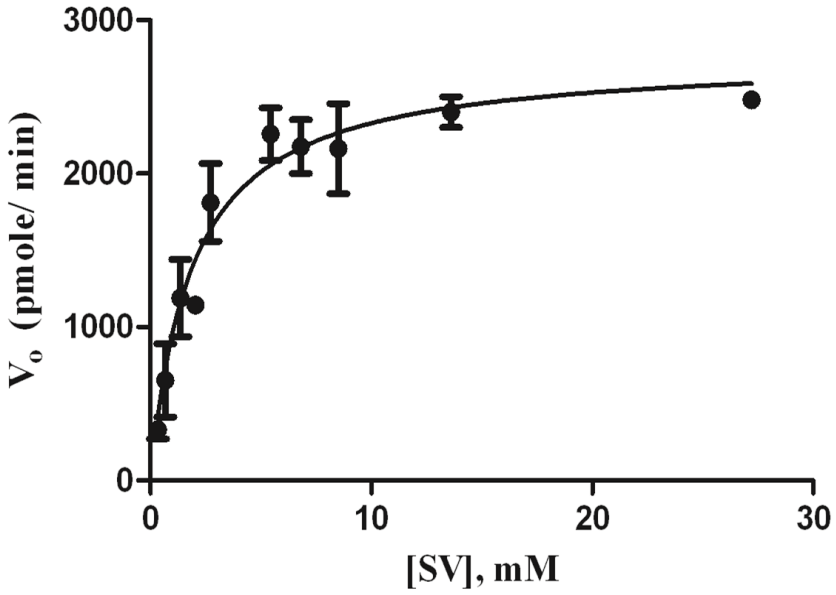


Figure 4.20 Michaelis-Menten plots of EcBgl 4 on stevioside.

The enzyme was incubated with stevioside concentrations ranging from 0.5 to 30 mM in 50 mM sodium acetate buffer (pH 6.0). The Michaelis–Menten curve was plotted using initial velocity (v_0) versus stevioside concentrations by a curve-fitting function of Graphpad Prism 5.0 software.

Table 4.4 Kinetic parameters of EcBgl 4 towards *p*NPG and stevioside.

Kinetic parameters, K_m and V_{max} were calculated from the Michaelis-Menten equation using a curve-fitting function of Graphpad Prism 5.0 software. The apparent k_{cat} values were calculated by dividing the V_{max} by total enzyme concentration.

Substrate	K_m (mM)	V_{max} (pmol/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
*p*NPG	0.3700±0.1100	1007±91.50	3176	8583
stevioside	1.82±0.510	2753±221.8	173.6	95.41

CHAPTER V
DISCUSSION

Although previous studies on the metabolism of steviol glycosides by human intestinal microflora have shown only bacteroides, the most prevalent group of bacteria in the gut able to degrade steviol glycosides via their β-glucosidase activity and the rate of conversion of stevioside was higher than rebaudioside A (Koyama et al., 2003; Gardana, Simonetti, Canzi, Zanchi, & Pietta 2003). Conversely, our results indicating that *Enterococcus casseliflavus*, a bacterium isolated from bacterial contaminant of stevia leaf extract which in fact appears in the human gut as a minor enterococci group, exhibited stevioside hydrolyzing activity. Similar to those reported, the β-glucosidase possessed this role and stevioside was much more hydrolyzed than rebaudioside A. Thus, this is the first study elucidation of the role of *E. casseliflavus* with its β-glucosidase in the hydrolysis of stevioside. The cloning, expression in *E. coli*, and biochemical characterization of β-glucosidase from *E. casseliflavus* were reported herein.

In the present study, to investigate the enzyme from *E. casseliflavus* responsible for steviol glycosides hydrolyzing activity, full-length of the four *β-glucosidase* candidate genes were cloned and expressed in *E.coli* BL21(DE3). Among these genes, *EcBgl 1*, *EcBgl 2*, and *EcBgl 3* were GH 1 β-glucosidase and a GH 3 β-glucosidase, *EcBgl 4*. Even all β-glucosidases were overexpressed in soluble form with corresponding to the expected molecular mass but only an EcBgl 4 exhibited functionally active that provided both hydrolytic activities towards stevioside and its synthetic substrate, *p*NPG. To prelude the optimization for obtaining the activity of all three GH 1 β-glucosidases, the culture temperature was adjusted to 16°C to allow slowly grown of the cell as well as gradually folded of the target proteins. The reaction pH of the enzyme crude extracts on *p*NPG and stevioside was also varied. Not only preferable pH for general recombinant β-glucosidase between 6.0 and 7.0 were used, but also pH 8.5 that optimum pH of the reported recombinant GH 1 β-glucosidase of *Streptomyces* sp. GXT6 (Wang et al., 2015). In addition, the expression host was

changed from *E.coli* BL21(DE3) to *E.coli* Rosetta2(DE3). As this host strain was Tuner^{TM} derivatives containing the pRARE2 plasmid (p15A replicon), expresses seven rare tRNAs to facilitates expression of genes that encode rare *E. coli* codons which often improves the levels of protein production in many cases. However, they did not perform their function neither toward *p*NPG nor steviosides. Possible explanation of the inactive expressing proteins may due to three GH 1 *β-glucosidases* were pseudogenes, or *p*NPG was not their substrate, but rather than this, they had no activity on steviosides. The non performing enzymes may caused by improperly fold.

According to the HPLC analysis of reaction product upon incubating crude extract and purified EcBgl 4 with stevioside, the only one product, rubusoside was found. Also, the product was confirmed by NMR result of rubusoside production of β-glucosidase from *Bacteroides thetaiotaomicron* performed by Somsiri Udompisan as well as glucose released from the reaction was detected. This suggested that EcBgl 4 specifically hydrolyzed the glucose moiety of the sophoroside at C-13 in stevioside to produce rubusoside.

EcBgl 4 was expressed in form of His_6-tagged fusion protein at the C-terminus. The introduction of His_6-tagged was successful to facilitate purification of the target protein by using a single step of nickel affinity chromatography. Purified EcBgl 4 showed the optimum pH at 6.0 on both *p*NPG and stevioside. As well as most *β*-glucosidases from bacterial and fungal origins, they showed a pH optimum between pH 4 and 7.5, which in slightly acidic or neutral pH ranges depending on their source and cellular location. β-glucosidases were found to be stable over a range of pH from 4 to 9 (Ketudat Cairns & Esen, 2010), EcBgl 4 activity was also stabilized between pH 6.0 and pH 10.0. Towards both *p*NPG and stevioside, the enzyme optimal temperature was found at 37 °C which is physiological temperature and was inactivated at 60°C. In agreement with mesophilic β-glucosidases, they showed the highest activity at 30–65°C, but were generally inactivated at and above 55–70°C (Dhamawardhana, Ellis & Carlson 1995; Chuankhayan et al., 2005; Nagano, Matsushima & Hara-Nishimura 2005). Since the industrial application of β-glucosidase requires a thermostable enzyme, and temperature stability of EcBgl 4 was also measured and the enzyme performed poor thermostability. To overcome this obstracle, immobilized enzyme strategy should be established. According to the previous study demonstrated that

Thermus thermophiles immobilized lactase used for the production of rubusoside increased thermal stability with higher efficiency of hydrolysis compared to that using free enzyme (Nguyen et al., 2014). As well as cost effective, the immobilization procedure on alginate beads was very easily carried out under mild conditions, giving it considerable potential for industrial utilizations (Arya & Srivastava, 2006; Goel, Sharma & Tandon 2006). Moreover, temperature optima of over 100°C of β -glucosidases from a thermophilic bacterium, *T. maritima* BglA was reported (Kengen, Luesink, Stams & Zehnder 1993). By engineering of a bacterial β -glucosidase to contain the same N-terminal and C-terminal residues as *T. maritima* BglA, allowed hydrogen bonding between these termini which the interactions might be important for high stability lead to stabilization of the enzyme (Nam et al., 2008). This thermostability was also caused by an increase in number of proline residues, electrostatic bridges, and internal water molecules, and binding of more subunits in the quaternary structure compared to many mesophilic enzymes (Chi et al., 1999).

Despite, uninhibitory effect by most metal ions to β-glucosidases, the enzyme activity was completely inhibited by Hg^{2+}, Cu^{2+}, and Ag^{2+} that were reported as potent β-glucosidases inhibitors (Zollner 1989), Fe^{3+} was also reported as well but EcBgl 4 in contrast, was just partially inactivated. Hg^{2+} reacts with thiol groups of cysteines required for activity or cheated active site acidic amino acids resulting in enzyme inactivation (Tsakalidou & Kalantzopoulos 1992; Chich, Marchesseau & Gripon 1997). Cu^{2+} and Ag^{2+} strongly precipitated protein caused enzyme inactivation. Zn^{2+}, Na^{+}, K^{+}, Mg^{2+} Ca^{2+}, and Co^{2+} exhibited no significant decreased the enzyme activity which in accordance with *C. michiganense* β-glucosidase (Nakano, Okamoto, Yatake, Kiso, & Kitahata 1998). But, these metal ions except Co^{2+} in the final concentration of 10 mM were observed in enhance the enzymatic activity of β-glucosidase from a thermophilic fungus, *Melanocarpus* sp. MTCC 3922 (Kaur, Chadha, Kumar, Ghatora, & Saini, 2007). However, in this study only PMSF was found to slightly enhance the activity since it might increase stability of enzyme and prevented the degradation of enzyme from trace proteases. Mn^{2}+ performed different effects on β-glucosidases, it strongly inactivated EcBgl 4 but had positive effect on a β-glucosidase from environmental DNA (Kim et al., 2007), and provided no effect on C. *michiganense* β-glucosidase, stevioside-specific β-Glucosidase from *A. aculeatus*

(Ko et al., 2012), and *F. johnsonae* (Okamoto, Nakano, Yatake, Kiso, & Kitahata, 2000). Many reported β-glucosidases, were not denaturated by ionic detergents such as SDS, which allows extraction with buffers containing up to 3% SDS and zymogram development after SDS-PAGE when samples were run without heating (Ketudat Cairns & Esen, 2010). Conversely, the presenting of 1% SDS caused totally lost EcBgl 4 activity as well as strongly inhibited cytoplasmic rice β-glucosidase Os1BGlu4 (Rouyi et.al., 2014). Similar to the others β-glucosidases, EDTA had no effect on the enzyme activity.

For the substrate specificity profile, except *p*-Nitrophenyl β-D-glucopyranoside (*p*NPG) EcBgl 4 failed to show activity on other aryl-glycoside synthetic substrates, indicating it was specific not only to β-glycosidic linkage but also to glucose residue of the glycone moiety of the synthetic substrates. In case of aglycone specificity determination by using natural glycosides, the enzyme exhibited broad substrate catalysis with preferentially active against β-glycosides however it hydrolyzed α-glycosides towards α-(1→1) and α-(1→2)-glycosides; trehalose and sucrose, respectively. Interestingly, among the natural saccharides the enzyme showed the highest activity towards aryl-cyanogenic β-glucoside, amygdalin. Amygdalin is a natural product has been used as alternative drug for anti-tumor and cancer (Aritomi, Kumor, & Kawasaki, 1985; Chang, Shin, & Yang, 2006; Kwon, Lee, & Hong, 2010). Several studies have been carried out to investigate the anti-tumor mechanism and metabolism of amygdalin and showed that the compound itself has no anti-tumor activity, but rather the active ingredients were its degradation products. For example, the purified degradation products of amygdalin produced by the extracellular enzymes from *Aspergillus niger* performed high anti-tumor activity by significantly suppressed the growth of S-18 tumor cells (Chang & Zhang, 2012). Amygdalin activated with β-glucosidase with stronger effect than its natural form on growth inhibition and induction of apoptosis was found in HepG2 cells cytoactivity tested (Zhou et al., 2012). Hence, the ability to degrade amygdalin of EcBgl 4 may be useful for preparation of activated amygdalin in order to further use as anti-tumor agent. Our finding showed that EcBgl 4 has different substrate hydrolysis profile from other β-glycosidases, suggesting that it is a novel β-glycosidase.

Moreover, similar to most stevioside hydrolyzing β-glycosidases, EcBgl 4 rarely hydrolyzed rebaudioside A. The result was thought to be due to steric hindrance, as it has a β-glucosyl (1→3) derivative of the C-13-hydroxyl group of stevioside (Wang et al., 2015). Because the rate of hydrolysis of rebaudioside A monitored by glucose assay was significantly lower than that of stevioside and *p*NPG. However, no reaction product was observed by incubated the enzyme with rebaudioside A using HPLC. As glucose assay provided higher sensitivity than HPLC, resulting in the detectable glucose, thus, we judged EcBgl 4 has little activity against rebaudioside A.

The result of substrate specificity of EcBgl 4 was in agreement with the kinetic parameter on its substrates. The enzyme hydrolyzed *p*NPG more efficiently than stevioside with catatytic efficiently (k_{cat}/K_m) of 8583 $mM^{-1}s^{-1}$ for *p*NPG and 95.41 $mM^{-1}s^{-1}$ for stevioside. Unlike other β-glycosidases, which have relatively low k_{cat} values (~300 s^{-1} or lower) (Ketudat Cairns & Esen, 2010), whereas EcBgl 4 showed significantly higher values of 1848 s^{-1} for *p*NPG. Indeed, when compared to the stevioside hydrolyzing β-glycosidases previously reported, EcBgl 4 was found to be the highest efficient enzyme catalyzed both *p*NPG and stevioside (Table. 5.1).

To the best of our knowledge, a total of six steviosides hydrolyzing β-glucosidases have been repoted. β-glucosidases purified from *Clavibacter michiganense* (Nakano, Okamoto, Yatake, Kiso, & Kitahata 1998) and *Flavobacterium johnsoniae* (Okamoto et al., 2000) were investigated by the same group of researcher. Both enzymes cleaved the *β*-glucosidic linkage of the 19-carboxyl group of rebaudioside A, stevioside, and rubusoside but not degrade *β*-glucosidic linkages of the 13-hydroxyl group of rebaudioside B or steviol bioside. Two β-glucosidases from *Chryseobacterium* sp. (Jiang et al. 2011) and *Aspergillus aculeatus* (Ko et al., 2012) hydrolysis of stevioside into rubusoside were demonstrated. *Chryseobacterium* sp. β-glucosidase derived from the fermentation liquid with no detail of the enzyme chacterization. *A. aculeatus* β-glucosidase was screened from nine commercially providing glycosidases and was first characterized as a stevioside-specific β-glucosidase. This enzyme was proposed to be a potential enzyme for industrial production of rubusoside as it showed highly specific to clave a glucose moiety at 13-hydroxyl group of stevioside and had no hydrolytic activity on β-glucosidic linkage at the 19-carboxyl group of rebaudioside A or stevioside.

Production of steviol from stevioside by a β-glucosidase was isolated from *Penicillium decumbens* naringinase (Ko et al., 2012). Cleavage at both sides of β-glucosidic linkages at the 13-hydroxyl group and 19-carboxyl group of stevioside could produce steviol as a main product and provided steviolbioside and steviol monoglucoside by-products. For this reason, it was claimed that the multi-functional β-glucosidase enzyme produce steviol from stevioside. The biggest similarities between these five steviosides hydrolyzing β-glucosidases were the enzyme sources as they directly purified from their belonging organisms and all were GH 3 glucosidases. Currently, a recombinant stevioside hydrolyzing β-glucosidase from *Streptocomyces* sp. GTX6 was recently constructed (Wang et al. 2015). This enzyme and *A. aculeatus* β-glucosidase served to specifically hydrolyze the glucose moiety of the sophoroside at 13-hydroxyl group of stevioside to produce rubusoside as a final product. Thus, they were proposed to be a potential enzyme for industrial production of rubusoside. Nevertheless, this enzyme was more suitable for the application since it displayed high tolerance to glucose and its time consuming for the conversion of stevioside to rubusoside was less than 6 h whereas the former used 12 h. Together with the β-glucosidase from *E.casseliflavus* (EcBgl 4) presented herein, was shown to be a promising candidate to produce rubusoside. Not only the enzyme specifically converted stevioside to yield rubusoside as a final product but also it was the highest catalytic efficiency enzyme catalyzed stevioside as aforementioned before.

EcBgl 4 and previously reported stevioside-hydrolyzing β-glucosidase from *Streptocomyces* sp. GTX6 were similar in their function in order to hydrolyze the glucosyl residue of the sophoroside at 13-hydroxyl group of stevioside to produce rubusoside. However, the biggest different between these two recombinant enzymes was presented respect to the glycosyl hydrolase family. EcBgl 4 was identified as a GH 3 as well as the five stevioside-hydrolyzing β-glucosidases mentioned before. In contrast to *Streptocomyces* sp. GTX6 β-glucosidase, it was indicated as GH 1 hence it was named BGL1. EcBgl 4 presently exposed was very different in its biochemical properties and substrate specificities from BGL1. For example, the larger molecular mass of EcBgl 4 (79 kDa) than BGL1 (47 kDa) was found. EcBgl 4 showed the same optimum pH at 6.0 on both *p*NPG and stevioside that higher acidic pH than BGL1 optimum pH at 6.5 and 8.5 against *p*NPG and stevioside, respectively. The optimal

temperature (37 °C) of EcBgl 4 was also the same towards both *p*NPG and stevioside which a little higher than that of BGL1 optimal temperature towards *p*NPG (45 °C) and stevioside (50 °C). Nevertheless, poor thermostabilty was observed on both EcBgl 4 and BGL1 activity. Even EcBgl 4 and BGL1 hydrolyzed stevioside on the same glucose residue but they exhibited differ in substrate specificities. EcBgl 4 had high activity against amygdalin but not BGL1.

With regard to the β-glucosidase sources, almost enzymes mentioned above were derived from stepwises purification from the original microorganisms and expression of recombinant protein from the *β-glucosidase* encoding genes. The molecular cloning of genes has specific advantages over the purification procedure. First, it allowed the large production of enzymes for further purification and characterization. On the other hand, in some cases, low amount of enzyme was obtained from the purification lead to inadequate quantities for thorough characterization. As some enzymes were not abundant accumulated in their natural source and more purification steps, less enzyme remained. Second, full-length gene cloning offered the possibility of applying protein engineering methods. Both enzyme mechanism elucidation and catalytic efficiency improvement could be achieved by construction of chimeric and mutant of amino acids at the active site responsible for binding and catalysis (Cicek & Esen 1998).

Table 5.1 Kinetic parameters of four β-glucosidases toward *p*NPG and stevioside.

Enzyme	*p*NPG			stevioside		
	Km (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	*Km* (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
SSGase[a]	0.0500	4.800	95.60	3.600	30.40	8.500
SPGase[b]	1.300	3.200	2.500	10.50	10.20	1.000
BGL[c]	1.400	24.40	17.80	1.500	13.20	9.000
EcBgl 4[d]	0.370	3175	8583	1.800	173.6	95.40

[a] β-glucosidase from *Aspergillus aculeatus* (Ko et al., 2012)

[b] β-glucosidase from *Penicillium decumbens* (Ko et al., 2013)

[c] β-glucosidase from *Streptomyces* sp. GXT6 (Wang et al., 2015)

[d] β-glucosidase from *Enterococcus casseliflavus* (this study)

CHAPTER VI
CONCLUSION

This study aimed to elucidate stevioside hydrolyzing enzyme from an isolated bacterium *Enterrococcus casseliflavus* BO2. In summary, *E. cassiliflavus* BO2, a human intestinal microflora from bacterial contaminant of stevia leaf extract possesses stevioside degradation activity by a β-glucosidase was identified. Four steviol glycosides hydrolyzing β-glucosidase candidate genes were cloned and expressed in C-terminal His_6-tagged soluble forms using *E. coli* BL21(DE3). Only EcBgl 4 had both β-glucosidase activity towards its synthetic substrate, *p*NPG and stevioside hydrolyzing activity. Further hydrolyzed product identification indicated that EcBgl 4 performed catalytic activity by converting stevioside to rubusoside.

The purified enzyme showed the optimum pH and temperature at 6.0 and 37°C, respectively on both *p*NPG and stevioside. The enzyme was stable between pH 6.0-10.0 but was not thermostable. Almost tested metal ions had no significantly effects, however, Hg^{2+}, Cu^{2+}, Ag^{2+}, and 1% SDS completely inhibited and PMSF slightly enhanced the enzyme activity. Broad substrate specificities on natural saccharides were found with the highest activity towards amygdalin but no hydrolytic activity was observed on tested aryl-glycoside synthetic substrates. The kinetic studies revealed that *p*NPG was more efficiency hydrolyzed than stevioside. Importantly, EcBgl 4 appeared to be the most effective stevioside hydrolyzing β-glucosidase compare to the previous reported enzymes.

In conclusion, our results clearly demonstrated that *E. casseliflavus* BO2 possessed stevioside hydrolyzing activity via a β-glucosidase**,** EcBgl 4 and enzyme characterization implying that the novel β-glucosidase served as a potential enzyme for future applications.

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APPENDICES

APPENDIX A

Vector used

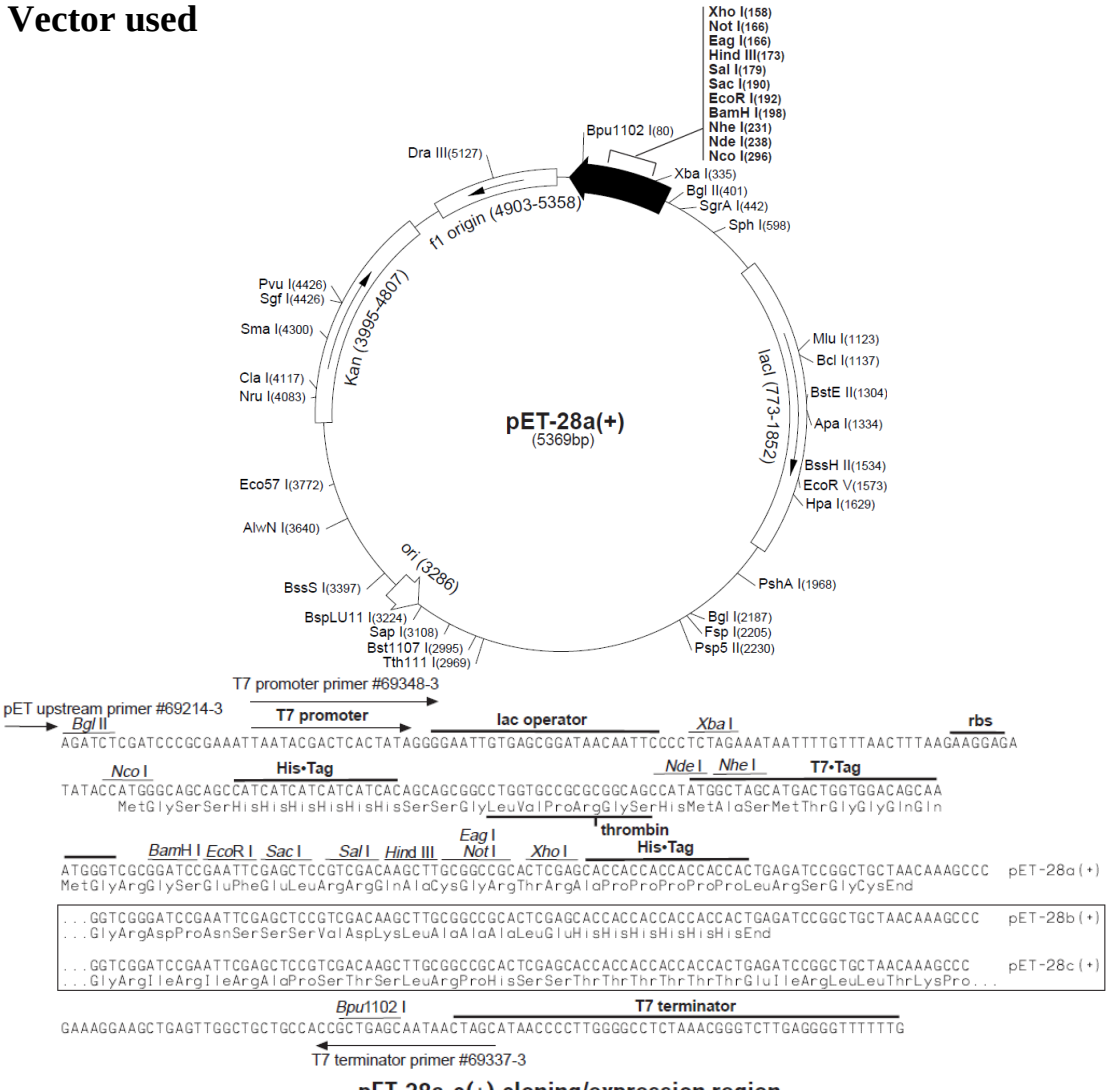

Figure A1. The pET-28a(+) plasmid

The pET-28a(+) plasmid map 5369 bp (upper) and the promoter and multiple cloning sequence (below) is from Novagen, Inc. The plasmid was used as expression vector to express the four β-glucosidase genes with His_6-tagged at C-terminal form under the control of T7 promoter. The vector also contains f1 origin, a ribosome binding site, a lac repressor gene, a kanamycin resistance gene, and multiple cloning site (MCS).

APPENDIX B

DNA sequence and the deduced amino acid sequence of *GH 1 β-glucosidase* genes

```
   1 ATGCAGACAG AAGGTGGTTG GAATGAAGGC GGTAAAAGCT TATCCGTTTA
     M  Q  T  E   G  G  W   N  E  G   G  K  S  L   S  V  Y
  51 TGATATACGG GAAGCATCGG AACAGGCTAG CGATTGGCAT GTAGCAAATG
     D  I  R   E  A  S  E   Q  A  S   D  W  H   V  A  N  D
 101 ATAACTATCA TGCGTATACA GAAGATTTTG ATTATATGCA AGATTTGGGG
     N  Y  H   A  Y  T   E  D  F  D   Y  M  Q   D  L  G
 151 ATGAACATGT ATCGTTTCCA AATTTCTTGG TCAAGGGTCG TTCACGATGG
     M  N  M  Y   R  F  Q   I  S  W   S  R  V  V   H  D  G
 201 AGATGGTGCC TTTAATGAAG AAGGGATCGC CTATTACGAT CGTTTTATTG
     D  G  A   F  N  E  E   G  I  A   Y  Y  D   R  F  I  D
 251 ATGATCTGTT AGCTCGAGGG ATCGAGCCTA TGATTTGTTT GTACCATTTT
     D  L  L   A  R  G   I  E  P  M   I  C  L   Y  H  F
 301 GATATGCCTC TGCATCTGGC GAAAACCTAT AACGGGTTCA TCAGCAAAGA
     D  M  P  L   H  L  A   K  T  Y   N  G  F  I   S  K  E
 351 AGTGAAGGAC GCATTCATTC GTTTTGGAAA AGAAATGGTG GATCGTTTTG
     V  K  D   A  F  I  R   F  G  K   E  M  V   D  R  F  A
 401 CGGATAAAGT CCATTATTGG TTAACCTTTA ACGAACAAAA TCTTTTCCAC
     D  K  V   H  Y  W   L  T  F  N   E  Q  N   L  F  H
 451 CAAGACCATT GTTTTAAGAT CAGCGGCTAT TTAGAAGGGG AAGAAACGAC
     Q  D  H  C   F  K  I   S  G  Y   L  E  G  E   E  T  T
 501 AGAAGAATTG TATCAGATTT TCCACAATGT CATGGTTTGC CATGCAGAAA
     E  E  L   Y  Q  I  F   H  N  V   M  V  C   H  A  E  I
 551 TCTGTAATTA TCTTCATGAA ACAACAAATG CGAAGATCGG CGGCATGCTG
     C  N  Y   L  H  E   T  T  N  A   K  I  G   G  M  L
 601 GCGTATTCTG AGGCGTATCC AGCGACTTGT CGTCCACAAG ACATCAAAGC
     A  Y  S  E   A  Y  P   A  T  C   R  P  Q  D   I  K  A
 651 AGCAAGAGAG TTTGATGAAT TTGCGAATTT TACTTTATTA GATTGCTATG
     A  R  E   F  D  E  F   A  N  F   T  L  L   D  C  Y  A
 701 CGCACGGCAA GTATTCGGCA GCACAAGACC GATACGTGAA AAATCATCAA
     H  G  K   Y  S  A   A  Q  D  R   Y  V  K   N  H  Q
 751 CTCAATATGA ATATTTTACC TGGGGAATTA GAAGCGATCG CAGCGCAGAA
     L  N  M  N   I  L  P   G  E  L   E  A  I  A   A  Q  K
 801 AAATGATTAC ATTGCCTTTA GCTATTATGC TTCTTCTACC TTGAACGCTG
     N  D  Y   I  A  F  S   Y  Y  A   S  S  T   L  N  A  E
 851 AAAAAATTCC TGAAGGAACG CCGCCAAATC GGTACATGAC TTTTGGAAAA
     K  I  P   E  G  T   P  P  N  R   Y  M  T   F  G  K
 901 CAAGACAATC CGTATATTGA AACGACGGAA TGGAATTGGC AAATTGATCC
     Q  D  N  P   Y  I  E   T  T  E   W  N  W  Q   I  D  P
 951 ACTCGGCTTC CGTGATGTCT TAAATAAAGT CTATCAGCGG TACCGGTTGC
     L  G  F   R  D  V  L   N  K  V   Y  Q  R   Y  R  L  P
1001 CAGTGTTTCC GATTGAAAAC GGGATTGGTG TCAGAGAGCA TTGGAATGGC
     V  F  P   I  E  N   G  I  G  V   R  E  H   W  N  G
```

Figure B1 Nucleotide sequence and the deduced amino acid sequence of *EcBgl 1* gene showed an open reading frame of 1365 bp encoding 455 amino acids.

```
1051 AAAGAACCGA TTCAAGATGA TTACCGTATT GCCTATCATC AAGAGCATAT
     K  E  P  I   Q  D  D   Y  R  I   A  Y  H  Q   E  H  I
1101 TCGAGCGATG TTTGAAGCGA TGGTGGAAGA TGGAGTGGTC TGTCTTGGGT
      R  A  M   F  E  A  M   V  E  D   G  V  V   C  L  G  Y
1151 ATCTTGGTTG GGGGCTGATC GACATTTTGA GCTCGCAAGG TGATATGGAG
       L  G  W   G  L  I   D  I  L  S   S  Q  G   D  M  E
1201 AAACGATACG GACTCGTGTA TGTCAATCGT GGCAACCATG AGTTGCGAGA
     K  R  Y  G   L  V  Y   V  N  R   G  N  H  E   L  R  D
1251 TATGAAACGT GTACCGAAAA AATCGTATGA ATGGTTCAAA GAAGTGATCA
      M  K  R   V  P  K  K   S  Y  E   W  F  K   E  V  I  K
1301 AAACGAATGG TAGTACGATC TATACAAACC CATATACAGA ACTCGAGCAC
       T  N  G   S  T  I   Y  T  N  P   Y  T  E   L  E  H
1351 CACCACCACC ACCAC
     H  H  H  H   H
```

Figure B1 (cont) Nucleotide sequence and the deduced amino acid sequence of *EcBgl 1* gene showed an open reading frame of 1365 bp encoding 455 amino acids.

```
   1 ATGACTAACT TGCATTTTCC TAAAGATTTT TGGTGGGGAG CCGCTGCCAG
     M  T  N  L   H  F  P   K  D  F   W  W  G  A   A  A  S
  51 CGGTCCGCAA ACTGAAGGAC GAACGGCCGA TGATGGCAAA GGCGATTCGA
      G  P  Q   T  E  G  R   T  A  D   D  G  K   G  D  S  I
 101 TTTGGGACTA TTGGTACGCA ACGGAGCCAG AACGTTTTTA TCAAAAACGG
       W  D  Y   W  Y  A   T  E  P  E   R  F  Y   Q  K  R
 151 GGACCAAAAG ATACCGTCCA GCTGCTGCAG CGTTACCCAG AAGATGTGGC
     G  P  K  D   T  V  Q   L  L  Q   R  Y  P  E   D  V  A
 201 GTTGATGAAA GAGATCGGCT TTAATTCTTT TCGAACCTCG ATCCAGTGGA
      L  M  K   E  I  G  F   N  S  F   R  T  S   I  Q  W  S
 251 GTCGCTTGCT GCCAGAAGGA AGAGGGGCGG TCAATCAACA AGCGGTGGCG
       R  L  L   P  E  G   R  G  A  V   N  Q  Q   A  V  A
 301 TTTTATCGCG AGTACTTCGC CACTTTGATC GAAAATGGCA TCGAACCCTT
     F  Y  R  E   Y  F  A   T  L  I   E  N  G  I   E  P  F
 351 CATCAATTTG TATCATTTTG ATATGCCGAT GGCACTGCAA AAAGAAGGCG
      I  N  L   Y  H  F  D   M  P  M   A  L  Q   K  E  G  G
 401 GCTGGCTCAA TCGCGAGACT GTCACAGCAT TTGCAGATTA TGCTGCTTTG
       W  L  N   R  E  T   V  T  A  F   A  D  Y   A  A  L
 451 TGTTTTGAGT TGTTCGGTGA TCAAGTCTGT CATTGGTTCA CGCAAAATGA
     C  F  E  L   F  G  D   Q  V  C   H  W  F  T   Q  N  E
 501 ACCGATCGTG CCAGTGGAGA TGGGGTATTT GTATCAACAG CATTACCCAG
      P  I  V   P  V  E  M   G  Y  L   Y  Q  Q   H  Y  P  A
 551 CAGAAATCAA CTTGGCACAT GCGGTGCAAG TCGGCTACCA TGAAGCACTC
       E  I  N   L  A  H   A  V  Q  V   G  Y  H   E  A  L
 601 GCCAGCGCCT TAGCGATCCA ACGCTTCCGT CAATCTCAGC AATCAGGCGA
     A  S  A  L   A  I  Q   R  F  R   Q  S  Q  Q   S  G  E
 651 AATCGGCATC ATCTTGAATT TGACACCGAC TTATCCGAAA GATCCGAACG
      I  G  I   I  L  N  L   T  P  T   Y  P  K   D  P  N  D
 701 ATGAAAAAGA TGTCCAAGCG GCGCAATTGG CAGATGCCTT TTTTAACCGA
       E  K  D   V  Q  A   A  Q  L  A   D  A  F   F  N  R
 751 TCCTTCTTAG ATCCAGCGGT CAAAGGCACC TTTCCTGTAG AATTGATCGC
     S  F  L  D   P  A  V   K  G  T   F  P  V  E   L  I  A
 801 TGTCTTAAAC GAGTTGGCGA TCATGCCAGA AACCAGGGCA GAAGATTTGC
      V  L  N   E  L  A  I   M  P  E   T  R  A   E  D  L  Q
 851 AGATCATTAA AGAAAACACG GTCGATCTCT TGGGGGTCAA CTATTACCAG
       I  I  K   E  N  T   V  D  L  L   G  V  N   Y  Y  Q
 901 CCACGGCGGG TGCAGCAAAA GCGTTCTGCC AAAACAGCGG ACACACCAAT
     P  R  R  V   Q  Q  K   R  S  A   K  T  A  D   T  P  M
 951 GCCAGAAGAT TATTTCGATG TGTATGATTG GCCAGAAAAG AAAATCAATC
      P  E  D   Y  F  D  V   Y  D  W   P  E  K   K  I  N  P
1001 CCCATCGAGG CTGGGAGATC TATGAACGAG GCATCTATGA TACCTTGATC
       H  R  G   W  E  I   Y  E  R  G   I  Y  D   T  L  I
1051 AACCTACGAG ACAACTACGG CAATATCCCC TGTTATATTT CAGAAAACGG
     N  L  R  D   N  Y  G   N  I  P   C  Y  I  S   E  N  G
1101 CATGGGGGTC GAAGATGAAA CACGCTTTAT CGATGCCAGC GGTCAGATCC
      M  G  V   E  D  E  T   R  F  I   D  A  S   G  Q  I  Q
1151 AAGATGAATA CCGCATCACC TTTATCAAAG ATCATTTGCG GTATGTTCAC
       D  E  Y   R  I  T   F  I  K  D   H  L  R   Y  V  H
1201 CAAGCGATCC AAGAAGGCAG TCAGTGCCGC GGGTATCACA TGTGGACCTG
     Q  A  I  Q   E  G  S   Q  C  R   G  Y  H  M   W  T  C
1251 CATGGACAAT TGGTCTTGGC TGAATGAATT CAAAAATCGT TACGGGTTTA
      M  D  N   W  S  W  L   N  E  F   K  N  R   Y  G  F  I
1301 TTGCTGTCGA TTTGGCTACC CAAAAACGGA CCATCAAAAA GTCTGGTGAG
       A  V  D   L  A  T   Q  K  R  T   I  K  K   S  G  E
1351 TGGTTCCACG AAGTGATTCT ACAAAACGGC TTTGTCGACC TCGAGCACCA
     W  F  H  E   V  I  L   Q  N  G   F  V  D  L   E  H  H
1401 CCACCACCAC CAC
      H  H  H  H
```

Figure B2 Nucleotide sequence and the deduced amino acid sequence of *EcBgl 2* gene showed an open reading frame of 1413 bp encoding 471 amino acids.

```
   1 ATGGTAAAAA TACCTGAAAC ATTTATTTTA GGGGCTGCTT CGTCTGCTTG
     M  V  K  I     P  E  T     F  I  L     G  A  A  S     S  A  W
  51 GCAGACAGAA GGCTGGCAAG GAAAGAAAGA AGGGCAAGAT TCGTATCTCG
      Q  T  E     G  W  Q  G     K  K  E     G  Q  D     S  Y  L  D
 101 ACCGCTGGTA CAAAGAAGAA CGCTTTGTCT GGCATAATGG CTACGGGCCG
       R  W  Y     K  E  E     R  F  V  W     H  N  G     Y  G  P
 151 GCTGTTGCGA CGAATTTTAT GGAAAAGTTT TCAGAGGATG TTGAACTGAT
     A  V  A  T     N  F  M     E  K  F     S  E  D  V     E  L  M
 201 GCAGGAGGTT CATTTGACCC ATTACCGAAC ATCGATCAAT TGGGCGCGCT
      Q  E  V     H  L  T  H     Y  R  T     S  I  N     W  A  R  F
 251 TCTTTACCGA CCATGAACAG CTGATCGTTG ATGAAGATTA TGCTGCTCAT
       F  T  D     H  E  Q     L  I  V  D     E  D  Y     A  A  H
 301 ATCGATCAGC TTATCGACCA ATTGCTTGCG GCTGGGGTCG AACCGATGCT
     I  D  Q  L     I  D  Q     L  L  A     A  G  V  E     P  M  L
 351 TTGTTTGGAA CATTATGAAC TGCCAGCTAG TTTGATGGAT GCCTATGATG
      C  L  E     H  Y  E  L     P  A  S     L  M  D     A  Y  D  G
 401 GCTGGAGCTC GCGGAAAGTG GTCGATCTAT ATACTCGTTA TGCCGAAATC
       W  S  S     R  K  V     V  D  L  Y     T  R  Y     A  E  I
 451 GCCTTCGAGC GTTACGGTGA TCGAGTCAAA CAGTGGTTTA CCTTCAATGA
     A  F  E  R     Y  G  D     R  V  K     Q  W  F  T     F  N  E
 501 GCCGATCGTT ATTCAGACTC GTTGCTATCT CGATGCGATC CGCTGGCCCC
      P  I  V     I  Q  T  R     C  Y  L     D  A  I     R  W  P  H
 551 ATGAGCAAAA CACAAAAAAA TGGATGCTTT GGAACTATCA CAAAGTATTG
       E  Q  N     T  K  K     W  M  L  W     N  Y  H     K  V  L
 601 GCGTCCGCCC AAGCGGTGGC TGTCTATCAT GAAAAGGGCT ACGAGGGGCG
     A  S  A  Q     A  V  A     V  Y  H     E  K  G  Y     E  G  R
 651 CATCGGTTGT GTGCTAAATC CAGAAATGGT TTACGCACGC TCTTCCTCAC
      I  G  C     V  L  N  P     E  M  V     Y  A  R     S  S  S  P
 701 CAGGGGATCA AAACGCGCAA AAAATGTACG ATCTTTTTTT CAACCGAATC
       G  D  Q     N  A  Q     K  M  Y  D     L  F  F     N  R  I
 751 TTTTTTGATC CAATGGTTAA GGGGCATTAT CCAGATGAAC TTATCACACT
     F  F  D  P     M  V  K     G  H  Y     P  D  E  L     I  T  L
 801 TTGCCAAACC TATGACATCT ACTTTGATCC GACAGAAGAA GATTTGGAAA
      C  Q  T     Y  D  I  Y     F  D  P     T  E  E     D  L  E  T
 851 CGATCCGCAC CCATACGGTG GATTTCTTAG GTGTCAATCA GTACTACCCG
       I  R  T     H  T  V     D  F  L  G     V  N  Q     Y  Y  P
 901 AAACGCGTGC GGGCACCACG ATATCAATGG CATTCGGATA CACCATTTCA
     K  R  V  R     A  P  R     Y  Q  W     H  S  D  T     P  F  H
 951 TCCAGAAATG TTTTATGAGA CCTTTGATTT GCCAGGAAAA CAAATGAATA
      P  E  M     F  Y  E  T     F  D  L     P  G  K     Q  M  N  N
1001 ACTCCCGTGG CTGGGAAATC TATCCGAAGG TCATGTACGA TATGGCTAAT
       S  R  G     W  E  I     Y  P  K  V     M  Y  D     M  A  N
1051 TATCTTAAAG AAAATTATGG CAATATTCCA TGGCTGATCA CTGAAAATGG
     Y  L  K  E     N  Y  G     N  I  P     W  L  I  T     E  N  G
1101 TATGGGGCGG GAAAATGAAG AACAGTATAT GGATGCCACA GGTCAAGTCC
      M  G  R     E  N  E  E     Q  Y  M     D  A  T     G  Q  V  Q
1151 AAGATGATTA TCGGATTCAG TTTATTACCG AACATCTTTA CTGGCTGCTA
       D  D  Y     R  I  Q     F  I  T  E     H  L  Y     W  L  L
1201 AAAGCCGTCG AGGAAGGCGC CAACTGCGAA GGCTATATGC TTTGGGCATT
     K  A  V  E     E  G  A     N  C  E     G  Y  M  L     W  A  F
1251 TACCGATTGT GTTTCACCTA TGAATGCGTT CAAAAATCGC TATGGTCTCG
      T  D  C     V  S  P  M     N  A  F     K  N  R     Y  G  L  V
1301 TACGAATCGA GCTAAATGAA GAACGGACTC GGTCATTAAA AAAATCAGCC
       R  I  E     L  N  E     E  R  T  R     S  L  K     K  S  A
1351 GCTTGGTATC GTTCCCTGAT TGATGAACGG TTGCTTCCAT TTGAAGAAAA
     A  W  Y  R     S  L  I     D  E  R     L  L  P  F     E  E  K
1401 ACCATTGATT CTGCTCGAGC ACCACCACCA CCACCAC
      P  L  I     L  L  E  H     H  H  H     H  H
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Figure B3 Nucleotide sequence and the deduced amino acid sequence of *EcBgl 3* gene showed an open reading frame of 1437 bp encoding 479 amino acids.

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