Bioconversion of steviol glycosides into steviol by *Microbacterium barkeri**

Hui-Ling Jiang^a, Yan Xuan^a, Qian Zeng^a, Qi-Ming Jimmy Yu^b, Yu-Qian Zhang^c, Yu-Ru Chen^a, Hai-Bo Luo^a, He Huang^a and Qing Xu^a

^aSchool of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, China; ^bEnvironmental Engineering, Griffith School of Engineering, Griffith University, Brisbane, Queensland 4111, Australia; ^cTaizhou College, Nanjing Normal University, Taizhou 225300, China

*CONTACT: Yu-Ru Chen, chenyruru@njnu.edu.cn; Hai-Bo Luo, luohaibo_1216@126.com. School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Nanjing 210023, China

Bioconversion of steviol glycosides into steviol by *Microbacterium barkeri*

ABSTRACT

The strain which degraded steviol glycosides to steviol (STE) was screened and isolated from soil samples. A phylogenetic tree was constructed and used to determine the taxonomic status of the strain. 16S rDNA sequence was ultimately used to identify the strain as *Microbacterium barkeri* XJ. The transformation product was detected and identified as STE by HPLC/LC-MS/IR analysis. The bioconversion rate of 1% (v/v) steviol glycosides (stevioside, rebaudioside A, rebaudioside C) into STE in basic medium were 100% within 24 h, 84 h and 144 h, respectively. The results indicated XJ was more effective than mixed flora in the bioconversion of steviol glycosides to STE.

KEYWORDS

natural sweetener; stevioside; steviol; biotransform; *Microbacterium barkeri*

1. Introduction

Stevia rebaudiana Bertoni (Bertoni) is a genus of about 150 species of herbs and shrubs in the Asteraceae (Compositae) family, and native to subtropical and tropical in South and Central America [1], widely introduced to China since 1990s, owing to its high sweetness.

Steviol glycosides, no-calorie sweetener, are glycoside derivatives of steviol (STE) [2]. They are 150-300 times sweeter than sucrose and are the main components of sweeteners extracted from the leaves of *Stevia rebaudiana* Bertoni [3]. They are mainly composed of stevioside (STV) and rebaudioside A (RA), accounting for 3-10% (w/w) and 1% (w/w), together with other components such as rebaudioside C (RC), dulcoside A and the extremely minor components rebaudiosides B, D, E and steviolbioside (Table 1 and Figure 1). STE, the aglycone of STV, has broad application prospects in the field of medicine for good bioactivity. Structural modification, such as the adding of α -methylene cyclopentanone structure, can greatly increase the anti-tumor activity of STE [4].

The possible mutagenic and genotoxic effects of stevia extracts have been studied on bacterial cells and different mammalian species, and results demonstrated that stevia extracts are safe for use [5]. However, some aspects of the effects of stevia extracts on human health need further investigation [6, 7]. STE had a weak mutagenic activity in the forward mutation assays [8]. In contrast, STE decreased glucose production and inhibited oxygen uptake [9]. Interacting with some drug transporters, STE was also considered as a drug modulator. Furthermore, its effects were found to

be similar to those of gibberellin. It could regulate the growth of the plant, and stimulate seed germination and sprouting [10]. The metabolism and elimination of steviol glycosides in bacterial intestinal flora of rats [11], mice [12], pigs [2] and humans [13, 14] have been studied. In these processes steviol glycosides were converted into aglycone STE under the action of mixed flora *in vivo*. Researchers considered that the bioconversion of steviol glycosides by some bacterium species is the initial steps of the metabolism and elimination processes. It is therefore important to characterize the bioconversion processes of steviol glycosides by the bacterium strains involved in the metabolism and elimination processes. Based on previous studies, the biodegradation of STV and its related compounds by a newly identified *Microbacterium barkeri* was studied.

2. Results

2.1. Screening and identification of the strain XJ

It is generally recognized that two bacteria can be identified as the same strain when their sequence similarity is over 97%. As shown in Figure 2, the full length of 16S rDNA sequence of strain XJ was 1439 bp. Blast analysis in GeneBank showed that the strain XJ was the most similar of 99.86% to *M. barkeri*. Phylogenetic tree also showed that the strain XJ has clustered with *M. barkeri* DSM20145 (Figure 3). Therefore, the strain XJ is most likely to be *M. Barkeri* (X77446).

2.2. Analysis and identification of products

Samples of steviol glycosides (STV, RA and RC) before, during and after bioconversion were analysed with the HPLC method and typical HPLC

chromatograms were obtained (Figure 4). LC-MS analysis of the product ion scan spectrum was used to confirm the identity of the first hydrolysis product as shown in Figure 4a. In Figure 4b, two new peaks could be identified. The characteristic fragment ion of $[M-H]^-$ was identical to the STE standard that were all at m/z 317.3. The spectral data of IR (KBr flaking, cm^{-1}) for it were 1690, 2950, 3280, 3460. 3460 (-OH), 1690 (-COOH), identical to that of the STE standard (Figure 4c). Therefore, the new compound was identified as STE. There was another peak at m/z 665.2 (Figure 4b) which was an intermediate product of the biotransformation process and it was identified to be rubusoside (RS) as reported by Jiang et al [15].

2.3. Effects of inoculum concentration on conversion rate

The effects of inoculum concentration on STV conversion efficiency were studied using four different groups of PDA liquid medium containing 0.3%, 0.5%, 1%, 3% and 5% (v/v) of inoculum, respectively, to which 1% (v/v) of steviol glycosides (50 ml) was applied (Figure 5). As revealed in Figure 5, the highest conversion (71.9%) of STV was obtained after 12 h of treatment with 1% (v/v) inoculum concentration. This may be the sufficient oxygen and high activities of enzymes were presented at this concentration, which was favourable for STV conversion.

2.4. Biological transformation

The biological transformation of steviol glycosides were studied by determining the change of bioconversion efficiencies along with the treatment time prolongs (Figure 6). Figure 7 showed the chromatograms of the treatment medium at various times. The initial concentration of STV was 3.2 mg/ml, and the concentration of STV

decreased, while the conversion efficiency increased with the treatment time prolonging (Figure 7a). As mentioned earlier, there was an intermediate product of RS (Figure 7b) which indicated that the hydrolysis was achieved in a two-step process. The STV was completely eliminated and the conversion reached 100% after 24 h treatment. Similarly, an apparent decrease in RA concentration (from 3.2 mg/ml) was also observed and the conversion after 24 h treatment was 72%, and complete elimination was reached after 84 h (Figure 7c and 7d). In addition, the concentration of RC was almost unchanged and 98% of it still remained after 24 h treatment. 6 Days treatment period was needed to completely eliminate RC (Figure 7d). The relative elimination rates of substrates were in the order of STV > RA > RC (Figure 8).

2.5. Separation and purification of STE

The product STE was extracted by n-butanol, the organic phase was concentrated in vacuum, and the impurities such as esters were removed by petroleum ether/acetone=(85:15, v/v). Finally, the solvent was volatilized in water bath. The purity of STE was about 90%, and the yield was 81.6%.

3. Discussion

M. barkeri was a newly identified and highly efficient strain that can be used for the bioconversion of steviol glycosides to STE *in vitro*. In previous studies, it was shown that STV (2.5 mg/ml) and RA (3 mg/ml) can be completely degraded to STE by a rat whole cell suspension within 48 h and 144 h [16]. In this study, STV (3.2 mg/ml and RA (3.2 mg/ml) were completely eliminated after 24 h and 84 h of treatment, respectively. In addition, RC was eliminated after 6 days. In comparison,

the conversion rates were relatively high and the substrate concentrations were also relatively higher.

By using the methods of HPLC/LC-MS/IR, the identity of the intermediate and final hydrolysis products were determined. STV was shown to be hydrolysed to the intermediate product RS and then to STE. RA was also completely hydrolysed to STE by *M. barkeri*, but a longer time was required (84 h). During the period of 0-24 h, RA was first hydrolyzed to RS, and this intermediate product was rapidly converted into STE.

In summary, a strain with highly efficient and specific ability to bioconversion steviol glycosides was screened and identified from soil. It effectively bioconverted steviol glycosides (STV, RA and RC) into the final product STE at 24 h, 84 h and 144 h, respectively. The highest conversion rate to 1% STV was achieved when the inoculum of *M. barkeri* was 1% (V/V), which was advantageous in terms of time and energy consumption.

4. Experimental

4.1. Chemicals and medium

The potato dextrose agar (PDA) liquid medium contained 1% steviol glycosides (STV, RA, and RC). Steviol glycoside samples were purchased from Pusaike Biotechnology Co. Ltd, Jiangxi Province, China. Standard samples of STE (purity > 99%) were obtained from Zhangshu Chemical Technology Co. Ltd, Shanghai, China.

4.2. Analytical methods

Quantitative analysis of steviol glycosides was carried out by using high

performance liquid chromatography (HPLC) method on an Agilent 1100 HPLC system. STV, RA, RC and their metabolites were separated on a Kromasil-NH₂ column (250 mm×4.6 mm I.D., 5 μm) at a column temperature of 25 °C. The composition of the mobile phase was acetonitrile/H₂O (80:20, v/v) at a rate of 1.0 ml/min. UV detection was at 210 nm. Liquid chromatography-mass spectrometry (LC-MS) was conducted on an Agilent 6460 HPLC system, coupled with negative electrospray ionization (ESI) tandem mass spectrometry (MS/MS) method. Mass spectra in the negative ion mode were operated under the following conditions: fragmenter voltage=5 eV, voltage=3500 V, nebulizer pressure=45 psi, capillary temperature=300°C, *m/z* range=50-1000. The analytical column used was a Kromasil-NH₂ column (250 mm×4.6 mm I.D., 5 μm), and the flow rate was 0.5 ml/min. The injection volume was 20 μl. Infrared spectroscopy (IR) was conducted on Nexus 670. MID-IR, scanning range 4000-400 cm⁻¹.

4.3. Isolation and identification of the strain

Soil samples were collected from the campus of Nanjing Normal University, Jiangsu, China.

Genomic DNA of isolated strain was extracted using a SK1201-UNIQ-10 column type bacterial DNA Isolation Kit (Sangon, Shanghai, China). The 16S rDNA was amplified by polymerase chain reaction (PCR) using universal primers (27f, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r, 5'-GGTTACCTTGTTACGACTT-3'). The PCR system consisted of: 10 pmol of template; 1 μl of primer up (10 μM); 1 μl of primer down (10 μM); 1 μl of dNTP mix

(10 mM each); 5 μ l 10**Taq* reaction buffer; 0.25 μ l *Taq* DNA polymerase (5 U/ μ l) and adding water to 50 μ l. Amplification parameters were as follows: 5 min at 98 °C; 35 s at 95 °C, 35 s at 55 °C, and 1 min at 72 °C for 35 cycles; with a final extension step of 8 min at 72 °C. The 16S rDNA gene was amplified and submitted to National Center for Biotechnology Information (NCBI) GeneBank for comparison with blast sequence database. The highly similar 16S rDNA sequences were obtained from the database, and analyzed by MegAlign software to construct phylogenetic trees.

4.4. Bioconversion of steviol glycosides

Microbaterium barkeri were added to the PDA liquid medium contained 1% (v/v) of solutions of steviol glycosides (50 ml). The medium was treated at 37 °C and 220 rpm for 6 days under aerobic conditions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was financially supported by the National Key Research and Development Program of China (2019YFA0904900), the Program for Jiangsu Excellent Scientific and Technological Innovation Team(17CXTD00014) and the Special Projects of Introduction of Foreign Technical and Managerial Talents (BX2019014).

References

- [1] C. Rosaria, M. Francesco, P. Mario, and P. Mario, *Biofuel. Bioprod. Biorefin.* **13**, 445-452 (2019).
- [2] D.D. Soejarto, E.M. Addo, and A.D. Kinghorn, *J. Ethnopharmacol.* **243**, 112056 (2019).
- [3] O. Kim, C. Simon, S. Angelika, S. Ernesto, D.M. Michael, Birger, and L.M. Birger, *Microb. Cell. Fact.* **15**, 207-222 (2016).
- [4] D.Y. Zhang, Y. Tang, K. Wang, X.M. Wu, and W.Y. Hua, *J. Chin. Pharm. Univ.* **41**, 20-25 (2010).
- [5] S. Casas-Grajales, K. Reres-Gordillojales, C.M. Cerda-García-Rojas, V. Tsutsumi, M.R. Lakshman, and P. Muriel, *J. Appl. Toxicol.* **39**, 1118-1131 (2019).
- [6] J. Jiang, L. Qi, Q. Wei, and F. Shi, *Reprod. Toxicol.* **76**, 35-45 (2018).
- [7] C. Schiano, V. Grimaldi, M. Franzese, C. Fiorito, F.D. Nigris, F. Donatelli, A. Soricelli, M. Salvatore, and C. Napoli, *Toxicol. Vitro.* **62**, 104694 (2020).
- [8] T. Terai, H. Ren, G. Mori, Y. Yamaguchi, and T. Hayashi, *Chem. Pharm. Bull.* **50**, 1006-1010 (2002).
- [9] M. Ritu and J. Nandini, *J. Sci. Food. Agric.* **96**, 4231-4234 (2016).
- [10] B.H. De Oliveira, J.C. Stiirmer, J.D. de Souza Filho, and R.A. Ayub, *Phytochemistry.* **69**, 1528-1533 (2008).
- [11] R.E. Wingard, J.P. Brown, F.E. Enderlin, J.A. Dale, R.L. Hale, and C.T. Seitz, *Experientia.* **36**, 519-520 (1980).
- [12] A.M. Hutapea, C. Toskulkao, D. Buddhasukh, P. Wilairat, and T. Glinsukon, *J.*

- Clin. Biochem. Nutr.* **23**, 166-186 (1996).
- [13] E. Koyama, K. Kitazawa, Y. Ohori, O. Izawa, K. Kakegawa, A. Fujino, and M. Ui, *Food. Chem. Toxicol.* **41**, 359-364 (2003a).
- [14] E. Koyama, N. Sakai, Y. Ohori, K. Kitazawa, O. Izawa, K. Kakegawa, A. Fujino, and M. Ui, *Food. Chem. Toxicol.* **41**, 875-883 (2003b).
- [15] Z.Y. Jiang, Y.R. Chen, and H. Liu, *Acta Microbiol. Sin.* **51**, 43-49 (2011).
- [16] G.I. Kovylyaeva, G.A. Bakaleinik, I.Yu. Strobykina, V.I. Gubskaya, R.R. Sharipova, V.A. Al'fonsov, and A.G. Tolstikov, *Chem. Nat. Compd.* **43**, 81-85 (2007).

Table 1. Structures of steviol glycosides.

No.	Compounds	Abbreviation	R1	R2
1	Stevioside	STV	glc-	glc ² -glc-
2	Steviol	STE	H-	H-
3	Stevia monoglycoside	SM	H-	glc-
4	Steviolbioside	SB	H-	glc ² -glc-
5	Rubusoside	RS	glc-	glc-
6	Rebaudioside A	RA	glc-	glc ² -[glc ³]-glc-
7	Rebaudioside B	RB	H-	glc ² -[glc ³]-glc-
8	Rebaudioside C	RC	glc-	rha ² -[glc ³]-glc-
9	Rebaudioside D	RD	glc ² -glc-	glc ² -[glc ³]-glc-
10	Rebaudioside E	RE	glc ² -glc-	glc ² -glc-
11	Rebaudioside F	RF	glc-	xly ² -[glc ³]-glc-
12	Rebaudioside G	RG	glc-	glc ³ -glc-
13	Rebaudioside H	RH	glc-	glc ³ -rha ² -[glc ³]-glc-
14	Rebaudioside I	RI	glc ³ -glc-	glc ² -[glc ³]-glc-
15	Rebaudioside J	RJ	rha ² -glc-	glc ² -[glc ³]-glc-
16	Rebaudioside K	RK	glc ² -glc-	rha ² -[glc ³]-glc-
17	Rebaudioside L	RL	glc-	glc ⁶ -glc ² -[glc ³]-glc-
18	Rebaudioside M	RM	glc ² -[glc ³]-glc-	glc ² -[glc ³]-glc-
19	Rebaudioside N	RN	rha ² -[glc ³]-glc-	glc ² -[glc ³]-glc-
20	Rebaudioside O	RO	glc ³ -rha ² -[glc ³]-glc-	glc ² -[glc ³]-glc-
21	Dulcoside A	DA	glc-	rha ² -glc-
22	Dulcoside B	DB	H-	rha ² -[glc ³]-glc-

Figure captions

Figure 1. Backbone structures of steviol glycosides.

Figure 2. The 16S rDNA sequence of *M. barkeri* XJ.

Figure 3. Phylogenetic tree of *M. barkeri* XJ.

Figure 4. LC-MS and IR spectrum of product. (a) LC-MS of STE, (b) LC-MS of RS, (c) IR spectrum of new product and STE standard.

Figure 5. Effects of inoculum concentration on conversion of STV.

Figure 6. Time courses of conversion efficiencies from steviol glycosides.

Figure 7. Typical HPLC chromatograms obtained at different times of steviol glycosides solutions treated with *M. barkeri* XJ (a, HPLC chromatogram of 1% STE glycosides solution; b, HPLC chromatogram of conversion medium after 12h treatment; c, HPLC chromatogram of conversion medium after 24h treatment; d, HPLC chromatogram of conversion medium after complete conversion of steviol glycosides).

Figure 8. Deglycosylation pathways of steviol glycosides in *M. barkeri*.

Figure 1. Backbone structures of steviol glycosides.

```

1   CAGAGTTTGA TCCTGGCTCA GGATGAACGC TGGCGGCGTG CTTAACACAT
51  GCAAGTCGAA CGATGAAGCC CAGCTTGCTG GGTGGATTAG TGGCGAACGG
101 GTGAGTAACA CGTGAGCAAC CTGCCCCTGA CTCTGGGATA ACAGCCGGAA
151 ACGGTTGCTA ATACCGGATA TGCATCATGG CCGCATGGTC TGTGGTGGA
201 AAGATTTTTC GGTTGGGGAT GGGCTCGCGG CCTATCAGCT TGTGGTGAG
251 GTAGTGGCTC ACCAAGGCGT CGACGGGTAG CCGGCCTGAG AGGGTGACCG
301 GCCACACTGG GACTGAGACA CGGCCAGAC TCCTACGGGA GGCAGCAGTG
351 GGGAATATTG CACAATGGGC GGAAGCCTGA TGCAGCAACG CCGCGTGAGG
401 GATGACGGCC TTCGGGTTGT AAACCTCTTT TAGCAGGGAA GAAGCGTAAG
451 TGACGGTACC TGCAGAAAAA GCGCCGGCTA ACTACGTGCC AGCAGCCGCG
501 GTAATACGTA GGGCGCAAGC GTTATCCGGA ATTATTGGGC GTAAAGAGCT
551 CGTAGGCGGT TTGTCGCGTC TGCTGTGAAA ACCCGAGGCT CAACCTCGGG
601 CCTGCAGTGG GTACGGGCAG ACTAGAGTGC GGTAGGGGAG ATTGGAATTC
651 CTGGTGTAGC GGTGGAATGC GCAGATATCA GGAGGAACAC CGATGGCGAA
701 GGCAGATCTC TGGGCCGTTA CTGACGCTGA GGAGCGAAAG GGTGGGGAGC
751 AAACAGGCTT AGATACCTTG GTAGTCCACC CCGTAAACGT TGGGAACTAG
801 TTGTGGGGGC CTTTCCACGG TCTCCGTGAC GCAGCTAACG CATTAAAGTC
851 CCCGCCTGGG GAGTACGGCC GCAAGGCTAA AACTCAAAGG AATTGACGGG
901 GACCCGCACA AGCGGCGGAG CATGCGGATT AATTCGATGC AACGCGAAGA
951 ACCTTACCAA GGCTTGACAT ACACCGGAAA CGTCTGGAAA CAGTCGCCCC
1001 TTTTGGTTCG GTGTACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCTG
1051 AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCTCGTT CTATGTTGCC
1101 AGCACGTCAT GGTGGGAACCT CATGGGATAC TGCCGGGGTC AACTCGGAGG
1151 AAGGTGGGGA TGACGTCAAA TCATCATGCC CTTATGTCT TGGGCTTCAC
1201 GCATGCTACA ATGGCCGGTA CAAAGGGCTG CAATACCGTG AGGTGGAGCG
1251 AATCCCAAAA AGCCGGTCCC AGTTCGGATT GAGGTCTGCA ACTCGACCTC
1301 ATGAAGTCGG AGTCGCTAGT AATCGCAGAT CAGCAACGCT GCGGTGAATA
1351 CGTTCCCGGG TCTTGACAC ACCGCCCGTC AAGTCATGAA AGTCGGTAAC
1401 ACCTGAAGCC GGTGGCCTAA CCCCTTGTGG GAGGGAGC

```

Figure 2. The 16S rDNA sequence of *M. barkeri* XJ.

Figure 3. Phylogenetic tree of *M. barkeri* XJ.

Figure 4. LC-MS and IR spectrum of product. (a) LC-MS of STE, (b) LC-MS of RS, (c) IR spectrum of new product and STE standard.

Figure 5. Effects of inoculum concentration on conversion of STV.

Figure 6. Time courses of conversion efficiencies from steviol glycosides.

Figure 7. Typical HPLC chromatograms obtained at different times of steviol glycosides solutions treated with *M. barkeri* XJ (a, HPLC chromatogram of 1% steviol glycosides solution; b, HPLC chromatogram of conversion medium after 12 h treatment; c, HPLC chromatogram of conversion medium after 24 h treatment; d, HPLC chromatogram of conversion medium after complete conversion of steviol glycosides).

Figure 8. Deglycosylation pathways of steviol glycosides in *M. barkeri*.