A sustainable strategy for biosynthesis of Rebaudioside D using a novel glycosyltransferase of Solanum tuberosum

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A sustainable strategy for biosynthesis of Rebaudioside D using a novel glycosyltransferase of *Solanum tuberosum*

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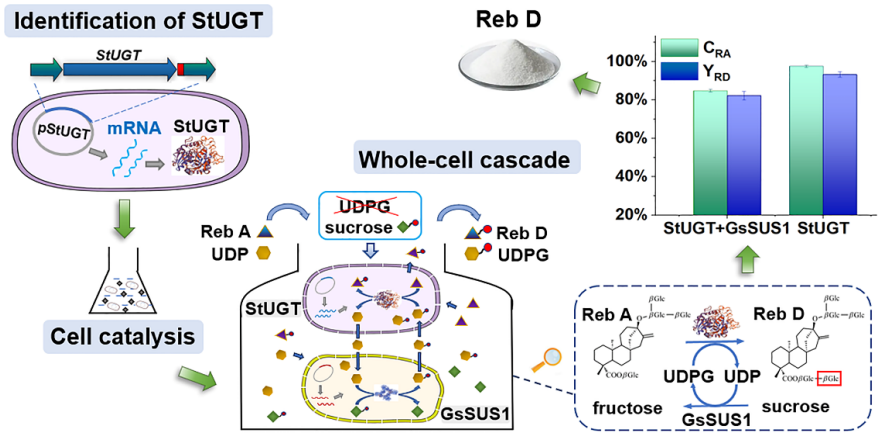
with sucrose. A StUGT-GsSUS1 system exhibited high catalytic capability, and 5.27 g/L Rebaudioside D was achieved finally without UDPG addition by systematic optimization. This is the best performance reported in cell-cascaded biosynthesis, which paves a new cost-effective strategy for sustainable synthesis of scarce premium sweeteners from biomass.

Key words: UDP-glycosyltransferase, StUGT, Rebaudioside D, whole-cell catalysis, cascade reaction



Graphical Abstract and Lay Summary

Bioconversion of Rebaudioside D faces high-cost obstacles. This study screens and characterizes a novel high-efficient glycosyltransferase StUGT converting Rebaudioside A to Rebaudioside D. And a new cascade process utilizing this recombinant strain and *E. coli* expressing sucrose synthase is established to reduce cost through replacing expensive UDPG with sucrose.

1. Introduction

Steviol glycosides (SGs) isolated from the leaves of *Stevia rebaudiana* consist of a diterpenoid steviol backbone that are decorated with various sugar moieties at the steviol C13-hydroxyl and/or C19-carboxylic acid positions. They are popular, near-zero calory, high-stability, and high-sweetness (250–450 times sweetness than sucrose) sweeteners. As an ideal substitute for sucrose, SGs are widely used as sweeteners in the food, pharmaceutical and cosmetic industry. In addition, they have various health functions such as improving immunity, lowering blood pressure, and inducing an antidiabetic effect.[1] They have been approved a Generally Recognized as Safe (GRAS) status by the Food and Drug Administration (FDA) of the United States and also allowed to be used in other countries, including European Union, Japan, China, and Australia.[2] Stevioside (Stv, 5–10% of leaf dry weight) and Rebaudioside A (Reb A, 2–4%) are the major SGs in stevia leaves. Others such as Rebaudioside D (Reb D) and Rebaudioside M (Reb M) have a lower content (0.4–0.5%).[3] Among SGs, Reb D has a cleaner sweet taste, better sugar properties, and a higher market value when compared with Reb A and Stv. [4,5]

The SGs on the market are typically extracted from Stevia leaves by maceration and heat extraction.[6]The isolated single components of Reb A or Reb D are more popular as advanced food additives, due to their improved sweetness characteristics and ease of use. However, the extraction process of single target components consumes large amounts of water, chemicals, and time. Large-scale production of Reb D by traditional extraction is difficult due to its low content in stevia leaves and poor water solubility.[7] Therefore, biocatalytic production of Reb D has been paid attention to reduce its environmental impact and increase production throughput.

In *S. rebaudiana* , UDP-glycosyltransferases (UGTs) catalyze the synthesis of SGs.[8] The diterpene steviol produced from the gibberellic acid (GA) biosynthetic pathway is sequentially glucosylated by UGT74G1, UGT85C2 and UGT91D2 to form Stv. Then, UGT76G1 and UGT91D2 sequentially convert Stv to Reb A followed by a conversion to Reb D.[9] The Reb A is relatively cheap and easy to be obtained, and Reb D production from Reb A catalyzed by UGTs is studied. The engineered *Oryza sativa* EUGT11 was shown to convert Reb A to Reb D.[10] *Solanum lycopersicum* UGTSL2 was also identified to glycosylate Reb A and produce Reb D, but with a byproduct of Reb M2.[7] Currently, only a few UGTs can synthesize Reb D from Reb A via glycosylation at C19. The UGTs belong to the Glycosyltransferases 1 (GT1) family consisting of 26,825 members, and their actual functions still remain obscure.[11] Therefore, it is essential and challenging to explore novel and highly efficient UGTs from nature for improving the biosynthesis of Reb D.

SGs production using crude enzyme usually increase the time and cost due to the complex preparation process of crude extracts. The whole-cell catalysis is a simple, rapid, cost-effective, and environmentally friendly process. [12]However, the literature hosts few reports with respect to highly efficient whole-cell catalysis for Reb D. We engineered a *P. pastoris*secretively expressing EUGT11, which can catalyze Reb A to produce Reb D with a 95% conversion rate. The secreted extracellular enzyme is likely to contact the substrate to increase the reaction rate. But, an intracellular enzyme has better stability but relatively low efficiency due to the cellular surface barrier.[7]Therefore, a whole-cell catalytic system with intracellular enzymes is also worthy being investigated and developed, nevertheless, the relevant research is still lack at present. Besides, in the reported whole-catalysis system, only 1 g/L substrate was conducted and the titer of the product is relatively low,[10,13] which would result in a relatively high product recovery cost.[14] Therefore, the improvement of product concentration should be considered for industrial-scale production.

Transglycosylation catalyzed by UGTs requires an expensive uridine diphosphate glucose (UDPG) as a sugar donor. Sucrose synthase (SuSy) converts sucrose and uridine diphosphate (UDP) to UDPG and fructose. [15,16] As such, the UGT and SuSy can be coupled to generate UDPG providing sugar donors to reduce the production cost of Reb D.[17]However, the synthesis of Reb D using a cell mixture of UGT and SuSy has not been used, to the best of our knowledge. A mixed whole-cell strategy would decrease the costs of feedstocks and enzyme preparation and reduce the production time.

In general, Reb D bioconversion faces serious bottlenecks. Only a few UGTs may catalyze the reaction, which causes a necessity to explore novel high-efficiency enzymes. The Reb D biosynthesis cost is relatively high due to time-consuming and complicated enzyme preparation procedures, low concentrations of the final product, and the high cost of reagents in the reaction system. Therefore, exploration of new and highly efficient UGTs and catalytic strategies is imperative for large-scale production of Reb D.

To reach this goal, this study screened and characterized a novel glycosyltransferase for glycosylation of Reb A to Reb D. A high-efficiency whole-cell catalytic technology for Reb D was then developed using a mixed recombinant *E. coli* expressing StUGT and GsSUS1. As a whole, this study provides a novel bioconversion technology for Reb D synthesis. The technology may decrease wastewater discharge, increase cost efficiency, and establish a foundation for green and scalable industrial production of premium sweeteners.

2. Materials and Methods

2.1 Chemicals and reagents

Reb A and Reb D were supplied by Sinochem Health Company Ltd. (Qingdao, China). UDPG was obtained from Canspec Inc. (Beijing, China). HPLC grade of acetonitrile was purchased from Fisher Scientific (Beijing, China). All other reagents were analytical grade and commercially available.

2.2 Protein sequence and structure analysis

UGTSL2 (GenBank: XP_004250485.1) and EUGT11 (GenBank: XP_015629141.1) was used as a query to search against the National Center for Biotechnology Information (NCBI) database to retrieve the homology sequences, respectively. Multiple sequence alignment was performed with ClustalX software (EMBL, Heidelberg, Germany). The secondary structure of the StUGT (XM_006367619.1) was analyzed by the online software ESPript3 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Functional domains were predicted using conserved domain database (CDD). A three-dimensional structure of StUGT was simulated by the SWISS-MODEL online server (https://swissmodel.expasy.sorg/) using the crystal structure of the OsUGT91C1 (PDB: 7ES2) as a template. And the structure of StUGT was analyzed and visualized using Pymol software (DeLano Scientific LLC). Model refinement and docking of the Reb A to StUGT were performed using AutoGrid4 and AutoDock4, respectively.

2.3 Plasmid and strains

The StUGT, CaUGT and BdUGT genes were codon-optimized and synthesized by GenScript (Nanjing, China), and the synthesized genes were then inserted into *Nco* I and*Xho* I sites of expression vector backbone pET28a (+) using Gibson Assembly according to the manufacturer's procedure (NEB, America), respectively. The resulting recombinant vectors were named pETStUGT, pETCaUGT and pETBdUGT, and then transformed into competent *E. coli* BL21 (DE3) cells (Biomed, Beijing). The transformants were selected on Luria Bertani (LB) plates supplemented with 50 μg/mL kanamycin. And the positive colonies were verified by PCR using T7 primers (T7 forward: 5'-TAATACGACTCACTATA-3' and T7 reverse: 5'-TAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT TTTTGA-3') and sequencing (Sangon, Shanghai). The resulting recombinant strains were named BL21 (pETStUGT), BL21 (pETCaUGT) and BL21 (pETBdUGT), respectively. All plasmids and strains used in this study are listed (Table S1).

2.4 Expression and preparation of the glycosyltransferases

The three engineered *E. coli* strains were inoculated into 5 mL of LB medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of NaCl) containing 50 μg/mL of kanamycin, and cultured at 37 °C with 220 rpm overnight, respectively. One mL of overnight cultures was transferred into 100 mL of Terrific Broth (TB) for culture at 37 °C. When the OD_{600} of cultures reached 0.6–0.8, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added with a final concentration of 0.5 mM for induction of expression of target proteins.

When they were cultured for 12 h, 100 mL culture broth was taken and centrifuged at 12,000 rpm for 10 min at 4 °C for collection of the corresponding cells. And the cell pellets were washed once with 10 mM of pH 7.2 phosphate buffer solution (PBS, 50 mM NaH_2PO_4, 300 mM NaCl) and suspended in pH 7.2 PBS with a final concentration of 30 OD_{600}. The suspensions were ultrasonicated by an Ultrasonic Cell Disruptor (Model JY92-2D, Scientz Industry, Ningbo, China) for 1 h (5 s plus on and 5 s off, 300 W) on ice. Then, the supernatants were collected and used for enzyme assay.

2.5 Expression optimization and purification of StUGT

The strain BL21 (pETStUGT) was inoculated and cultured until the OD_{600} reached 0.6–0.8 as described aforementioned. The IPTG was then added with a final concentration of 0.5 mM and the cultures were incubated at 18°C, 25°C, and 30 °C for 5, 12 and 17 h to optimize the expression level of StUGT. The 20 μL and 50 μL of crude enzyme solution was subjected to SDS-PAGE analysis and detection of protein concentration, respectively. The 150 μL of crude enzyme preparation was used for enzyme assay.

The strain BL21 (pETStUGT) was cultured in LB medium containing 0.5 mM IPTG at 18 °C for 17 h. Cells were then collected by centrifugation at 12,000 rpm for 10 min and washed once with 10 mM of pH 7.2 PBS. To optimize purification efficiency, pH 4.0, 6.0, 7.0 and 8.0 PBS was prepared for affinity purification. The cell pellets were suspended in pH 4.0–8.0 binding buffer (PBS with 2 mM imidazole) with a final concentration

of 30 OD_{600}. The suspensions were ultrasonicated by an Ultrasonic Cell Disruptor for 1 h (5 s plus on and 5 s off, 300 W) on ice. The supernatants were collected and then loaded onto a Ni-NTA column (GE Healthcare, America). The target enzyme was then washed twice using the pH 4.0–8.0 washing buffer (PBS containing 20 mM imidazole) and eluted with the same pH of the elution buffer (PBS containing 150 mM imidazole) as described previously.[11] Subsequently, the elution fractions containing the purified StUGT were desalted twice to remove the imidazole and NaCl by using the PD-10 column (Merk, Germany). The purified enzyme was then subjected to 10% SDS-PAGE analysis and protein concentration determination by Bradford method.[18]

2.6 Enzymatic assays

The glucosyltransferase assay was performed in 0.5 mL mixture containing 1 mM Reb A, 1 mM UDPG, 3 mM $MgCl_2$, 50 mM pH 7.2 PBS and 2 nmol of purified protein (102 μg StUGT) or 150 uL crude enzyme. The mixture was incubated at 30 °C for 24 h, and 0.5 mL of 60% (v/v) acetonitrile was then added to quench the reaction. The Reb A and Reb D contents were determined by High Performance Liquid Chromatography (HPLC). The molar conversion rate of Reb A(C_{RA}) and molar yield of Reb D (Y_{RD}) were then calculated as follows:

C_{RA} (%) = (RA_{t0}-RA_t) / RA_{t0}

Y_{RD} (%) = RD_t / RA_{t0}

where RA_{t0} represented the initial molar concentration of Reb A, and the RA_t and RD_trepresented the final molar concentration of Reb A and Reb D after reaction, respectively.

To determine the effect of reaction condition on catalytic efficiency, the reaction parameters such as temperature, pH, metal ions and UDPG were varied separately, while the others except the investigated parameter were kept constant. And the relative activity was calculated using the ratio of C_{RA} each condition to the highest that to compare the catalytic activities under different conditions. The optimal temperature for StUGT was investigated at temperature ranging from 25 °C to 50 °C. The optimal pH of StUGT was determined using 50 mM citrate buffer (pH 5.0–6.0), potassium phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0) and glycine buffer (pH 9.0–10.5). The concentrations of UDPG were adjusted to 1, 2, 3, 4 and 6 mM to test the influence of the glycosyl donor concentration on glycosylation efficiency. Divalent metal ions, including $MgCl_2$, Ca $(NO_3)_2$, $CuSO_4$, Pb $(CH_3COO)_2$, $FeSO_4$, $BaCl_2$, $ZnSO_4$, $CoCl_2$ and $MnCl_2$ were added to the reaction mixtures at a final concentration of 1 mM, 3 mM and 6 mM to optimize the yield of Reb D. One unit (U) of glucosyltransferase activity was defined as amount of enzyme catalyzing the bioconversion of 1mmol of Reb A per minute. The specific activity was expressed as units per gram of protein.

2.7 HPLC and LC-MS analysis

The 0.5 mL reaction system was mixed with 0.5 mL 60% (v/v) acetonitrile and centrifuged at 12,000 rpm for 10 min. The supernatants were taken up and then filtered using a 0.22 μm nylon membrane filter for HPLC analysis. HPLC was performed on an Agilent 1200 Series equipped with a UV-vis detector (wavelength = 210 nm) and carried out at 40 °C on a column of LunaC18 column (5 μm, 100 Å, Phenomenex). The mobile phase consisting of acetonitrile and 10 mM sodium dihydrogen phosphate buffer (pH 2.6) in the ratio of 32:68 (v/v) was used at a flow rate of 1 mL/min. The contents of Reb A and Reb D were detected based on an external standard method. The HPLC fractions at the retention time of 4 min were collected and submitted to Liquid Chromatograph Mass Spectrometer (LC-MS) analysis. Mass spectrometry for identification was carried out on a LCMS-IT-TOF (Shimadzu, Japan) equipped with an Electron Spray Ionization (ESI). ESI-MS was performed in negative ion mode under the following operating parameters: a capillary voltage of 1.75 kV, an atomizing gas flow of 10 L/min, a heating module temperature of 200 °C, a CDL temperature of 200 °C and a drying gas pressure of 100 kPa.

2.8 Kinetic analysis of glucosyltransferase StUGT

The kinetic characteristics of purified StUGT were determined by varying the Reb A concentration in the reaction mixture. The glycosylation reaction was carried out in 0.5 mL mixture containing 3 mM UDPG, 3 mM $MgCl_2$, 2 nmol of purified protein, 50 mM pH 8.0 potassium phosphate buffer and different concentrations of Reb A (0.1–1.0 mM) at 40 °C. After incubation for 1 hour, the reaction was stopped and the supernatant was taken up for the component determination. The reaction rate was calculated as micromoles of Reb A converted per milligram of the purified StUGT per minute (μmol/mg/min). The Reb D production rate versus substrate concentration was fitted to Michaelis-Menten kinetics, and the K_m and K_{cat} of StUGT for Reb A was calculated according to the Lineweaver-Burk plot.[19]

2.9 Whole-Cell catalysis Assay

The strain BL21 (pETStUGT) was cultured and collected as described above. Then, the cells were washed once and resuspended in 10 mM phosphate buffer (pH 7.2). The suspensions as whole-cell catalysts were added to 0.5 mL of above reaction mixture with a final cell density of 60 OD_{600} (22.8 g DCW/L). The reaction was conducted at 30 °C for 24 h. In order to enhance the permeability of cell membrane, the washed cells were suspended in 0.1–0.3 g/L surfactants (Triton-X-100, CTAB and Tween-80) and incubated at 30 °C for 30 min. The cells were then collected and washed once, and then resuspended to an OD_{600} of 100 (38 g DCW/L) in 10 mM PBS (pH 7.2). These treated cells were added to aforementioned reaction mixture at a final cell density of 60 OD_{600} to investigate the catalytic efficiency.

The cells treated with 0.2 g/L CTAB were added to the above reaction system at a final cell density of 60 OD_{600} to investigate the influence of reaction parameters on catalytic efficiency. The reaction parameters such as temperature, pH, and the reaction time, StUGT biomass/RA ratio, shaking speed or RA/UDPG ratio varied separately, while the others except the investigated parameter were kept constant. The activity was measured at temperature ranging from 25 °C to 50 °C. For determination of optimal reaction pH, the reactions were performed in the four kinds of buffer with pH 5.5 to 10.5. The optimal reaction time was examined in the range of 2–48 h. To optimize the ratio of StUGT biomass/RA, 24–96 OD_{600}cells were used with 1, 2 and 3 g/L Reb A as substrate, respectively. The shaking speed was set at 0, 50, 100, 150, 200 and 250 rpm, respectively. The ratio of RA/UDPG was set up ranging from 2:1 to 1:4.

In order to determine the optimum combination of reaction conditions in whole-cell bioconversion, four factors each at three levels were first considered and an orthogonal layout of L9 (3^4) was performed (Table S4). And then the catalytic parameters, including the biomass, shaking speed, the ratio of RA/UDPG and reaction time of the whole-cell catalysis were also optimized by the orthogonal test (Table 1). The influence of various parameters on Reb D yield was assessed by the range analysis.

2.10 Fed-batch synthesis of Reb D by whole-cell catalysis

The fed-batch reaction was carried out in optimal conditions as described above. The initial reaction mixture (0.5 mL) containing 5 g/L Reb A, 12.5 g/L UDPG, 50 mM PBS (pH 8.0), and 72 OD_{600}(27.36 g DCW/L) StUGT cell catalysts was incubated at 30°C for 60 h. In the fed-batch reaction, 5 g/L RA was added once at 16 h; in another reaction, 2.5 g/L Reb A was added twice at 16 and 30 h. Samples were collected at different time intervals (16, 30, 36, 42, 48, 52, and 60 h) to measure Reb A and Reb D concentrations.

2.11 Whole-cell cascade reaction

The strain BL21 (pETStUGT) and BL21 (pETAtSUS1) expressing the sucrose synthase AtSUS1 from *Arabidopsis thaliana* as well as BL21(pETGsSUS1) expressing GsSUS1 from *Gossypium schwendimani* were cultured and induced for expression as described above. The cells were then suspended in 0.15 g/L CTAB and incubated at 30 °C for 30 min for the improvement of cell permeability. The cells were collected and

resuspended to an OD_{600} of 300 (114 g DCW/L) in 10 mM phosphate buffer (pH 7.2) as whole-cell catalyst. The StUGT cell catalysts were added to reaction mixture with a final cell density of 54 OD_{600}(22.8 g DCW/L), and GsSUS1/AtSUS1 cell catalysts (27–270 OD_{600}) were added to the reaction mixture in the ratios of 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5, respectively. And the 0.5 mL of reaction mixture containing 2 g/L Reb A, 5 mM UDP, 50 mM PBS (pH 8.0), 2.1 mM sucrose and the mixed cells was incubated at 30 °C for 24 h. Reactions were quenched by addition of 0.5 mL 60% (v/v) acetonitrile. The cascade efficiency was calculated as follows:

The cascade efficiency (%) = Y_{RDt} / Y_{RDt0}

where Y_{RDt} indicated the Y_{RD} in the StUGT-SUS1 cascade system, and the Y_{RDt0} indicated the Y_{RD} in the only StUGT system under the same reaction conditions.

To further increase the Reb D yield by orthogonal test, four factors each at three levels were first considered (Table S5). And then the catalytic parameters, including the ratio of StUGT/GsSUS1, Reb A/sucrose, and sucrose/UDP were also optimized by the orthogonal test (Table S6).

3. Results and discussion

3.1 Screening of novel UGT for Reb D synthesis by bioinformatics analysis

The glycosyltransferases UGTSL2 and EUGT11 catalyzing the bioconversion of Reb A to Reb D were used as a query to search homologous glycosyl-transferase. The UDP-glucuronosyltransferase from *Solanum tuberosum* (StUGT) and *Capsicum annuum* (CaUGT) have 84.16% and 74.66% homology with UGTSL2, respectively. *Brachypodium distachyon* UDP-glucuronosyltransferase (BdUGT) shared the highest homologous with the EUGT11 (Table S2). These three candidate enzymes were expressed in *E. coli* to characterize whether they could convert Reb A to Reb D.

Their crude enzyme extracts were prepared for conversion of Reb A. No any product was formed using BdUGT as a catalyst. Not only a 4-minute product like Reb D but also other by-products were detected in the CaUGT-catalyzed reaction mixture. Nevertheless, the peak of Reb A catalyzed by the StUGT was disappeared completely and a new peak was appeared at the retention time of 4 min (Figure S1). The 4-min fractions were thus collected and analyzed by the LC-MS. The peak appearing at m/z = 1151.4670 [M + H + Na]+ matched the Reb D peak (m/z = 1152.15 [M + H + Na]+). This indicates the product was Reb D with no evidence of by-products forming. This is the first report on how glycosyltransferase StUGT can specifically catalyze the bioconversion of Reb A to Reb D. Therefore, the StUGT arouses our great interest.

For a better understanding of the StUGT function, bioinformatics analysis of StUGT was conducted. Multiple sequence alignment and CDD analyses revealed that StUGT belonged to the glycosyltransferase 1 (GT1) family (Figure 1a). The model in Figure 1b indicates StUGT has N-terminal and C-terminal domains. The C-terminal domain has a plant secondary product glycosyltransferase (PSPG) motif composed of 44 amino acid residues (residues 318–361), which involves the binding of UDP-sugar donors (Figure 1a). [20] The more flexible N-terminal domain participates in the binding of various acceptors of sugar residues. [21] The amino acid residues Glu67 and Glu70 at the StUGT N-terminus form bidentate hydrogen bonds with the hydroxyl groups of the Reb A (Figure 1c). The analysis of the secondary and spatial model also suggests StUGT has the potential to transfer glucose from the glycosyl donor UDPG to Reb A to produce Reb D (Figure 1d).

3.2 Functional characterization of StUGT in*E. coli*

The expression of StUGT was induced under various conditions and the crude enzyme was all prepared from the same numbers of cells for SDS-PAGE analysis and protein concentration determination (Figure S2). The most intense band of StUGT was observed in the sample taken from cultures incubated at 18°C for 17 h.

Under these culture conditions, the total protein concentration and conversion rate of Reb A (C_{RA}) were the highest. All subsequent inductions were carried out at 18°C for 17 h to maximize conversion.

We purified the StUGT by affinity chromatography (Figure 2a) using a pH 4.0–8.0 buffer to optimize the purification efficiency. Each purified StUGT showed a single band on the SDS-PAGE gel with an approximate molecular mass of 51 kDa, which was consistent with the predicted value. C_{RA} from the StUGT purified at pH levels of 4.0, 6.0, 7.0, and 8.0 were 85.40%, 89.36%, 70.48%, and 64.39%, respectively (Figure S3). When the pH of the purification buffer was 4.0 or 6.0, the catalytic activity was significantly higher than in other conditions. This may be due to the distribution of total net charges on the StUGT at those pH levels where the pH of the purification buffer is close to the isoelectric point (pI = 5.18) of the target protein. As a result, the distribution maintains the catalytic activity of active sites.[22] Due to the increased activity, the StUGT was purified using a pH 6.0 buffer for subsequent experiments. The effect of purification buffer pH on purification efficiency has rarely been investigated; thus, our investigation of this effect in this study may provide valuable information that can be used for the improvement of purification efficiency.

3.3 Biochemical characteristics of the StUGT

The biochemical characteristics of enzymes are very crucial for their industrial application. The activity of purified StUGT gradually increased when the temperature was increased from 25°C to 40°C, but it reached a peak at 40°C (Figure 3a). The catalytic activity at 45–50°C decreased to 14.96–38.17% of the highest catalytic activity. This indicates a loss of activity at a higher temperature. The optimal temperature of 40°C for StUGT converting Reb A is similar to both UGTSL2 and EUGT11.[7,10]

The pH of the reaction impacts structural stability and catalytic activity of the enzyme. The activity was increased with a pH ranging from 5.0 to 8.0, with the maximum activity occurring at pH 8.0. The activity then showed a downward trend from pH 8.0 to 10.5 (Figure 3b). The dissociation state of the active groups at pH 8.0 may be optimum for the combination of enzymes and substrates.

UDPG is a glycosyl donor in the glycosylation reaction for Reb D, so the catalytic activity of StUGT was enhanced significantly when the concentration of UDPG increased from 1 mM to 3 mM (Figure 3c). The 3 mM UDPG concentration reached 98% of the highest catalytic activity. The maximum C_{RA} of 100% was observed at 4 mM UDPG, and the maximum activity still remained at 4–6 mM UDPG. However, a 3 mM concentration of UDPG is appropriate due to cost considerations.

Metal ions form complexes with enzymes and act as structural regulators and usually play a key role in protein folding and catalysis.[23]Therefore, metal ions were added to the reaction system to explore and expand the catalytic efficiency. Some metal ions, Cu^{2+}, Zn^{2+}, and Co^{2+}, severely inhibited the catalytic activity of the StUGT (Figure 3d). However, the addition of Pb^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, and Mn^{2+} improved the Reb D production by 5.28%, 10.01%, 7.63%, 9.86%, and 9.86%, respectively. This indicates these ions exhibit a positive effect on the enzymatic activity. The influence of concentrations of these ions on activity was thus also tested (Table 2). The Reb D yield (Y_{RD}) reached a maximum of 86.61% in the presence of 6 mM $MgCl_2$. Mg^{2+} as a cofactor promotes the binding of pyrophosphate groups in the sugar group donor and the active site of the enzyme to improve the catalytic activity of StUGT.[24]

Based on the optimal conditions discussed above, the specific activity and kinetic parameters of purified StUGT toward Reb A glycosylation were determined (Figure S4). The specific activity of the purified StUGT was 1024.53 U/g protein, and the maximum velocity (V_{max}) and the Michaelis constant (K_m) value were 0.1174 μmol min^{-1} mg^{-1} and 0.1073 mM, respectively. Since the literature lacks reports discussing glycosyltransferases catalyzing Reb A to Reb D, these parameters enable more functional studies of UGTs. A low K_m value represents a high affinity for the enzyme with a substrate. The K_m of StUGT for Reb A was the lowest compared with those of wild-type glycosyltransferases Yojk, UGTSL2, and EUGT11 (Table S3).[25-27] As such, StUGT might have a higher affinity for Reb A, and the reaction by StUGT exhibits a relatively high catalytic efficiency without by-products. The high efficiency and lack of by-products suggest

that StUGT has favorable product specificity and is a potential candidate for the process of producing Reb D for the food industry.

3.4 Construction of the *E. coli*whole-cell biocatalyst system

The strain expressing StUGT was directly used to construct a catalysis system for Reb D synthesis (Figure 2b), where C_{RA} of 13.45% by the cells was lower than that by the purified StUGT. The poor permeability of cell may limit enzyme–substrate contact to decrease catalytic efficiency.[28] As such, we treated cells using surfactants (CTAB, SDS, Tween-80, and Triton-X-100) and organic solvents (acetone, isopropyl alcohol, ethanol, and toluene) to enhance permeability. Treatment with 0.1–0.3 g/L of surfactants, except for SDS, significantly improved catalytic activity (Figure 4a), while the treatment with the organic solvents and SDS reduced catalytic activity (data not presented). The CTAB enhanced cell activity more than Tween 80 and Triton-X-100 at the same concentrations (Figure 4a). The cells treated with 0.2 g/L CTAB exhibited the highest catalytic efficiency (62.83% of C_{RA}), which was 367% higher than that of the control.

The cell permeability is crucial for catalytic function. An appropriate permeability improvement is difficult to obtain due to the divergent composition of the cell wall/membrane of different cell catalysts.[29] Therefore, proper cell treatment should be considered to establish an efficient whole-cell catalytic system.

3.5 Optimization of whole-cell catalysis efficiency

The biological reaction conditions have considerable influence on the biocatalytic activity.[30] Tracking the effect of reaction temperature, pH, and reaction time on catalytic efficiency enables optimal performance. The highest catalytic activity was obtained at 30°C (Figure 3a). The catalytic activity decreased when the temperature increased from 30°C to 50°C. The higher temperatures may contribute to cell degradation and inactivation.[31] The optimal temperature of 30°C can easily be maintained during industrial processes.

The peak activity by the whole-cell catalyst was observed at a pH of 8.0, which coincides with the optimal pH used with purified StUGT (Figure 3b). In the pH range of 6.5–9.5, the whole-cell biocatalysts could retain more than 70% of its optimal activity, while the purified enzyme only retained about 30% of its optimal activity. As such, the whole-cell catalysis has a wider pH tolerance than the purified enzyme due to how the outer barrier of cell wall and membrane protects enzymes from adverse experimental conditions.[32]

With regard to reaction time, the catalytic activity increased as the time increased from 0 to 14 h, with the highest C_{RA}obtained at 14 h (Figure 4b). The activity at 20 h retained 98.8% of the maximum, which was deemed insignificant ($P > 0.05$). This result demonstrates the catalysis reaction is rapid and stable.

The interactive effects of the abovementioned reaction condition parameters were further investigated by the L9 (3^4) orthogonal test. The range value R indicated the order of significance for each factor upon Y_{RD} was as follows: pH > CTAB concentration > temperature > reaction time (Table S4). Accordingly, the optimum reaction condition was pH 8.0, 30°C, 0.15 g/L CTAB, and 16 h reaction time. Under the optimal conditions, C_{RA} and Y_{RD} could reach 90.07% and 68.53%, respectively, suggesting adequate Reb D production. Cell catalysis is more easily influenced by factors such as cell density and stirring speed than a purified enzyme is, but the production is rapidly optimized using statistical methods. In short, C_{RA} improved by 34.32% more than the maximum value of the single-factor experiments. These results highlight the importance and impact of a systematic and efficient statistical strategy to optimize complex catalytic systems.

3.6 Whole-cell catalytic synthesis of high-titer Reb D

To obtain high-titer products, the substrate concentration was correspondingly increased from 1g/L to 3 g/L, and the effect of the ratio of catalyst and glycosyl donor to substrate on catalytic efficiency was investigated. When the substrate concentration was constant, C_{RA} increased gradually with increasing biomass of StUGT where it reached a maximum of 96 OD_{600}. An increase in C_{RA} had no significant impact in the range of

72–96 OD_{600} (Figure 4c), possibly due to that the excessive accumulation of cells in the reaction system obstructs the movement of substances into and out of the cells.[33]

With substrate concentrations of 1, 2, and 3 g/L, the catalytic efficiency reached the maximums at the 72:1, 48:1, and 36:1 ratios of StUGT biomass/Reb A (Figure S5a), respectively. This demonstrates the ratio of catalyst to substrate for obtaining a maximum C_{RA} decreased as the substrate concentration increased. This relationship highlights how the StUGT cells exhibit good catalytic performance for high-concentration substrates. The ratio of UDPG to substrate is important for glycosylation. C_{RA} reached a maximum with a RA/UDPG mass ratio of 1:2 (Figure S5b). The low content of UDPG possibly leads to insufficient glycosyl donors, while excessive high-concentration UDPG inhibits the activity of UGTs.[34] The catalytic efficiency at 0–250 rpm was also investigated. C_{RA} reached a maximum at 200 rpm and then gradually decreased with increasing speeds (Figure S5c). This may be due to how the increased shaking speed disrupts the structure of the enzyme.[31] When the system contains high-titer substrates, the cell catalysts and glycosyl donors increase proportionally with respect to the substrate concentration making the interaction space between substrate and enzyme closer. Accordingly, the shaking speed and reaction time need to be adjusted to obtain highly efficient conversion.

To investigate the reciprocal effect of high-titer substrate, cell catalyst concentration (biomass), and shaking speed, an L9 (3^4) orthogonal design was performed using 3 g/L Reb A as the substrate (Table 1). Based on the range analysis, the factor that exhibited the greatest influence on the Reb D production was RA/UDPG ratio, followed by the StUGT biomass, shaking speed, and reaction time. The optimal combination for the maximum production was a RA/UDPG ratio of 1:2, 72 OD_{600} cells, a shake speed of 0 rpm, and a 20 h reaction time (Table 1). The confirmatory tests under these optimal conditions highlight the highest Y_{RD} could reach was 94.53%, which is significantly higher than that of the first orthogonal test (68.53% of Y_{RD}).

A total of 2–10 g/L Reb A was then added in the optimal system for high titer production of Reb D. Y_{RD} increased gradually with Reb A amounts increasing from 1 to 6 g/L (Figure 4d) to reach a maximum yield of 6.12 g/L (Y_{RD} of 94.41%). The yield then exhibited a downward trend with increasing Reb A concentrations (Figure 4d). When the formed Reb D exceeded 4 g/L, it would precipitate due to the low solubility. Reb D found in solution and precipitate form were both calculated as part of the yield.

The Reb D production using over 8 g/L Reb A was significantly lower than when 6 g/L of Reb A was utilized as the substrate. This phenomenon stems from substrate inhibition. Two fed-batch reactions were initiated to avoid substrate inhibition. A 5 g/L Reb A sample was used as the initial substrate, and 5 g/L Reb A was added subsequently a single time or two doses of 2.5 g/L Reb A were added twice to achieve a final concentration of 10 g/L (Figure 5). Y_{RD} in the two fed reactions was increased to 5.88 g/L, which was 68% higher than the yield in the batch reaction using 10 g/L Reb A as a substrate (3.50 g/L). As such, the inhibitory effects might be circumvented by a fed-batch reaction.

In the two fed-batch reactions, Reb D titer, before feeding, reached 80% of the maximum, and the production rate (0.28 g/L/h) before feeding was significantly higher than that after feeding (Figure 5). Basically, the production process of Reb D became sluggish after feeding. Whether in a batch or feed-batch reaction, the product was no longer formed when Reb D reached a concentration close to 5.8 g/L, indicating that it is a threshold for dynamic equilibrium and the feeding does not significantly improve the yield. This stems from how excessively high concentrations of Reb D inhibit the reaction process, but the product inhibition could be alleviated by simultaneously removing the fermentation products.[35]

The maximum Reb D yield of 6.12 g/L was obtained from 6 g/L Reb A in the batch reaction. Presently, whole cell systems can convert 0.12–1.11 g/L Reb D from Reb A and with a Reb D yield of 10%–98.27%.[13,36] The higher Reb D production in this system (6.12 g/L) supports the use of this new system as an optimized and efficient catalytic system. High-titer products are difficult to gain successfully because the system usually contains plentiful substances such as substrates, catalysts, and ions that harmfully contribute to volume exclusion and Donnan equilibrium effects.[37] To overcome these challenges, we developed a cell catalytic platform to produce high-titer Reb D, which would promote high-titer production using cell catalysts.

3.7 Reb D production by StUGT and sucrose synthase

To reduce production costs, a system using whole-cell catalysts of StUGT and sucrose synthase was constructed to regenerate UDPG as donors for glycosylation (Figure 6a). The SuSy has a relatively broad substrate spectrum, and the candidate SuSy can convert sucrose and UDP to eliminate the need to synthesize costly UDPG.[17]Among the 48,300 different types of SuSy, only a few, such as AtSUS1, exhibit the proper function.[38] Therefore, the AtSUS1 sequence was used as a query to obtain a homologous SuSy from*Gossypium schwendimani* (GsSUS1). This shares the highest amino acid sequence identity (85%) with AtSUS1 and is a novel putative unknown function SuSy.

The authors previously constructed recombinant *E. coli* expressing AtSUS1 and GsSUS1 (unpublished) to develop the coupled system. The StUGT cells were combinatorically used with the various amounts of biomass of AtSUS1 and GsSUS1 cells. The two cascade-reaction system converted Reb A to Reb D, and Y_{RD} in the StUGT-GsSUS1 was significantly higher than that in the StUGT-AtSUS1 system (Figure 6b). When the ratio of StUGT/SUS1 was changed from 2:1 to 1:3, the production showed a noticeable upward trend where it reached a maximum at the ratio of 1:3. This is 61.67% of the maximum Y_{RD} in the StUGT reaction (Figure 6c). The Reb D decreased slightly as the ratio decreased from 1:3 to 1:5. This trend may stem from excessive whole-cell catalyst. As a whole, a cascade system using sucrose as the glucosyl donor was developed, and the function of GsSUS1 was also revealed for the first time. However, the StUGT-GsSUS1 system could benefit from further optimizing the catalytic conditions.

The orthogonal design was performed using 3 g/L Reb A as a substrate to optimize the production. The optimal condition for Reb D production was a StUGT/GsSUS1 cell ratio of 1:2, a pH of 8.0, a 200 rpm shake speed, and a 20 h reaction time (Table S5). The reaction time had the largest effect on Y_{RD} as shown by its extreme value. The optimal reaction time of 20 h was the longest among the investigated reaction times, which may be because the cascade reaction requires multiple catalytic steps. A relatively long reaction time is conducive to high conversion. Therefore, extending the reaction time was considered in the following experiment.

The Reb A concentration was increased to 6 g/L to improve the economy of industrial production, and an orthogonal test was performed to investigate the effect of the ratio between the reaction components on the cascade reaction. The optimal combination for maximum production was a StUGT/GsSUS1 cell ratio of 1:2.5, a RA/sucrose ratio of 1:5, a sucrose/UDP ratio of 1:1, and a 28 h reaction time (Table S6). The maximum Y_{RD} was 3.57 g/L, and the cascade efficiency reached 84.98%, which was 37.79% higher than the single-factor experiments. The sucrose/UDP ratio affected the results more than any other factor. Y_{RD} was highest when using a 1:1 ratio of sucrose/UDP, but it decreased significantly when using a 1:2 ratio. This may be because the high concentrations of UDP inhibit catalytic activity.

As a final effort to maximize efficiency, the biomass of StUGT/GsSUS1 in the system was increased from 54/135 to 72/180 OD_{600}under the above optimal reaction conditions. Y_{RD}improved to 5.27 g/L and could reach 88.10% of the pure StUGT reaction system (Figure 6d). The high-performance conversion results from the high-activity catalyst and the other optimized parameters.

Developing an efficient cascade catalytic system consisting of the poorly studied enzymes is difficult. We overcame this difficulty by expanding new candidate analogous enzymes and accurately designing cell combinations according to the characterization of catalysts. This work provides the first example of efficient production of high-titer Reb D by a whole-cell cascade model using novel SuSy and UGT. This system may serve as a guideline to build other cascade glycosylation systems to establish green and scalable industrial production of Reb D.

4. Conclusions

A novel glycosyltransferase StUGT with transglycosylation function of Reb A to Reb D was identified. The StUGT exhibited high substrate specificity and strong Reb A affinity. A whole-cell catalytic platform for high-titer production was developed, and a final 6.12 g/L Reb D was achieved from a 6 g/L Reb A feedstock, which is the highest production by whole-cell catalysis to date. We also developed a StUGT-GsSUS1 whole-cell-based cascade catalysis using cheap sucrose as a glycosyl donor, and 5.27g/L Reb D was obtained by systematic optimization. This report revealed a novel glycosyltransferase and established a high-efficiency low-cost biotransformation technology.

AUTHOR CONTRIBUTIONS

Si-yuan Ma: Methodology, Formal analysis, Investigation, Writing – original draft. **Yuan-yuan Ma:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Validation, Resources, Supervision, Writing – review and editing.

CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

No data was used for the research described in the article.

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REFERENCES:

1. Yadav, S. K., & Guleria, P. (2012). Steviol glycosides from Stevia: biosynthesis pathway review and their application in foods and medicine. *Critical Reviews in Food Science and Nutrition* ,*52* , 988-998.
2. Basharat, S., Huang, Z., Gong, M., Lv, X., Ahmed, A., Hussain, I., Li, J., Du, G., & Liu, L. (2021). A review on current conventional and biotechnical approaches to enhance biosynthesis of steviol glycosides in *Stevia rebaudiana* . *Chinese Journal of Chemical Engineering* , *30* , 92–104.
3. 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* , S1–S10.
4. Allen, A. L., McGeary, J. E., & Hayes, J. E. (2013). Rebaudioside A and Rebaudioside D bitterness do not covary with Acesulfame K bitterness or polymorphisms in *TAS2R9* and *TAS2R31* .*Chemosensory Perception* , *6* , 109–117.
5. Prakash, I., Dubois, G. E., Clos, J. F., Wilkens, K. L., & Fosdick, L. E. (2008). Development of rebiana, a natural, non-caloric sweetener. *Food and Chemical Toxicology* , *46* , S75–82.
6. Scipioni, G. P., Ferreyra, D. J., Acuna, M. G., & Schmalko, M. E. (2010). Rebaudioside A release from matrices used in a yerba mate infusion. *Journal of Food Engineering* , *100* , 627–633.
7. Chen, L., Sun, P., Zhou, F., Li, Y., Chen, K., Jia, H., Yan, M., Gong, D., & Ouyang, P. (2018). Synthesis of rebaudioside D, using glycosyltransferase UGTSL2 and in situ UDP-glucose regeneration.*Food Chemistry* , *259* , 286–291.

8. Wang, J., Li, S., Xiong, Z., & Wang, Y. (2016). Pathway mining-based integration of critical enzyme parts for de novo biosynthesis of steviolglycosides sweetener in *Escherichia coli* . *Cell Research* , *26* , 258–261.
9. Olsson, K., Carlsen, S., Semmler, A., Simon, E., Mikkelsen, M. D., & Moller, B. L. (2016). Microbial production of next-generation stevia sweeteners. *Microbial Cell Factories* , *15* , 1–14.
10. Wang, Z., Hong, J., Ma, S., Huang, T., Ma, Y., Liu, W., Liu, W., Liu, Z., & Song, H. (2020). Heterologous expression of EUGT11 from*Oryza sativa* in *Pichia pastoris* for highly efficient one-pot production of rebaudioside D from rebaudioside A.*International Journal of Biological Macromolecules* , *163* , 1669–1676.
11. Zhang, P., Zhang, Z., Zhang, L., Wang, J., & Wu, C. (2020). Glycosyltransferase GT1 family: Phylogenetic distribution, substrates coverage, and representative structural features. *Computational and Structural Biotechnology Journal* , *18* , 1383–1390.
12. De Carvalho, C. C. (2011). Enzymatic and whole cell catalysis: finding new strategies for old processes. *Biotechnology Advances* ,*29* , 75–83.
13. Fei, Liwen., & Wang, Y. (2018). Whole cell catalysis of sweetener rebaudioside D by recombinant *E.coli* . *Food Fermentation Industry* , *44* , 1–7.
14. Bell, E. L., Finnigan, W., France, S. P., Green, A. P., Hayes, M. A., Hepworth, L. J., Lovelock, S. L., Niikura, H., Osuna, S., Romero, E., Ryan, K. S., Turner, N. J.,& Flitsch, S. L. (2021). Biocatalysis.*Nature Reviews Methods Primers* , *1* , 46.
15. De Bruyn, F., Maertens, J., Beauprez, J., Soetaert, W., & De Mey, M. (2015). Biotechnological advances in UDP-sugar based glycosylation of small molecules. *Biotechnology Advances* , *33* , 288–302.
16. Zhang, L., Gao, Y., Liu, X., Guo, F., Ma, C., Liang, J., Feng, X., & Li, C. (2019). Mining of sucrose synthases from glycyrrhiza uralensis and their application in the construction of an efficient UDP-Recycling System. *Journal of Agricultural and Food Chemistry* , *67* , 11694–11702.
17. Schmolzer, K., Gutmann, A., Diricks, M., Desmet, T., & Nidetzky, B. (2016). Sucrose synthase: A unique glycosyltransferase for biocatalytic glycosylation process development. *Biotechnology Advances* , *34* , 88–111.
18. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* ,*72* , 248–254.
19. Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society* , *56* , 658–666.
20. Speeckaert, N., Adamou, N. M., Hassane, H. A., Baldacci-Cresp, F., Mol, A., Goeminne, G., Boerjan, W., Duez, P., Hawkins, S., Neutelings, G., Hoffmann, T., Schwab, W., Jaziri, M. E., Behr, M., & Baucher, M. (2020). Characterization of the UDP-glycosyltransferase UGT72 family in poplar and identification of genes involved in the glycosylation of monolignols. *International Journal of Molecular Sciences* ,*21* , 5018.
21. Palcic, M. M. (2011). Glycosyltransferases as biocatalysts.*Current Opinion In Chemical Biology* , *15* , 226–233.
22. Ahmad, N., Xu, K., Wang, J.-N., & Li, C. (2020). Novel catalytic glycosylation of Glycyrrhetinic acid by UDP-glycosyltransferases from*Bacillus subtilis*. *Biochemical Engineering Journal* , 162.
23. Ishida, N., Okubo, A., Kawai, H., Yamazaki, S., & Toda, S. (1980). Interaction of amino acids with transition metal ions in solution (I) solution structure of L-lysine with Co (II) and Cu (II) ions as studied by nuclear magnetic resonance spectroscopy. *Agricultural and Biological Chemistry* , *44* , 263–270.
24. Hu, Y., & Walker, S. (2002). Remarkable structural similarities between diverse glycosyltransferases. *Chemistry & Biology* ,*9* , 1287–1296.
25. Chen, L., Cai, R., Weng, J., Li, Y., Jia, H., Chen, K., Yan, M., & Ouyang, P. (2020). Production of rebaudioside D from stevioside using a UGTSL2 Asn358Phe mutant in a multi-enzyme system. *Microbial Biotechnology* , *13* , 974–983.
26. Guo, B., Hou, X., Zhang, Y., Deng, Z., Ping, Q., Fu, K., Yuan, Z., & Rao, Y. (2022). Highly efficient

production of rebaudioside D enabled by structure-guided engineering of bacterial glycosyltransferase YojK.*Frontiers in Bioengineering and Biotechnology* , *10* , 985826.
27. Lin, M., Wang, F., & Zhu, Y. (2020). Modeled structure-based computational redesign of a glycosyltransferase for the synthesis of rebaudioside D from rebaudioside A. *Biochemical Engineering Journal* , *159* , 107626.
28. Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli* : advances and challenges.*Frontiers in Microbiology* , *5* , 172.
29. Cortez, D. V., & Roberto, I. C. (2012). CTAB, Triton X-100 and freezing–thawing treatments of *Candida guilliermondii* : effects on permeability and accessibility of the glucose-6-phosphate dehydrogenase, xylose reductase and xylitol dehydrogenase enzymes.*New Biotechnology* , *29* , 192–198.
30. Wu, C., Ma, C., Li, Q., Chai, H., & He, Y. C. (2023). Efficient production of hydroxymethyl-2-furfurylamine by chemoenzymatic cascade catalysis of bread waste in a sustainable approach. *Bioresource Technology* , *385* , 129454.
31. Gao, R., & Li, Z. (2021). Biosynthesis of 3-Hydroxy-3-Methylbutyrate from l-Leucine by Whole-Cell Catalysis. *Journal of Agricultural and Food Chemistry* , *69* , 3712–3719.
32. Wu, S., & Li, Z. (2018). Whole-cell cascade biotransformations for one-pot multistep organic synthesis. *ChemCatChem* , *10* , 2164–2178.
33. Duan, P., Long, M., Zhang, X., Liu, Z., You, J., Pan, X., Fu, W., Xu, M., Yang, T., Shao, M., & Rao, Z. (2023). Efficient 2-O-α-D-glucopyranosyl-sn-glycerol production by single whole-cell biotransformation through combined engineering and expression regulation with novel sucrose phosphorylase from *Leuconostoc mesenteroides* ATCC 8293. *Bioresource Technology* , *385* , 129399.
34. Wang, Y., Chen, L., Li, Y., Li, Y., Yan, M., Chen, K., Hao, N., & Xu, L. (2016). Efficient enzymatic production of rebaudioside A from stevioside. *Bioscience, Biotechnology, and Biochemistry* ,*80* , 67–73.
35. Lye, G. J., & Woodley, J. M. (1999). Application of in situ product-removal techniques to biocatalytic processes. *Trends in Biotechnology* , *17* , 395–402.
36. Yang, Y., Fei, L., Li, J., Yang, J., & Wang, Y. (2017). Production of rebaudioside D by recombinant *Escherichia coli* whole cell catalyst [J]. *Industrial Microbiology* , *47* , 1–7.
37. Holstein, M., Hung, J., Feroz, H., Ranjan, S., Du, C., Ghose, S., & Li, Z. J. (2020). Strategies for high-concentration drug substance manufacturing to facilitate subcutaneous administration: A review.*Biotechnology and Bioengineering* , *117* , 3591–3606.
38. Tao, Y., Sun, P., Cai, R., Li, Y., & Jia, H. (2022). Exploring the strategy of fusing sucrose synthase to glycosyltransferase UGT76G1 in enzymatic biotransformation. *Applied Sciences* , *12* , 3911.

Table 1 Orthogonal results for Reb D production by StUGT cells

Number	Number	A (biomass)	A (biomass)	B (shaker speed)	B (shaker speed)	B (shaker speed)	C (RA/UDPG)	C (RA/UDPG)	D (reaction time)	D (reaction time)	D (reaction time)	Results	Resu
												RA conversion	RA conv
1	OD_{600} = 72	OD_{600} = 72	OD_{600} = 72	OD_{600} = 72	0 rpm	1:1.5	1:1.5	12 h	12 h	12 h	84.24%	84.24%	73.42
2	OD_{600} = 72	OD_{600} = 72	OD_{600} = 72	OD_{600} = 72	100 rpm	1:2	1:2	16 h	16 h	16 h	93.01%	93.01%	84.87
3	OD_{600} = 72	OD_{600} = 72	OD_{600} = 72	OD_{600} = 72	200 rpm	1:2.5	1:2.5	20 h	20 h	20 h	97.42%	97.42%	91.88
4	OD_{600}=96	OD_{600}=96	OD_{600}=96	OD_{600}=96	0 rpm	1:2	1:2	20 h	20 h	20 h	92.67%	92.67%	83.87

Number	Number	A (biomass)	A (biomass)	B (shaker speed)	B (shaker speed)	B (shaker speed)	C (RA/UDPG)	C (RA/UDPG)	D (reaction time)	D (reaction time)	D (reaction time)	Results	Resu
5	OD_{600}=96	OD_{600}=96	OD_{600}=96	OD_{600}=96	100 rpm	1:2.5	1:2.5	12 h	12 h	12 h	96.77%	96.77%	89.1
6	OD_{600}=96	OD_{600}=96	OD_{600}=96	OD_{600}=96	200 rpm	1:1.5	1:1.5	16 h	16 h	16 h	65.11%	65.11%	57.5
7	OD_{600} = 120	OD_{600} = 120	OD_{600} = 120	OD_{600} = 120	0 rpm	1:2.5	1:2.5	16 h	16 h	16 h	96.54%	96.54%	87.4
8	OD_{600} = 120	OD_{600} = 120	OD_{600} = 120	OD_{600} = 120	100 rpm	1:1.5	1:1.5	20 h	20 h	20 h	62.49%	62.49%	52.7
9	OD_{600} = 120	OD_{600} = 120	OD_{600} = 120	OD_{600} = 120	200 rpm	1:2	1:2	12 h	12 h	12 h	76.56%	76.56%	63.5
K_I	K_I	92.92%	92.92%	88.98%	88.98%	88.98%	68.26%	68.26%	81.71%				
K_{II}	K_{II}	83.65%	83.65%	81.96%	81.96%	81.96%	86.55%	86.55%	83.20%				
K_{III}	K_{III}	73.49%	73.49%	79.12%	79.12%	79.12%	95.24%	95.24%	85.15%				
R	R	19.43%	19.43%	9.86%	9.86%	9.86%	26.98%	26.98%	3.45%				

K_I, K_{II} and K_{III} were the average values of the results at each level in the orthogonal experiment. The range R is the absolute value of the difference between the maximum value and the minimum value of K_{ij}.

Table 2 Relative catalytic activity of StUGT at different concentrations of metal ions

Concentration Metal Ion	1mM	3mM	6mM
Ca^{2+}	103.2±1.5	103.4±0.1	66.5±2.6
Pb^{2+}	109.7±0.4	101.2±3.4	19.4±1.3
Ba^{2+}	112.9±3.9	105.5±0.1	109.9±0.3
Mn^{2+}	114.6±0.8	105.6±0.1	103.3±0.3
Mg^{2+}	92.1±0.2	115.1±1.4	115.6±1.0
CK		100	

Figure legends

Figure 1 : Bioinformatics analysis of StUGT. (a) Multiple sequence alignment of StUGT and its homologues. The sequences of UGTSL2 (XP_004250485.1), EUGT11 (XP_015629141.1), and UGT91D2 (KAG6582627.1) were from NCBI. The predicted secondary structure of StUGT is shown above each line of the alignment. α, β, and η indicate alpha-helix, beta-sheet, and 310-helix, respectively. Conserved residues are highlighted, the identical residues are colored in red, while similar residues are in the blue frame. The PSPG motif (residues 318–361) is labeled by a single blue bracket. (b) Structure modeling of StUGT. (c) Molecular docking of StUGT with Reb A. Green and red sticks indicate Reb A. Yellow dashed lines show the hydrogen bonds between Reb A and amino acid residues Glu67 and Glu70 (grey). (d) Synthetic pathway of Reb D from Reb A with StUGT.

Figure 2 : Schematic illustration of expression, purification, and catalysis of StUGT. (a) The expression and purification of StUGT. (b) Strategy for whole-cell production of Reb D from Reb A.

Figure 3 : Biochemical properties of the StUGT. Relative activity of StUGT at different temperatures (a)

and pH (b) by two forms of catalytic reaction. (c) Y_{RD} under various metal ions. Blank group refers to the control without the addition of any metal ions. (d) C_{RA} of reaction at different UDPG concentrations.

Figure 4 : Optimization of Reb D synthesis using whole-cell catalysis. (a) Relative activity of StUGT cells treated by CTAB, Tween-80, and Triton-X-100. Relative activity at various reaction times (b) and biomass (c). C_{RA} and Y_{RD} of StUGT at various Reb A concentrations (d).

Figure 5 : Fed-batch strategies of whole-cell bioconversion for the production of Reb D from Reb A. The 5 g/L Reb A was added once at 16 h (a), and 2.5 g/L Reb A was added twice at 16 h and 30 h (b), respectively.

Figure 6: Cascade reaction for the Reb D synthesis using sucrose as the glycosyl donor. (a) A scheme of whole-cell cascade reaction using StUGT and GsSUS1. (b) The Reb D yield under various cell density ratios of StUGT/SUS1. (c) CRA and YRD of the StUGT-GsSUS1 reaction at high substrate concentration (6g/L). Control group refers to the reaction system without the addition of GsSUS1 cells.