

Directional bioconversion and optimization of stevioside into rubusoside by *Lelliottia* sp. LST-1

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Abstract

Steviol glycosides, mostly extracted from *Stevia rebaudiana* Bertoni, are non-caloric, safe natural sweeteners. Steviosides make up more than 50% of total steviol glycosides but possess a bitter aftertaste, which limits their application as food additives. Trace amounts of rubusosides are acceptable sweeteners, but their supply is limited. In this study, we screened an endophytic bacteria (*Lelliottia* LST-1) that can specifically convert stevioside into rubusoside and reach a conversion rate of 75.4% after response surface optimization. Potential β -glucosidases GH3-1, GH3-2, GH3-3, and GH3-4 based on whole genome sequencing were expressed and interacted with steviol glycosides to explore principle and process of biological transformation. High-performance liquid chromatography revealed that all enzymes hydrolyzed stevioside and generated rubusoside, but substrate specificity analysis indicated that GH3-2 is the most suitable choice for the durative, steady, and high-efficiency production of rubusoside

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Abstract

Steviol glycosides, mostly extracted from *Stevia rebaudiana* Bertoni, are non-caloric, safe natural sweeteners. Steviosides make up more than 50% of total steviol glycosides but possess a bitter aftertaste, which limits their application as food additives. Trace amounts of rubusosides are acceptable sweeteners, but their supply is limited. In this study, we screened an endophytic bacteria (*Lelliottia* LST-1) that can specifically convert stevioside into rubusoside and reach a conversion rate of 75.4% after response surface optimization. Potential β -glucosidases GH3-1, GH3-2, GH3-3, and GH3-4 based on whole genome sequencing were expressed and interacted with steviol glycosides to explore principle and process of biological transformation. High-performance liquid chromatography revealed that all enzymes hydrolyzed stevioside and generated rubusoside, but substrate specificity analysis indicated that GH3-2 is the most suitable choice for the durative, steady, and high-efficiency production of rubusoside.

Key words: stevioside, rubusoside, bioconversion, response surface optimization, β -glucosidases

Introduction

Steviol glycosides (SGs) are natural low-calorie sweeteners found in the leaves of *Stevia rebaudiana* Bertoni, which has been approved as a food additive in many countries (Ahmad et al., 2020). They belong to a group of ent kaurene diterpene glycosides (Lopez-Carbon et al., 2019), that is, having the same backbone and differing in carbohydrate residues (Fig. 1a). SGs have more than 30 types, and the major components are stevioside (ST), rubusoside (RS), rebaudiosides A–F (RA–F), and dulcoside A (Table 1). The use and transport costs of SGs account for only 1/3 and 1/200 of sucrose, respectively, but its sweetness and calorific value are 300–450 times and 1/300 those of sucrose respectively. The lack of accumulation, toxicity, and carcinogenicity of SGs in human body (Zhao et al., 2020) make them highly recommended as natural substitutes of sucrose for individuals with diabetes or carbohydrate-controlled diets (Typek et al., 2020).

Steviosides are the most abundant component of SGs whereas are responsible for the characteristic bitter aftertaste (Bhardwaj et al., 2020; Chaturvedula & Prakash, 2011; Hajihashemi & Geuns, 2017). Comparatively, rubusoside (13-O-glucosyl-19-O-d-glucosyl-steviol) possesses more marvelous sweetness and flavor-enhancing properties, earning itself praises as the most acceptable sweeteners. At present, rubusoside is mostly extracted and purified using chemical or mechanical methods from *Rubus suavissimus*, an endemic and rare shrub in Guangxi Province, directly leading to limited area of cultivation and climate fluctuation, along with economic and environmental burdens (Boonkaew et al., 2019).

Currently, food-grade SGs cost about 100 Yuan per kg at market rates, while extracts of *R. suavissimus* (rubusoside 20%) cost 500–600 Yuan per kg. The price gap implies the great demand and expansive market prospects. In summary, innovation of the biotransformation methods of rubusoside would be advantageous for its large-scale production and broad application in the food and medical industries. The present study investigates the isolation, identification, and optimization of microorganisms directly transforming stevioside into rubusoside and analyzes the mechanism of enzymes in the transformation process to obtain the optimal enzyme.

2. Materials and methods

Isolation and identification of endophytic bacterium

Inorganic salt medium (KH_2PO_4 3 g/L, MgSO_4 0.1 g/L, K_2HPO_4 1.5 g/L, CaCl_2 0.01 g/L, EDTA disodium 0.01 g/L, pH 7.5) containing only 10 g/L stevioside was adopted as the selection medium to screen a mass of endophytic bacterium from *S. rebaudiana* Bertoni. Considering that the isolates were able to utilize steviosides and the release of D-glucose residue indicating the capacity of producing β -glucosidase, esculin-MS medium was used for a second screening campaign in accordance with the method described by Xuan et al. with some modifications (Xuan, 2012). MS medium with double agar was supplemented with 0.2% esculin (6,7-2-hydroxy-coumarin- β -D-glucoside) and 0.6% FeCl_3 of the same volume. Observation of the wells surrounded by black or dark brown, indicating that esculin was hydrolyzed by β -glucosidase and underwent chromogenic reaction with Fe^{3+} .

16S rDNA was amplified with the universal primer set 27F, 1492R and sequencing analysis was conducted by Sangon Biotech. A phylogenetic tree was constructed using the neighbor-joining method of the MEGA 6 program, and the bootstrap value was calculated based on 1000 replicates to obtain confidence levels for the branches. Scanning electron microscopy (SEM) of the bacterium was performed for species identification. The bacteria samples were observed by COXEM EM-30 (8.0nm @30KV SE, 20x-100,000x) after pre-treatment including surface cleaning, fixation, rinsing and dehydration.

2.2 Stevioside conversion experiment

Isolates were precultured in Luria broth medium at 220 rpm for 12 h at 37 °C, transferred into a fermentation medium (peptone 10 g/L, KH_2PO_4 5 g/L, K_2HPO_4 5 g/L, NaCl 2 g/L, MgSO_4 0.4 g/L, stevioside 10 g/L), and then incubated at 37 °C, 220 rpm for 2 days. Then, 1 mL broth was collected every 24 h and centrifuged. Supernatants were preserved at -80 °C as test samples.

2.3 Detection of rubusoside by thin-layer chromatography and

high-performance liquid chromatography

Preliminary screening by TLC was performed with Silica gel G plates and butanol: acetic acid: diethyl ether: water (9:6:3:1, v/v/v/v) as its developing solvent (Teng et al., 2007). Standards of ST, RA, RS (10 mg/mL), and samples were sequentially pointed on the activated silica plate with capillary tubes. Spots on TLC plates were detected by spraying 10% (v/v) H₂SO₄ in ethanol after adequately closed expansion and heated at 110 °C for 10 min. Black spots appeared on the silicone plate in different positions as a result of carbonization.

After centrifugation and filtration, samples were reconfirmed using an HPLC system (Elite, Dalian, China) and a Agilent HC-C₁₈ column (4.6×150 mm, ID 5 µm) with NaH₂PO₄ solution (10 g/L): acetonitrile (32:68, v/v). The flow rate was 1 mL/min and the detection level was UV 210 nm as described by Li et al with slight modifications (Li, 2010). Stevioside and rubusoside standards with different concentration gradients were also detected to draw standard curves.

2.4 Experimental design for statistical analyses and optimization

Medium with major inorganic salts means low yield and conversion efficiency. Response surface optimization was conducted to obtain the optimum medium composition and culture conditions. The impact of carbon sources (soluble starch, glucose, wheat bran, sucrose, lactose, corn pulp dry powder, 10 mg/mL), nitrogen sources (peptone, beef extract, ammonium sulfate, fermented soybean meal, soy powder, yeast extract, 10 mg/mL), temperatures, and pH were preliminarily validated to reduce interference factors. Then, Min Run Res IV was performed using Design-Expert® Software version 6.0.8 to select significant parameters. Twelve trial runs and two different levels were performed following the design as shown in Table S2. Manual mode and Main effect were used to analyze the interaction between variables on the results. Then design of the steepest ascent method was conducted (Table S4) followed by the above experimental results to enable the response value to quickly approach the maximum response center.

A Box-Behnken experiment was used to design a response surface analysis test (Table S5), the rubusoside yield was taken as the response value (Y), and these significant parameters were set as central points (Ko et al., 2012; Saikia et al., 2018). Experimental results were fitted to a second-order polynomial equation using multiple regression analysis. The accuracy of the design was expressed as the variation coefficient (CV). The lower the CV value, the higher the reliability of the design.

2.5 Complete genome sequence analysis

Complete genome sequence analysis was performed by Biomarker Technologies to elucidate the mechanism of stevioside bioconversion. The whole genome sequence has been uploaded to NCBI (<https://www.ncbi.nlm.nih.gov/>) and the accession number is CP063663.

2.6 Enzyme expression and purification

According to the complete genome sequence of *Lelliottia* sp. accompanying with annotation and analysis based on Carbohydrate-Active enZYmes Database (CAZY), four possible genes named GH3-1 (2298 bp), GH3-2 (1428 bp), GH3-3 (2376 bp), and GH3-4 (3078 bp) were amplified with respective primers (Table S1) that introduced *Hind* III and *Eco*R I restriction sites. Amplification products with pet28a vectors were double-digested with restriction enzymes *Hind* III and *Eco*R I and purified for ligation. The recombinants were transformed into *Escherichia coli* DH5α strain. Subsequently, colonies on kanamycin media were verified and confirmed by colony PCR with sequencing primers (T7, T7-Ter) and plasmid DNA sequencing (Tatielle et al., 2018).

The recombinant plasmids were transformed into *E. coli* BL21, then for incubation with 0.5 mM IPTG at 37 for 8 h (Udompaisarn et al., 2017). Bacterial cells were collected by centrifugation, and resuspended in 5 mL of Tris-HCl buffer with 1 mM phenylmethanesulfonyl fluoride, then disrupted by sonication in an ice-water bath. The cell lysates were centrifuged and then purified using Ni-NTA His-binding Resin (7Sea Pharmatech Co., Ltd).

2.7 Enzyme assay

2.7.1 Effect of pH and temperature on enzyme activity

β -glucosidase activity was carried out photometrically using *p*-nitrophenyl- β -D-glucopyranoside (pNPG) as a substrate. A 300 μ L mixture was composed of 260 μ L of PBS buffer, 30 μ L of 2 mM pNPG, and 10 μ L of diluted enzyme. The reaction was stopped by adding 300 μ L of 1 M NaCO₃ solution, and the amount of *p*-nitrophenol (pNP) was measured by the absorbance at 405 nm on a UV spectrophotometer (Dong et al., 2017). One unit of enzyme activity (U) was defined as the amount of enzyme that produced 1 μ mol of either pNP per minute.

The relative enzyme activity at different temperatures was compared varying from 25 °C to 65 °C, and then the influence of pH on enzyme activity was investigated in the pH range of 3.0–10.0 with respective buffers. The result is expressed as the relative activity given the initial activity of 100% (Ko et al., 2013; Wang et al., 2015).

2.7.2 Effects of metal ions on enzyme activity

The residual enzyme activities of the reaction systems with PBS buffer, enzyme, and metal ions (5 mM/L) were measured under optimal conditions, whereas those of others without metal ions served as the control group (Ikezawa et al., 1986).

2.8 Substrate specificity

The assay of substrate specificity was performed by determining the enzyme activity in buffers containing 2 mM gentiobiose, trehalose, lactose, sucrose, maltose, salicin, chondroitin disaccharide, melibiose, and amygdalin at 50 °C for 20 min. ST, RS, RA, RD, and RM were also selected as substrates to explore the mutual transformation of stevia glycosides (Boonkaew et al., 2019; Hu et al., 2012).

Results and Discussion

3.1 Screening and identification of transformed bacterium

A total of 46 isolates were preliminarily screened and purified using steviosides as the sole carbon source. In microorganisms, β -glucosidases (β -D-glucoside glucohydrolase, E.C. 3.2.1.21) are mainly involved in glucose metabolism in vivo. They catalyze the hydrolysis of β -glucosidic linkages of various oligosaccharides and aryl-glycosides, thus releasing the sugar moiety and the aglycone (Yoshiara et al., 2018). Esculin generates glucose and esculetin (6, 7-2-hydroxy-coumarin) under the action of β -glucosidase, and the interaction of esculetin with Fe³⁺ appears black or dark brown. β -glucosidases come from a wide range of sources and can be detected almost in any carbohydrate-based organism with a cellular structure. Therefore, esculin-MS medium was used to screen isolates producing β -glucosidases, and 34 isolates appeared black or dark brown (Fig. 1b).

3.2 Thin-layer chromatography and high-performance liquid chromatography detection

Given the wide range of activity of β -glucosidases, fermentation liquids of isolates were detected by TLC preliminarily. This method is reliable to some extent, considering the distinct differences among various standards (Fig. 1c). Finally, HPLC was performed for reconfirmation. Fig.1d show the disappearance of ST and occurrence of rubusoside without obvious byproducts, indicating that LST-1 was the target strain that specifically converts stevioside into rubusoside. Despite the temporary low conversion rate (12%–17%) due to the inappropriate culture medium, the unique specific transformation of LST-1 makes its hidden potential worth unlocking.

Standard curves were obtained according to the peak area with different concentrations of stevioside and rubusoside, where Y represents the peak area and x represents the concentration. The functions were used to calculate the conversion rate.

3.2 Species identification and complete genome sequence analysis

Results of 16S rDNA sequence analysis indicated that LST-1 shared high sequence similarities to *Lelliottia jeotgali* (99.85%), *Lelliottia nimipressuralis* (98.82%), *Lelliottia amnigena* (98.54%). Morphological identification suggested that LST-1 is Gram-negative, atrichous, short rod-shaped bacteria (Fig. 2d). Combined with comparative phylogenetic tree analysis based on 16S rDNA gene sequences and multilocus sequence typing (MLST) (Fig. 2e, 2f), LST-1 was classified as *Lelliottia* genus.

The genome of *Lelliottia* sp. LST-1 is composed of a chromosome with 4,611,055 base pairs (Fig. 2a) and a plasmid with 39,319 base pairs (Fig. 2b). The outermost circle represents genome size, and each scale is 5 KB. The second and third circles are genes on the plus and minus chains of the genome, respectively, and different colors represent different functional classifications of Cluster of Orthologous Groups (COG). The fourth circle is the repetition sequence. The fifth circle represents tRNA (blue) and rRNA (purple). GC content is displayed on the sixth circle. The light yellow part indicates that the GC content in this region is higher than the average GC content of the genome, while the blue part indicates that the GC content in this region is lower than the average GC content of the genome. The total length of the predicted coding gene sequence is 4,140,156. Gene function annotation analysis was conducted after Blast with functional databases, such as COG, Kyoto Encyclopedia of Genes and Genomes, Swiss-Prot, Non-Redundant Protein Database.

3.3 Optimization of rubusoside production

The effects of diverse carbon sources, nitrogen sources, temperatures, and pH were examined to enlarge rubusoside production. Simple sugars readily hydrolyzed to glucose are not conducive to the conversion because of glucose inhibition effect (Pang et al., 2017), whereas organic nitrogen sources favor the conversion process because of their abundant nutrients. As shown in Fig. 3, LST-1 largely accumulated rubusoside in the presence of wheat bran, corn pulp dry powder, fermented soybean meal, and soy powder under the conditions of 37 and pH 7.0. This result is consistent with common theory.

These particular carbon and nitrogen sources were selected to perform Min Run Res IV. Multiple regression equation obtained is $R1 = +2833.96 + 532.71 A + 297.58 B + 23.02 C + 391.28 D - 69.00 E$. Analysis of variance results of the Min Run Res IV model fitting are presented in Table S3. The determination coefficient (R^2) was 0.9683, indicating that the model does not explain only 3.17% of the total variations. The Model F-value of 27.73 implies the model is significant. Wheat bran, fermented soybean meal, and corn pulp dry powder efficiently promoted the bioconversion of stevioside to rubusoside. Subsequent design of the steepest ascent method approximated significant factors to the optimal response region. Apparently, the highest yield condition was 1.1% wheat bran, 0.5% fermented soybean meal, and 0.7% corn pulp dry powder (Table S4). Thus, it was taken as the central point of subsequent tests with some modifications.

A Box-Behnken experiment was used to design a response surface analysis with three factors and three levels, and 1% wheat bran, 0.5% fermented soybean meal, and 0.7% corn pulp dry powder were set as the central point. The experimental results were fitted with a quadratic polynomial model by using design-Expert 12.0, and its mathematical model was $R1 = +4481.08 + 396.11A + 65.86 B + 45.07C - 63.51AB - 101.89A C + 222.72BC - 603.94A^2 - 395.03B^2 - 291.54C^2$.

Variance analysis results of Box-Behnken model fitting are presented in Table S6. A relatively lower value of CV (1.34%) indicates better precision and reliability of experiments. The Model F-value of 184.11 implies the model is significant, the predicted R^2 of 0.9402 is in reasonable agreement with the adjusted R^2 of 0.9904, and the difference is less than 0.2, indicating the good reliability and fitness of the model. The 3D response surface plots (Fig. 6a) show that the interaction between each independent variable is good and that the best prediction point is within the scope of experimental investigation. The maximum predicted response was 5.012 mg/mL, followed by wheat bran (1.374%), fermented soybean meal (0.337%), and corn pulp dry powder (0.464%).

In the current research, the conversion of stevioside is defined as the consumption rate of stevioside, and the output rate of rubusoside is defined as the rate of rubusoside production with stevioside consumption. Purely from the conversion rate, LST-1 completely utilized stevioside, as evidenced by its absence in the

fermentation broth by HPLC within 24 h. The output rate was approximately 75.4%, illustrating the high efficiency, utilization level, and specificity of the substrate. Compared with the initial conversion rate (8%–12%), the optimized conversion rate increased up to six to nine times. Few publications described rubusoside biotransformation by bacterium. Jiang et al. identified a *Chryseobacterium* species that converts stevioside into rubusoside by intracellular enzyme, obtaining 5.7 g/L rubusoside solution after 48 h with 10 g/L stevia glycosides, including 7.2 g/L stevioside (Jiang et al., 2011). Nevertheless, LST-1 has a distinct advantage because of its high-to-excellent yields and short reaction times.

3.4 Enzyme purification and properties

Approximately 1 mL of enzymes were obtained after centrifugation through an ultrafiltration device for desalinization. The molecular masses of the purified enzymes on SDS-PAGE were about 85, 55, 86, and 116 kDa, respectively (Fig.4), which is consistent with the calculated molecular mass of the predicted amino acid sequence. The concentrations of the purified enzymes were 129.77, 225.53, 119.15, and 172.34 $\mu\text{g/mL}$.

The enzymatic activities of the recombinants were measured at various temperatures (30–70 °C) using pNPG as the substrate. Results showed that the activity increased within the temperature range of 30 °C–50 °C and gradually decreased as the temperature was increased; the optimal temperature of the recombinants was approximately 50 °C (Fig. 5a). This result is roughly coincident with the optimal temperature of known β -glucosidases at 40–110 °C (Pan & Luo, 2006). In general, four enzymes retained over 80% of their activity for 3 h at 40 °C and exhibited poor thermostability at 60 °C (Fig. 5e, 5f, 5g, 5h). By comparison, GH3-1 and GH3-2 stabilized weekly in excessive temperatures than the other two. In general, the optimum temperatures of mesophilic β -glucosidases range from 30 °C to 65 °C, and inactivation mostly occurs at 55 °C to 70 °C (Dharmawardhana et al., 1995; Nagano et al., 2005).

The enzymatic activities of recombinants were measured at various pH values (pH 2–10.0). The optimum pH value of the four enzymes was 6 and displayed high activity (over 80%) in weak acid to neutral conditions (Fig.5c). The enzymes also showed good and extensive pH stability, retaining over 80% of its original activity between pH 4 and 8 (Fig. 5d). However, under strong acid and strong base conditions, the enzyme activity and stability obviously reduced and declined. As reported in the literature, most β -glucosidases exhibit good acid and alkaline resistance, scilicet good viability and stability in a wide range of pH (Yao et al., 2018).

Metal ions (5 mmol/L) displayed different influences on the four enzymes. SDS, Cu^{2+} , and Co^{2+} remarkably inhibited the activity of the enzymes. This result may be ascribed to the fact that heavy metal ions inactivate enzymes by binding or reacting with their related groups or destroy some of the forces that stabilize the enzyme molecules (Hu et al., 2016). Mn^{2+} , Ca^{2+} , and Zn^{2+} exerted minimal effect, whereas Fe^{2+} and Mg^{2+} significantly promoted their activity up to 1.75 times (Fig. 5b). The reaction solution shows that proteins formed complexes with metal ions, thus accelerating the reaction. However, the effects of metal ions on different enzymes varied greatly because of the differences in their spatial structure and composition.

3.5 Assay of substrate specificity

The substrate specificity of the four enzymes with different glycosides and saccharides were determined. As shown in Table 2, the enzymes showed the broad substrate hydrolysis of α (α 1-1, α 1-4, α 1-6)- and β (β 1-2, β 1-4, β 1-6)-glycosidic bonds. The rare catalytic activities of rebaudioside A, rebaudioside D, and rebaudioside M can be ascribed to the steric hindrance of a β -glucosyl (1-3) derivative of the C_{13} -hydroxyl group (Wang et al., 2015), which fit to most reported steviosides hydrolyzing β -glucosidases. Meanwhile, no hydrolytic activity was detected for amygdalin and salicin compared with other β -glucosidases hydrolyzing stevioside (Boonkaew et al., 2019; Qing et al., 2019; Wang et al., 2015), signifying higher specificity to glycosides than reported. Relatively weak activity for melibiose and specific hydrolysis of trehalose by GH3-3 were also observed. In sum, high specificity for glycosides and broad hydrolysis of saccharides make the four enzymes competitive in actual production.

All enzymes hydrolyzed stevioside and generated rubusoside (Fig.6b). The substrate stevioside can be completely consumed within 2 h, and the yields of rubusoside were 2.49 ± 0.19 , 2.40 ± 0.11 , 2.96 ± 0.07 , and

1.49±0.036 mg/mL. In theory, the presumable yield of rubusoside was 3.32 mg/mL, and the output rates were respectively 75%, 72.29%, 89.16%, and 44.88%. GH3-3 had the highest output rate, but only GH3-2 displayed no activity for rubusoside, implying specific cleavage of glycosidic bond and glucose residue.

Only a few β -glucosidases convert stevioside to rubusoside, including S-producing β -glucosidase (SPGase), SPBGL1, and BGL1 (Jin-A Ko et al., Qing Lan et al., Zilong Wang et al.). As shown in Table 3, GH3-2 displayed higher output rate (72.29%) with 23.83 μ g/mL enzyme than SPGase (64%, 33.2 μ g/mL), BGL1 (78.8%, 55 μ g/mL). SPBGL1 (99%, 600 μ g/mL) had the highest conversion rate, but the substrate concentration and enzyme amount considerably increased with the longest reaction time in reverse. SPBGL1 showed high activity (>80%) in very narrow ranges (pH5.0–6.5) and greatly restricted the large-scale fermentation production. In addition, GH3-2 could improve the output rate by adjusting the ratio of substrate concentration to enzyme. The above analysis indicates that GH3-3 is a promising candidate in industrial production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of Tables

Table 1 Structure and characteristics of steviol glycosides

Name	R ₁ (C ₁₉)	R ₂ (C ₁₃)	Molecular weight	Sweetness	Proportion of total glycosides
Stevioside	Glc-	Glc-2Glc-	804.88	100–300	50%–70%
Rebaudioside A	Glc-	Glc-3Glc-Glc-	967.01	300–400	15%–20%
Rebaudioside B	H-	Glc-3Glc-2Glc	804.88	10–15	less
Rebaudioside C	Glc-	Glc-3Glc-2Rha	951.03	40–60	10%–15%
Rebaudioside D	Glc-2Glc	Glc-3Glc-Glc-	1129.15	200–250	less
Rebaudioside E	Glc-2Glc	2Glc-Glc-	967.01	100–150	less
Rebaudioside G	Glc-	Glc-2Glc-	804.88	Not clear	less
Rebaudioside M	Glc-2Glc -3Glc	Glc-2Glc-3Glc	1291.29	160–500	less
Durcoside A	Glc-	Glc-2Rha-	788.88	40–60	less
Steviolbioside	H-	Glc-2Glc-	642.74	10–15	less
Rubusoside	Glc-	Glc-	642.74	[?]300	less

Table 2 Substrate specificity of four enzymes

Substrate	Linkage of glycosyl group	GH3-1	GH3-2	GH3-3	GH3-4
ST	Glc β -(1-2) Glc	100	100	100	100
RA	Glc β -(1-2)/Glc β -(1-3) Glc	0	0	0	0
RD	Glc β -(1-2)/Glc β -(1-3) Glc	0	0	0	0
RM	Glc β -(1-2)/Glc β -(1-3) Glc	0	0	0	0
RS	β -Glc	24.78%±0.53	0	40.71%±0.23	37.21%±0.58
Amygdalin	β -Glc	0	0	0	0
Salicin	β -Glc	0	0	0	0
Lactose	Gal β -(1-4) Glc	42.59%±0.17	64.96%±0.08	49.80%±0.29	200.87%±0.44
Sucrose	Glc α -(1-2) Fru	0	0	0	0
Gentiobiose	Glc β -(1-6) Glc	61.11%±0.41	69.81%±0.40	48.98%±0.30	133.91%±0.19
Trehalose	Glc α -(1-1) Glc	0	0	36.68%±0.41	0
Maltose	Glc α -(1-4) Glc	58.20%±0.20	48.72%±0.34	46.61%±0.42	207.83%±0.22
melibiose	Glc α -(1-6) Gal	22.22%±0.35	49.29%±0.58	0	50.43%±0.15
Chondroitin	Glc β -(1-3) Glc	0	0	0	0

*Activities on glycosides and saccharides were determined as relative values of the percentage activity obtained with stevioside.

Table 3 Enzymatic ability of β -glucosidases on stevioside.

Enzyme	Substrate	The amount of enzyme	Reaction Time	Conversion rate	Yield rate
SPGL1	24% ST (W/V)	600 $\mu\text{g/mL}$	6h	98.6%	99%
BGL1	1% ST (W/V)	30 $\mu\text{g/mL}$	6h	98.2%	78.8%
GH3-2	1% ST (W/V)	23.83 $\mu\text{g/mL}$	2h	100%	72.29%

List of figure legends

Figure .1a General formula of steviol glycosides. R1 represents substituents in C₁₉, R2 represents substituents in C₁₃.

Figure .1b Color reactions of esculin hydrolysis. CK: distilled water. LST-1:100 μL fermentation broth. Esculetin interacting with Fe^{3+} appears black or dark brown.

Figure .1c TLC detection of steviol glycoside. Spots of rebaudioside A, stevioside, and rubusoside are standards (10 mg/mL), LST-1 is fermented broth, and negative control is the fermentation medium.

Figure .1d HPLC profiles of the hydrolysis product from LST-1. Dotted lines depict standards of ST and RS, and solid lines represent consumption of ST and production of RS.

Figure. 2a,b Representation of the LST-1 chromosome. Predicted coding regions on the strand were classified as functions according to color

Figure. 2c Results of CAZy annotation. The database consists of the following enzyme families: glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary activities (AAs), carbohydrate-binding modules (CBMs).

Figure. 2d Scanning electron microscope observation

Figure. 2e, 2f Phylogenetic tree of *Lelliottia* and *Enterobacter* strains based on 16S rDNA sequences by the maximum-likelihood method (e). MLST analysis using concatenated partial recA, atpD, and infB gene sequences (f).

Figure. 3 Optimization of different components of medium and various culture conditions. (a) Different carbon sources. (b) Different nitrogen sources. (c) Optimization of pH. (d) Optimization of temperature.

Figure. 4 SDS-PAGE analysis of recombinants. M: molecular mass marker. 1: vector control; 2: crude enzyme of GH3-1; 3: crude enzyme of GH3-2; 4: crude enzyme of GH3-3; 5: crude enzyme of GH3-4.

Figure. 5a, 5b, 5c, 5d Effect of temperature and pH on enzyme activity. Results are expressed as the relative activity given the initial activity of 100%. (a) Temperature optimum, (b) Effects of metal ion, (c) pH optimum, and (d) pH stability.

Figure. 5e, 5f, 5g, 5h Thermal stability of GH3-1, GH3-2, GH3-3, GH3-4 respectively. Enzymes were incubated at 40 °C to 60 °C, and activities were detected at pH 6.0 and 50 °C.

Figure. 6a Response surface plot of A (wheat bran), B (fermented soybean meal), C (corn pulp dry powder) on responses (R2)

Figure. 7b HPLC analysis of stevioside hydrolysis. a: Standard stevioside, rubusoside. b: Stevioside hydrolysate by GH3-1. c: Stevioside hydrolysate by GH3-2. d: Stevioside hydrolysate by GH3-3. e: Stevioside hydrolysate by GH3-4.

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