

O-FNN-03

Screening and Isolation of Stevioside and Rebaudioside A Degrading Bacteria from Forest Soil

Tanatchakorn Asawasriworanan¹, Phurt Harnvoravongchai¹, Jamorn Samana²,
Puey Ounjai^{1*}, Surang Chankhamhaengdecha^{1*}

¹Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand

²Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand

*Corresponding author. E-mail: puey.oun@mahidol.ac.th, surang.cha@mahidol.ac.th

DOI :

ABSTRACT

Stevioside and rebaudioside A are the two major steviol glycosides found in *Stevia rebaudiana* leaves. These glycosides have been widely utilized as natural sugar substitutes in food and beverage industries. Unfortunately, the steviol glycosides quantification are still relied on laborious techniques and expensive analytical instruments, such as mass spectrometry and high-performance liquid chromatography. These analytical techniques are not only time-consuming but also requiring skilled technicians. Previously, an established enzymatic method was developed as a more convenient and inexpensive alternative assay to evaluate stevioside quantity in plant specimen. This study was aimed to screen and identify bacteria with enzymatic capabilities to degrade stevioside and rebaudioside A from soil. The soil samples were collected from the deciduous forests in Nan province, Thailand. A total of 108 bacterial strains were isolated, using minimal salt medium supplemented with stevia leave extract as a sole carbon source. The stevioside and rebaudioside A degradation abilities of each bacterial isolate were investigated using thin-layer chromatography (TLC). Stevioside degrading activity was observed in 3 bacterial isolates. One of them appears to be able to degrade both substrates. The identification based on the sequence of 16S rRNA gene suggested that the bacterial isolates belong to genus *Burkholderia*, *Brevibacillus* and *Bacillus* respectively. Whole genome sequencing of the bacterial isolate capable of degrading both stevioside and rebaudioside A was further performed. The candidate genes responsible for the glycoside degrading activity were preliminarily annotated. While there were a few previous reports

of steviol glycosides degrading bacteria, the enzymatic activity reported here appeared to be unique. The glycoside degrading enzymatic activity revealed in this work could be fruitful for broadening the applicability of the enzymatic approach to determine the content of both stevioside and rebaudioside A in *Stevia* samples.

Keywords: Rebaudioside A, Steviol glycosides, Steviol glycosides degrading bacteria, stevioside

INTRODUCTION

The demand of sugar substitutes or low-calorie sweeteners have rapidly risen in recent years and have become one of a prime concern in food and nutrition science. Among various sugar substitutes, natural sweeteners have gained increased popularity across the world. (Brandle, Starratt & Gijzen, 1998).

Stevia or *Stevia rebaudiana* Bertoni, a tropical plant that was used for centuries to sweeten food and beverages in many parts of the world, has been considered as a promising natural source of plant-derived sweeteners. The compounds contributed in sweetness of *S. rebaudiana* are known as steviol glycosides, which contains stevioside (5-10% w/w) and rebaudioside A (2-4% w/w) as two major components. Some minor sweet compounds for examples, rebaudioside B, C, D, E and ducoside A (0.1–1% w/w) were also found (Brandle, Starratt & Gijzen, 1998; Ceunen & Geuns, 2013; Puri, Sharma & Tiwari, 2011).

Purified steviol glycosides have been approved as safe food and beverage additives in several countries as there was no health effect reported for steviol glycosides in human at an approved daily intake (Carakostas *et al.*, 2008; Chatsudthipong & Muanprasat, 2009; Lemus-Mondaca *et al.*, 2012; Puri, Sharma & Tiwari, 2011).

It should be noted that the sweetness of stevioside and rebaudioside A are around 300 and 350–450 times sweeter than sucrose, respectively. This make them particularly appealing as natural high-intensity sweeteners (Puri, Sharma & Tiwari, 2011).

However, *stevia* plant has been known to have high variability in different phenotypes including total sweetener contents. Various attempts have been made to setup a high throughput program to screen the *stevia* germplasm in many geographical areas to select the lineages of plants with high stevioside and rebaudioside A content. Therefore, one challenge in the improvement of

production of stevia related products in the industrial scale is to develop a rapid screening program to evaluate stevioside and rebaudioside A content in each stevia germplasm so that the program for selectively breeding new strains of stevia with high steviol glycosides content can be established. Therefore, a cheap and time-saving approach to measure the steviol glycosides components in the plant is needed.

However, current technology for the quantification of steviol glycosides content in stevia samples are largely relied on complex and time-consuming analytical methods, such as mass spectrometry and high-performance liquid chromatography (HPLC) (Bartholomees *et al.*, 2016; Gardana, Scaglianti & Simonetti, 2010; Montoro *et al.*, 2013). Moreover, the mentioned techniques also require skilled technicians and expensive instrumental facilities. These reasons could be obstacles hindering the development of rapid approach for the steviol glycosides-rich specimen screening or quality control of stevia products.

In previous studies, various enzymatic methods have been reported as a more convenient way to measure steviol glycosides content in Stevia samples particularly when compared with the common analytical methods (Mizukami, Shiiba & Ohashi, 1981; Udompaisarn, Arthan and Somana, 2017). Mizukami, Shiiba and Ohashi (1981) reported that the hydrolysis of both stevioside and rebaudioside A by crude hesperidinase from *Aspergillus niger* release free glucose molecules. Therefore, a measurement of the released glucose molecules could reflect the amount of stevioside and rebaudioside A content (Mizukami, Shiiba & Ohashi, 1981). Additionally, an enzymatic approach to determine stevioside content from *S. rebaudiana* leaves and their products was recently reported (Udompaisarn, Arthan and Somana, 2017). The approach exploited a recombinant β -glucosidase from *Bacteroides thetaiotaomicron* HB-13 that specifically convert stevioside into rubusoside and glucose. The released glucose molecules can subsequently be quantitated to reveal the stevioside content (Udompaisarn, Arthan and Somana, 2017). However, due to the specificity of the enzyme, such method is limited to only the quantification of stevioside content.

Notably, the rebaudioside A, another major sweet compound in Stevia plant, is also of great interest to be used as a sweetener as it has little to no bitter aftertaste and has greater stability in high temperature and acidic condition when compare to the stevioside (González *et al.*, 2014; Singla & Jaitak, 2016). Hence, a novel enzyme that can hydrolyze rebaudioside A would be fruitful for expanding the application of enzymatic assay.

The objectives of this study were therefore to screen and identify stevioside and rebaudioside A degrading bacteria from Thai forest soil. The enzymes identified from this work could be useful in the development of a new enzymatic approach for determination of steviol glycosides content in stevia specimen and related products.

MATERIAL AND METHODS

Plant Materials

Dried Stevia plants and Stevia leaves extract used as substrates for bacterial cultivation and enzymatic characterization were obtained from Sugavia Co., Ltd. (Nakhonratchasima, Thailand).

Soil sampling

Soil samples were collected from the deciduous forests in Nan province, Thailand. The soil sampling was performed in the monsoon season of Thailand during late July 2017. The top 30 centimeters of soil from 16 different sites were collected to a sterile micro-aerobic containment. All samples were kept at 4 °C within 72 hours after collection.

Screening for stevioside and rebaudioside A degrading bacteria

Selective enrichment culture of the stevioside and rebaudioside A degrading bacteria was performed by mixing 1 g of soil sample with 9 ml of modified minimal salt medium supplemented with 0.1% Stevia leaves extract (MMSS medium) and amphotericin B (0.25 mg/ml). The mixtures were incubated under micro-aerobic condition at 40°C for 7 days. The enriched sample was then serially diluted by 0.85% saline and the suspension was spread on MMSS agar plate, incubated at 40°C for 48 h. Bacterial colonies of different morphology were randomly selected and sub-cultured into the MMSS broth and incubated under micro-aerobic condition at 40°C for 2 days before further characterization. The culture supernatants were examined for stevioside and rebaudioside A hydrolytic activity by thin-layer chromatography (TLC) using a mobile phase consisting of ethyl acetate:isopropanol:acetone:water (53:30:2:15 v/v) (Udompaisarn, Arthan & Somana, 2017). TLC plates were subsequently dipped in 5% sulfuric acid solution followed by heating until the brown spots of products were observed.

Bacterial characterization

The candidate strains exhibiting hydrolytic activity against both stevioside and rebaudioside A were studied for their growth conditions and growth characteristics under different culture media, including MMSS, Luria-Bertani (LB) and LB supplemented with 0.2% glucose. growth of the candidate strain was characterized by determination of optical density using Tecan Magellan infinite 200 Pro microplate reader machine. The bacterial culture was incubated inside the microplate reader machine in Thermo Fisher Scientific-Nunclon 24-well plate at 37°C for 24 hours with hourly agitation and optical density measurement. All tests were conducted in triplicates. The morphology of the isolate was visually observed both by naked eyes and compound light microscope.

Bacterial identification and whole genome sequencing

After the candidate isolates of bacteria were selected based on their stevioside and rebaudioside A degrading activities, the genomic DNA of the candidate isolates were extracted by using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek). The 16S rRNA gene from gDNA of the selected isolates was amplified by polymerase chain reaction (PCR) using universal primers 27F (5'-AGAGTTTGGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplicons were sequenced (Bioneer Inc., Korea) and the obtained results were analyzed using BLASTN against Genbank 16S rRNA sequences (Bacteria and Archaea) database and EzBioCloud 16S database. The gDNA of the selected isolate which exhibited rebaudioside A degrading activity was sent for whole genome sequencing, assembly and annotation at the Omics Science and Bioinformatics Center (Thailand).

Identification of candidate enzymes

The candidate enzymes were predicted using data from whole genome annotation of the selected isolate obtained from the Omics Science and Bioinformatics Center (Thailand). In the first step, only the enzymes related to glucosidase and galactosidase families were studied for their potential function and mechanisms in comparison with the similar enzymes in KEGG pathways database.

RESULTS

Screening for stevioside and rebaudioside A degrading bacteria

A total of 108 bacterial isolates with the ability to grow in the minimal medium containing stevioside and rebaudioside A as the sole carbon sources were collected from the 16 soil samples. The isolates were further tested for stevioside and rebaudioside A degradation using thin-layer chromatography (TLC) method. As indicated by band shift on TLC plates, 4 isolates exhibited stevioside and rebaudioside A hydrolytic activities after incubation in MMSS medium for 2 days (Fig 1a-b). The 3 isolates, namely, T4S1, T4S3 and N1S8, were able to degrade stevioside (Fig 1a-b). Only T4S4 was able to digest both stevioside and rebaudioside A (Fig 1a, c). Notably, T4S4 isolate could completely deplete stevioside and rebaudioside A in the medium after 7 days of incubation (Fig 1c).

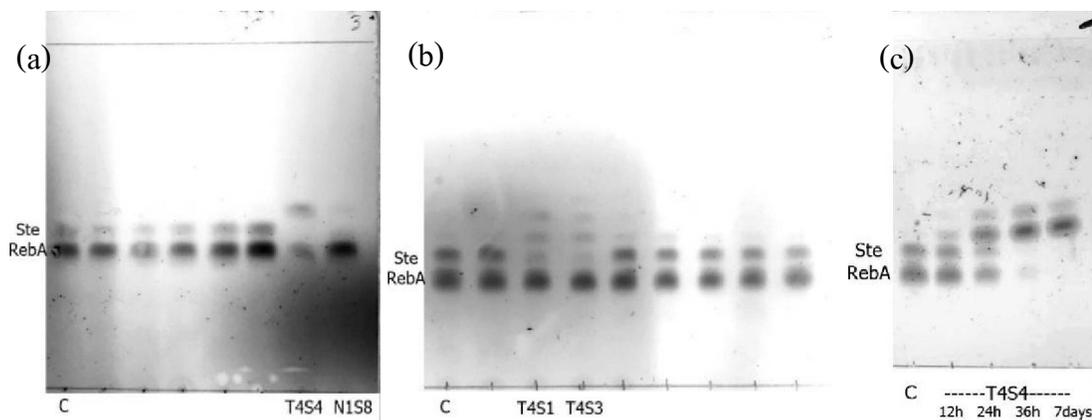


Figure 1. The TLC plates revealing stevioside and rebaudioside A hydrolysis by different bacterial isolates. The chromatogram in (a-b) revealed the stevioside and rebaudioside A degradation ability of different bacterial isolates during the screening process. Chromatogram in (c) showed the hydrolytic activity of T4S4 isolate at different time of incubation. C = Control (MMSS medium) and h = hours.

Characterization of bacterial growth

All bacterial isolates were screened in a micro-aerobic condition at 40°C. However, the 4 selected strains can also grow well under aerobic condition in LB and LB supplemented with 0.2% glucose media at 37°C. Due to the fact that the T4S4 was the only isolate of bacteria exhibiting dual

degradation abilities, the isolate was chosen for further analysis. The growth characteristics of T4S4 in different media including MMSS, LB and LB supplemented with 0.2% glucose were shown in Fig 2.

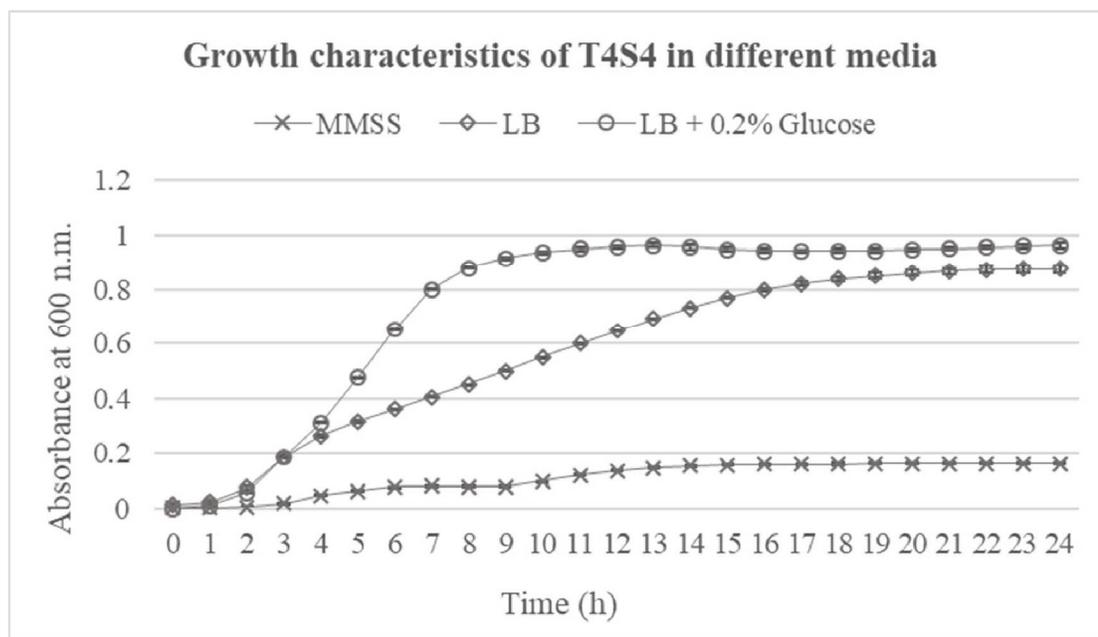


Figure 2. Growth characteristics of T4S4 strain in different media. The result revealed that the T4S4 can be cultivated in all bacterial media tested including MMSS, LB and LB + 0.2% glucose. The growth curves were generated by monitoring the optical density of culture at 600 nm using Tecan Magellan microplate reader machine at 37°C for 24 hours. The cultures were shaken hourly.

Validation of steviol glycosides degrading capability of T4S4

To confirm the steviol glycosides degradation ability of T4S4, subsequent TLC characterization was performed. Dramatic shift in the band pattern of steviol glycosides in the TLC plate was observed both in aerobic and micro-aerobic conditions, consolidating that the ability of T4S4 in utilizing stevioside and rebaudioside A. It should be noted that the T4S4 was able to completely degrade both steviol glycosides (stevioside and rebaudioside A) after 7 days of incubation in MMSS medium in both aerobic and micro-aerobic conditions (Fig 3).

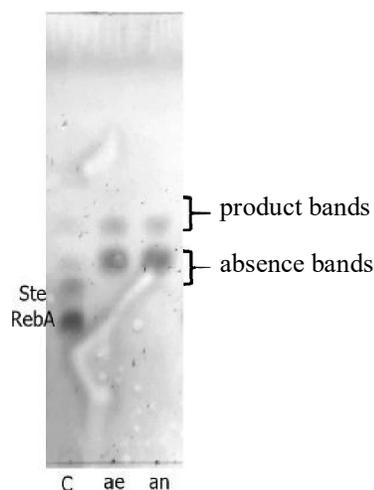


Figure 3. TLC plate revealed the result of stevioside and rebaudioside A hydrolysis of the T4S4 bacterial isolate when incubated in a MMSS medium at 37°C for 7 days in different conditions. C = control (culture medium), ae = aerobic condition, an = micro-aerobic condition.

Morphological characterization of T4S4 bacterial isolate

The colony morphology of T4S4 strain was observed by eyes and under stereomicroscope after an overnight incubation on LB agar plate. The results revealed that T4S4 can form colonies of approximately 3-4 mm in diameter (Fig 4). The T4S4 appeared to form slightly yellow colored, entire circular shaped colony with opaque appearance. The microscopic visualization of the T4S4 under light microscope displayed rod shaped bacteria of about 2.3 – 4 µm in size (Fig 5). Characterization using Gram staining suggested that the T4S4 can be classified in the group of gram-positive bacteria (Fig 6).

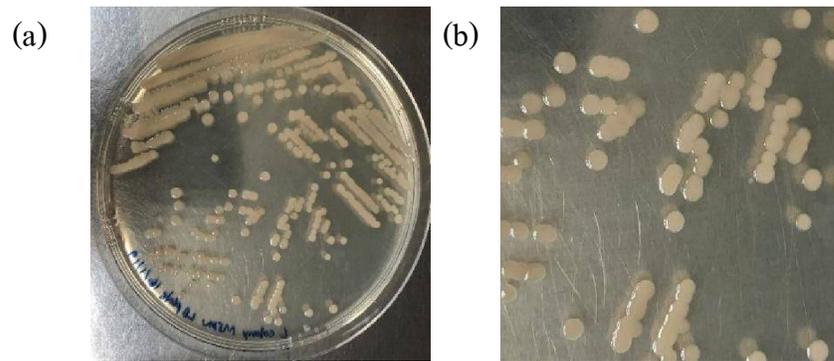


Figure 4. Colony characteristic of T4S4 on LB agar plate (a); an expand view of the T4S4 colony (b).



Figure 5. T4S4 cells under a compound light microscope (the scale bar indicates 10 μm).



Figure 6. Gram stain of T4S4 cells (the scale bar indicates 50 μm).

Bacterial identification and whole genome sequencing

The 16S rRNA gene (1,400 bases) of the N1S8, T4S1, T4S3 and T4S4 were successfully sequenced. The BLAST result showed that the N1S8 and T4S3 isolates were assigned to genus *Burkholderia* and *Brevibacillus*, respectively while T4S1 and T4S4 were assigned to genus *Bacillus*.

Afterwards, only the T4S4 isolates exhibiting stevioside and rebaudioside A degrading activities was submitted for whole genome sequencing. The results of the whole genome sequencing revealed that the genome size of T4S4 isolate was 5,978,437 bases with 38.9% of GC content and 5,908 genes. The result is well in accordance with other *Bacillus* genomes, with an average genome size of around 5-6 Mbps with approximately 40% of GC content.

Identification of candidate enzymes responsible for the steviol glycoside degradation

The candidate enzymes potentially responsible for the observed degradation of stevioside and rebaudioside A were predicted using data from whole genome annotation with an initial focus on the enzymes in glucosidase and galactosidase family, as shown in Table 1. The enzymes were further bioinformatically characterized for their function and their potential to degrade stevioside and rebaudioside A by comparing with the enzymes of similar family available in the KEGG pathways database.

Of all 15 enzymes found in the T4S4 genome as shown in Table 1, most of them except enzymes number 3, 4, 9 and 10 were found to have potential enzymatic function to digest and release glucose or galactose from polymeric sugar chain.

Table 1. The candidate enzymes from whole genome annotation.

No.	COG	EC number	Gene	Product	Length (bp)
1	COG1472	3.2.1.21		Beta-glucosidase BoGH3B	2,160
2	COG1472	3.2.1.21	bglB	Thermostable beta-glucosidase B	2,397
3	COG2313	4.2.1.70	psuG	Pseudouridine-5'-phosphate glycosidase	915
4	COG2723	3.2.1.86	bglH	Aryl-phospho-beta-D-glucosidase	1,437
5		3.2.1.22	agaA_1	Alpha-galactosidase	2,190

Table 1. The candidate enzymes from whole genome annotation (cont.).

No.	COG	EC number	Gene	Product	Length (bp)
6		3.2.1.23	cbgA	Beta-galactosidase	3,030
7		3.2.1.22	agaA_2	Alpha-galactosidase	2,238
8	COG3250	3.2.1.23	ebgA	Evolved beta-galactosidase subunit alpha	3,057
9	COG3250	3.2.1.31	uidA_1	Beta-glucuronidase	1,758
10	COG3250	3.2.1.31	uidA_2	Beta-glucuronidase	1,791
11	COG0366	3.2.1.10	malL_1	Oligo-1,6-glucosidase	1,671
12	COG0366	3.2.1.10	malL_2	Oligo-1,6-glucosidase	1,665
13	COG0366	3.2.1.10	malL_3	Oligo-1,6-glucosidase	1,608
14	COG0366			Beta/alpha-amylase	1,533
15	COG0366		apu	Amylopullulanase	4,344

DISCUSSION

From tropical decidual forest soil, we isolated 3 different bacterial isolates with ability to degrade steviol glycosides. The analysis of 16sRNA gene sequence of the three isolated revealed that the isolates belong to genus *Burkholderia*, *Bacillus* and *Brevibacillus*, respectively. Although all isolates can utilize stevioside, only T4S4 isolates belonging to the genus *Bacillus* contained the specific activity against rebaudioside A.

It should be noted that although various microorganisms have been proposed to have ability to degrade steviol glycosides including fungi, archaea and bacteria, only handful studies actually discussed about the natural enzymes found to be able to utilize both stevioside and rebaudioside A.

So far, only five microorganisms have been reported to contain the enzymes that can utilize both stevioside and rebaudioside A including β -glucosidase of Ascomycota fungus *Aspergillus niger* (Mizukami, Shiiba & Ohashi, 1981) and β -galactosidase from *Sulfolobus solfataricus* with specific ability to hydrolyze stevioside and rebaudioside A molecule at the glycosidic linkages of C-13 site and ester linkages of C-19 (Chen *et al.*, 2014; Nguyen *et al.*, 2016). The two β -glucosidase enzymes from *Clavibacter michiganense* and *Flavobacterium johnsonae* that are able to cleave stevioside and rebaudioside A molecules at the ester linkages of C-19 (Nakano *et al.*, 1998; Okamoto *et al.*,

2000). Moreover, the ability of bacteroidaceae in human intestine in converting stevioside and rebaudioside A into steviol was also previously described (Gardana, *et al.*, 2003). To our knowledge, our study is the first to report this unique hydrolytic activity in bacteria of genus *Bacillus*.

There was also a few reports of stevioside degrading activities from microbes, including β -glucosidase from *Aspergillus aculeatus* (Ko *et al.*, 2012), *Streptomyces* sp. GXT6 (Wang *et al.*, 2015), *Bacteroides thetaiotaomicron* HB-13 (Udompaisarn, Arthan & Soman, 2017), *Sphingomonas elodea* ATCC 31461 (Lan *et al.*, 2019), β -galactosidase from *Thermus thermophilus* (Nguyen *et al.*, 2014) and *Aspergillus* sp. (Tao, Kim & Xia, 2012). These enzymes were reported to cleave stevioside at the glycosidic linkages of C-13 site. Furthermore, β -galactosidase of *Kluyveromyces lactis* (Chen *et al.*, 2016) was reported to but stevioside molecule at the ester linkages of C-19. In addition, the *Penicillium decumbens* β -galactosidase enzyme was shown to be able to hydrolyze stevioside at the glycosidic linkages of C-13 and ester linkages of C-19 site (Ko *et al.*, 2013).

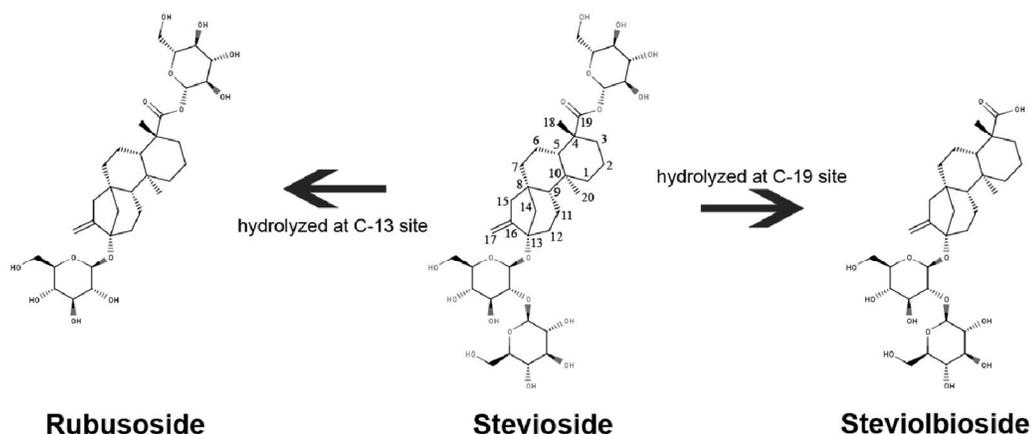


Figure 7. Major pathways of degradation of stevioside

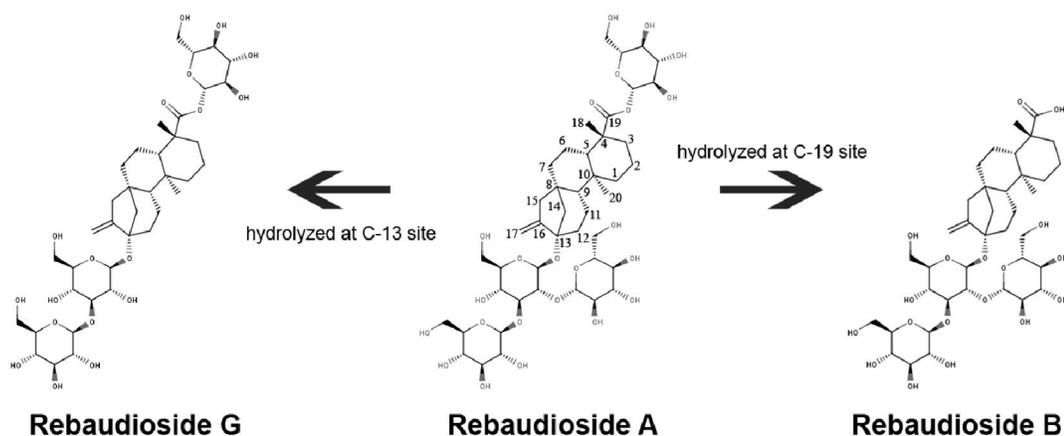


Figure 8. Major pathways of degradation of rebaudioside A

We gathered all the information regarding the aforementioned enzymes. It was clear that all of them can be classified in the group of either β -glucosidase or β -galactosidase. We therefore further scrutinized on the candidate enzymes from these two families from whole genome data of T4S4 as listed in Table 1. In any case, further attempts still need to be conducted to further identify the key enzymes behind the degradation of both steviol glycoside molecules in T4S4. Characterization of the hydrolytic products from degradation of stevioside and rebaudioside A from T4S4 could also further lead to a better understanding of the enzyme mechanism. Possible hydrolytic products were shown in figure 7 and 8.

Our work thus revealed a novel source of enzymes that can be beneficial for the improved screening and identification of stevioside and rebaudioside A-rich plants, evaluation of raw material quality in the production of stevia-based sweeteners, steviol glycoside extraction efficiency and quality control of steviol glycoside products.

CONCLUSION

In this work, we screened environmental bacteria from deciduous forest soil in Thailand for the stevioside or rebaudioside A degradation abilities. Our results demonstrated that three bacterial isolates belonging to the *Burkholderia*, *Bacillus* and *Brevibacillus*, can utilize stevioside in the medium. While only one isolate, T4S4, belonging to the genus *Bacillus* displayed the ability to degrade both stevioside and rebaudioside A. Candidate genes responsible for the activity was analyzed based on the comparison with KEGG pathway

database. 15 potential enzymes from β -glucosidase and β -galactosidase family were identified as candidate enzymes responsible for steviol glycosides degradation from whole genome sequencing of the T4S4 strain. Further characterization will be conducted to specifically identify and elucidate the mechanism of the key enzymes behind this unique degradation. This work not only provided information on a new alternative source of enzymes for the steviol glycoside hydrolysis but also set stage for further development of enzymatic as say for determination of stevioside and rebaudioside A content in plant and in the industrial production of steviol glycosides.

ACKNOWLEDGEMENTS

This research has been supported by Center of Excellence on Biodiversity (BDC), Office of Higher Education Commission (BDC-PG1-160004). The authors would like to thank the Faculty of Science, Mahidol University for providing equipment used in this study.

REFERENCES

- Bartholomees, U., Struyf, T., Lauwers, O., Ceunen, S., & Geuns, J. M. (2016). Validation of an HPLC method for direct measurement of steviol equivalents in foods. *Food chemistry*, 190, 270-275.
- Brandle, J. E., Starratt, A. N., & Gijzen, M. (1998). *Stevia rebaudiana*: Its agricultural, biological, and chemical properties. *Canadian journal of plant science*, 78(4), 527-536.
- Carakostas, M. C., Curry, L. L., Boileau, A. C., & Brusick, D. J. (2008). Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. *Food and Chemical Toxicology*, 46(7), S1-S10.
- Ceunen, S., & Geuns, J. M. (2013). Steviol glycosides: chemical diversity, metabolism, and function. *Journal of natural products*, 76(6), 1201-1228.
- Chatsudthipong, V., & Muanprasat, C. (2009). Stevioside and related compounds: therapeutic benefits beyond sweetness. *Pharmacology & therapeutics*, 121(1), 41-54.
- Chen, J. M., Ding, L., Sui, X. C., Xia, Y. M., & Lu, T. (2016). Production of a bioactive sweetener steviolbioside via specific hydrolyzing ester linkage of stevioside with a β -galactosidase. *Food chemistry*, 196, 155-160.

- Chen, J. M., Xia, Y. M., Wang, H. J., & Liu, X. (2014). A complete specific cleavage of glucosyl and ester linkages of stevioside for preparing steviol with a β -galactosidase from *Sulfolobus solfataricus*. *Journal of Molecular Catalysis B: Enzymatic*, *105*, 126-131.
- Gardana, C., Scaglianti, M., & Simonetti, P. (2010). Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. *Journal of chromatography A*, *1217*(9), 1463-1470.
- Gardana, C., Simonetti, P., Canzi, E., Zanchi, R., & Pietta, P. (2003). Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. *Journal of agricultural and food chemistry*, *51*(22), 6618-6622.
- González, C., Tapia, M., Pérez, E., Pallet, D., & Dornier, M. (2014). Main properties of steviol glycosides and their potential in the food industry: a review. *Fruits*, *69*(2), 127-141.
- Ko, J. A., Kim, Y. M., Ryu, Y. B., Jeong, H. J., Park, T. S., Park, S. J., ... & Lee, W. S. (2012). Mass production of rubusoside using a novel stevioside-specific β -glucosidase from *Aspergillus aculeatus*. *Journal of agricultural and food chemistry*, *60*(24), 6210-6216.
- Ko, J. A., Ryu, Y. B., Kwon, H. J., Jeong, H. J., Park, S. J., Kim, C. Y., ... & Kim, Y. M. (2013). Characterization of a novel steviol-producing β -glucosidase from *Penicillium decumbens* and optimal production of the steviol. *Applied microbiology and biotechnology*, *97*(18), 8151-8161.
- Lan, Q., Tang, T., Yin, Y., Qu, X., Wang, Z., Pang, H., ... & Du, L. (2019). Highly specific sophorose β -glucosidase from *Sphingomonas elodea* ATCC 31461 for the efficient conversion of stevioside to rubusoside. *Food chemistry*, *295*, 563-568.
- Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, *132*(3), 1121-1132.
- Mizukami, H., Shiiba, K., & Ohashi, H. (1982). Enzymatic determination of stevioside in *Stevia rebaudiana*. *Phytochemistry*, *21*(8), 1927-1930.
- Montoro, P., Molfetta, I., Maldini, M., Ceccarini, L., Piacente, S., Pizza, C., & Macchia, M. (2013). Determination of six steviol glycosides of *Stevia rebaudiana* (Bertoni) from different geographical origin by LC-ESI-MS/MS. *Food chemistry*, *141*(2), 745-753.
- Nakano, H., Okamoto, K., Yatake, T., Kiso, T., & Kitahata, S. (1998).

- Purification and characterization of a novel β -glucosidase from *Clavibacter michiganense* that hydrolyzes glucosyl ester linkage in steviol glycosides. *Journal of fermentation and bioengineering*, 85(2), 162-168.
- Nguyen, T.T. H., Jung, S. J., Kang, H. K., Kim, Y. M., Moon, Y. H., Kim, M., & Kim, D. (2014). Production of rubusoside from stevioside by using a thermostable lactase from *Thermus thermophilus* and solubility enhancement of liquiritin and teniposide. *Enzyme and microbial technology*, 64, 38-43.
- Nguyen, T. T. H., Kim, S. B., Kim, N. M., Kang, C., Chung, B., Park, J. S., & Kim, D. (2016). Production of steviol from steviol glucosides using β -glucosidase from *Sulfolobus solfataricus*. *Enzyme and microbial technology*, 93, 157-165.
- Okamoto, K., Nakano, H., Yatake, T., Kiso, T., & Kitahata, S. (2000). Purification and some properties of a β -glucosidase from *Flavobacterium johnsonae*. *Bioscience, biotechnology, and biochemistry*, 64(2), 333-340.
- Puri, M., Sharma, D., & Tiwari, A. K. (2011). Downstream processing of stevioside and its potential applications. *Biotechnology Advances*, 29(6), 781-791
- Singla, R., & Jaitak, V. (2016). Synthesis of rebaudioside A from stevioside and their interaction model with hTAS2R4 bitter taste receptor. *Phytochemistry*, 125, 106-111.
- Tao, G. J., Kim, D., & Xia, Y. M. (2012). Enzymatic preparation of a natural sweetener rubusoside from specific hydrolysis of stevioside with β -galactosidase from *Aspergillus* sp. *Journal of Molecular Catalysis B: Enzymatic*, 82, 12-17.
- Udompaisarn, S., Arthan, D., & Somana, J. (2017). Development and validation of an enzymatic method to determine stevioside content from *Stevia rebaudiana*. *Journal of agricultural and food chemistry*, 65(15), 3223-3229.
- Wang, Z., Wang, J., Jiang, M., Wei, Y., Pang, H., Wei, H., ... & Du, L. (2015). Selective production of rubusoside from stevioside by using the sophorose activity of β -glucosidase from *Streptomyces* sp. GXT6. *Applied microbiology and biotechnology*, 99(22), 9663-9674.