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Supplementary Information

An efficient whole-cell platform for Rebaudioside M biotransformation: cascade design, expression regulation, process engineering

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1. **Experimental section**

1.1. Strains, Vectors, and Materials

The dual-expression vector pRSFDuet-1 was used for the overexpression of *UGT76G1* and *GmSuSy* genes. *E. coli* DH5α was used for plasmid construction, and BL21(DE3) served as the expression host. Restriction enzymes were purchased from Takara (Beijing, China). FastPfu Fly DNA Polymerase for gene amplification and MultiF Seamless Assembly Mix for plasmid assembly were obtained from TransGen Biotech (Beijing, China) and ABclonal (Wuhan, China), respectively. Other molecular biology reagents and kits were purchased from Sangon Biotech (Shanghai, China).

Standard compounds Reb-M and Reb-D, as well as the substrate Reb-D for whole-cell catalysis, were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China) and Green Biological Preparation Co., Ltd. (Xinghua, China). Ultrapure water for HPLC was purchased by Wahaha Group Co., Ltd. (Hangzhou, China), and acetonitrile was from Meryer (Shanghai, China). All other analytical-grade chemicals were obtained from Aladdin (Shanghai, China) and Sigma-Aldrich (USA).

1.2. Plasmid construction

The gene encoding *UGT76G1* from *S. rebaudiana* (accession number: Q6VAB4), containing the structure-guided mutations T284S, M88L, and L200A [1], and the *GmSuSy* gene from *Glycine max* (accession number: AAC39323), were codon-optimized for *E. coli* using the online tool ExpOptimizer (NovoPro Bioscience, Shanghai, China) and synthesized by Sangon Biotech (Shanghai, China). The codon-optimized gene fragments were amplified by PCR and assembled into the multiple cloning sites (MCS) of pRSFDuet-1 via Gibson assembly. *UGT76G1* was inserted into MCS1 and *GmSuSy* into MCS2, yielding the recombinant plasmid pRSF-UGT76G1-*Gm*SuSy.

Based on this plasmid, a series of constructs carrying dual or triple copies of the UGT76G1 expression cassette were generated via PCR amplification and Gibson assembly. To further enhance UDP-Glc regeneration and modulate the expression level of *Gm*SuSy, seven ribosome binding site (RBS) sequences with varying predicted

translation initiation strengths were selected based on annotation and reported performance in the MIT BioStandard Parts Registry (https://parts.igem.org/Main_Page). Primers containing the corresponding RBS sequences were synthesized by Sangon Biotech (Shanghai, China) and used to replace the original RBS upstream of *Gm*SuSy in the pRSF-UGT76G1-*Gm*SuSy plasmid via PCR, followed by Gibson assembly, generating seven additional plasmid variants.

Colony PCR and Sanger sequencing verified all recombinant plasmids, and then transformed them into *E. coli* DH5α for amplification, and subsequently introduced into *E. coli* BL21 (DE3) to obtain engineered strains BL0-BL9 for expression and catalytic evaluation.

1.3. Enzyme Activity Assays

Enzymatic activities of GmSuSy and UGT76G1 were determined using crude enzyme extracts. Sucrose synthase activity was quantified based on the formation of a purple fructose-bicinchoninic acid complex as described by Waffenschmidt and Jaenicke (1987) with minor modifications. The standard reaction mixture (1.0 mL) contained 50 mM phosphate buffer (pH 6.5), 100 mM sucrose, and 10 mM UDP. Reactions were initiated by enzyme addition, incubated at 40 °C for 10 min, and terminated by boiling at 100 °C. After centrifugation (12,000 rpm, 5 min), 25 μL of the supernatant was mixed with 150 μL of chromogenic reagent (Solution A : Solution B : ethanol = 23 : 1 : 9), where Solution A contained 62.3 g/L Na_2CO_3 and 1.5 g/L 2,2'-bicinchoninic acid dipotassium salt, and Solution B contained 33 g/L Na_2CO_3, 7.3 g/L $CuSO_4$, and 23 g/L aspartic acid. The mixture was incubated at 70 °C for 30 min, cooled to room temperature, and absorbance was recorded at 560 nm using a microplate reader. One unit of sucrose synthase activity (1 U) was defined as the amount of enzyme required to produce 1 μmol fructose per minute under assay conditions.

UGT76G1 activity was determined based on Reb-D conversion to Reb-M following Guo et al. (2022) with minor modifications. A 200 μL reaction system containing 2 mM Reb-D, 5 mM UDP-Glc, and 50 mM Tris-HCl (pH 8.0) was pre-equilibrated at 40 °C, initiated by addition of crude enzyme, and incubated for 20 min. Reactions were

terminated at 95 °C for 5 min, diluted five-fold with methanol, centrifuged at 20,000 × g for 5 min, and filtrated through a 0.22 μm organic membrane. Reb-M was quantified by UPLC, and one unit (1U) of UGT76G1 activity was defined as the amount of enzyme required to form 1 μmol Reb-M per minute under the above conditions.

1.4. Analytical methods

At designated time points, 1 mL of the whole-cell reaction mixture was withdrawn and centrifuged at 12,000 × g for 10 minutes at 4 °C. The resulting supernatant was diluted 5-fold with deionized water, filtered through a 0.22 μm cellulose membrane (Millipore, USA) prior to HPLC analysis.

Reb-D and Reb-M were quantified using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector. Chromatographic separation was employed on a Poroshell 120 EC-C18 column (4.6 mm × 150 mm, 2.7 μm) maintained at 40 °C. Detection was set at 210 nm. Isocratic elution used methanol (solvent A, 68%) and 0.1% phosphoric acid in water (solvent B, 32%) at a flow rate of 1.0 mL/min. The injection volume was 10 μL. Reb-D and Reb-M were identified and quantified by external calibration against standard curves generated from commercial standards. The conversion rate of Reb-D and the yield of Reb-M were calculated as follows:

$$\text{The conversion of Reb-D (\%)} = {}^{(C_{d0} - C_{dt})}/_{C_{d0}} \times 100\%$$

Where C_{dt} (mg/L) was the concentration of Reb-D after the reaction, C_{d0} (mg/L) was the concentration of Reb-D before the reaction. Unless otherwise stated, all experiments were performed in at least three independent biological replicates ($n \geq 3$), and data are reported as mean ± SD. Group differences were evaluated using Duncan's multiple range test in SPSS v23.0 (IBM, USA), with $p < 0.05$ considered statistically significant.

2. Supplemental tables and figures

Tabe S1. Primers used in this study.

Plasmids	Characteristics	Source
pRSFDuet-1	RSF1030；Kan^R；Two T7；MCS1 & MCS2；His-tag (MCS1)；	Lab stock
pRSF-DTE	pRSFDuet-1 ligated with genes *DTE*	Lab stock
pRSF-UGT76G1	pRSFDuet-1 ligated with genes *UGT76G1*	This study
pRS-*Gm*SuSy	pRSFDuet-1 ligated with genes *GmSuSy*	This study
pRSF-UGT76G1-*Gm*SuSy	Co-expression of UGT76G1 (MCS1) and *Gm*SuSy (MCS2) in pRSFDuet-1	This study
pRSF-$(UGT76G1)_2$-*Gm*SuSy	Co-expression of two copies of UGT76G1 and GmSuSy in pRSFDuet-1	This study
pRSF-$(UGT76G1)_3$-*Gm*SuSy /pRSF-U_3*S	Co-expression of three copies of UGT76G1 and *Gm*SuSy in pRSFDuet-1	This study
pRSF (BBa_B0030)-U_3*S	pRSF-U_3*S with RBS (BBa_B0030) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_B0031)-U_3*S	pRSF-U_3*S with RBS (BBa_B0031) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_B0032)-U_3*S	pRSF-U_3*S with RBS (BBa_B0032) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_B0033)-U_3*S	pRSF-U_3*S with RBS (BBa_B0033) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_B0034)-U_3*S	pRSF-U_3*S with RBS (BBa_B0034) replacing the native RBS of *Gm*SuSy	This study

pRSF (BBa_B0035)-U_3*S	pRSF-U_3*Swith RBS (BBa_B0035) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_B0064)-U_3*S	pRSFDuet-1 with RBS (BBa_B0064) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_J61100)-U_3*S	pRSFDuet-1 with RBS (BBa_J61100) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_J61101)-U_3*S	pRSFDuet-1 with RBS (BBa_J61101) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_J61102)-U_3*S	pRSFDuet-1 with RBS (BBa_J61102) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_J61104)-U_3*S	pRSFDuet-1 with RBS (BBa_J61104) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_J61107)-U_3*S	pRSFDuet-1 with RBS (BBa_J61107) replacing the native RBS of *Gm*SuSy	This study
pRSF (BBa_J61117)-U_3*S	pRSFDuet-1 with RBS (BBa_J61117) replacing the native RBS of *Gm*SuSy	This study
pRSF(BBa_J61127)-U_3*S	pRSFDuet-1 with RBS (BBa_J61127) replacing the native RBS of *Gm*SuSy	This study

Table S2. Primers used in this study.

Primers	Sequence (5′-3′)
Primers for *Co-expression of two copies of UGT76G1 and GmSuSy*	
UGT76G1-F	CACCACAGCCAGGATCCGATGGAAAACAAAACCGAAACCA
UGT76G1-R	AAGCATTATGCGGCCGCAAGCTTTTACAGGCTGCTGATATA
GmSuSy-F	TATAAGAAGGAGATATACATAATGGCTACCGATCGTCTGACCCGTG
GmSuSy-R	TTACCAGACTCGAGGGTACCTTATTCCGCCGCCAGCGGCACGGAT
MCS1-F	AAGCTTGCGGCCGCATAATGCTTAA
MCS1-R	CGGATCCTGGCTGTGGTGATGATGG
MCS2-F	GGTACCCTCGAGTCTGGTAA
MCS2-R	TATGTATATCTCCTTCTTATACTTAACTAATATAC
Primers for multi-copy UGT76G1	
(*UGT76G1*)$_2$-F1	TATAAGAAGGAGATATACATATGATGTCCCTGGCAATCATCCCAG
(*UGT76G1*)$_2$-R1	TTACCAGACTCGAGGGTACCTTACAGGCTGCTGATATAGC
(*UGT76G1*)$_2$-F2	TATGTATATCTCCTTCTTATACTTAACTAATATAC
(*UGT76G1*)$_2$-R2	GGTACCCTCGAGTCTGGTAA
(*UGT76G1*)$_3$-F1	TCGAACAGAAAGTAATCGTTGCGACTCCTGCATTAGGAA
(*UGT76G1*)$_3$-R1	ATGCGGCCGTGTACAATCTTCTCAAATGCCTGAGGTT
(*UGT76G1*)$_3$-F2	ACGATTACTTTCTGTTCGACTTAAG
(*UGT76G1*)$_3$-R2	ATTGTACACGGCCGCATAATCG
Primers for the RBS of GmSuSy	
BBa_B0029-F	TTCACACAGGAAACCATATACATAATGGCTACCGATCGTCTGA
BBa_B0029-R	TATGTATATGGTTTCCTGTGTGAAAACTAATATACTAAGATG
BBa_B0030-F	AATTAAAGAGGAGAAAATATACATAATGGCTACCGATCGTCTGA
BBa_B0030-R	TATGTATATTTTCTCCTCTTTAATTAAACTAATATACTAAGATG
BBa_B0031-F	TCACACAGGAAACCATATACATAATGGCTACCGATCGTCTGA
BBa_B0031-R	TATGTATATGGTTTCCTGTGTGAAACTAATATACTAAGATG
BBa_B0032-F	TCACACAGGAAAGATATACATAATGGCTACCGATCGTCTGA
BBa_B0032-R	TATGTATATCTTTCCTGTGTGAAACTAATATACTAAGATGAAGATG

BBa_B0034-F	AAAGAGGAGAAAATATACATAATGGCTACCGATCGTCTGA
BBa_B0034-R	TATGTATATTTTCTCCTCTTTAACTAATATACTAAGATG
BBa_B0035-F	ATTAAAGAGGAGAAATATACATAATGGCTACCGATCGTCTGA
BBa_B0035-R	TATGTATATTTCTCCTCTTTAATAACTAATATACTAAGATG
BBa_B0064-F	AAAGAGGGGAAAATATACATAATGGCTACCGATCGTCTGA
BBa_B0064-R	TATGTATATTTTCCCCTCTTTAACTAATATACTAAGATG

Table S3. Strains used in this study.

Strains	Characteristics	Resources
E. coli DH5α	F^-, *φ80d*, *lacZΔM15* Δ*(lacZYA-argF)* U169, *endA1*, *recA1*, *hsdR17*(r_K^- m_K^-), *supE44*, *thi-1*, *gyrA96*, *relA1*	Lab stock
E. coli BL21 (DE3)/BL	F^-, *ompT*, *hsdSB* (r_B^- m_B^-), *gal*, *dcm*, *rne131* (DE3)	Lab stock
BL0	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy	This study
BL1	BL harboring plasmid pRSF-$(UGT76G1)_2$-*Gm*SuSy	This study
BL2	BL harboring plasmid pRSF-$(UGT76G1)_3$-*Gm*SuSy	This study
BL3	BL harboring plasmid pRSF-$(UGT76G1)_3$-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0029	This study
BL4	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0030	This study
BL5	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0031	This study
BL6	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0032	This study
BL7	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0033	This study
BL8	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0035	This study
BL9	BL harboring plasmid pRSF-UGT76G1-*Gm*SuSy, *GmSuSy* RBS replaced with BBa_B0036	This study
BL10	BL harboring plasmid pRSF-DTE,	Lab stock

Table S4. Composition of culture media used for expression screening.

Medium	Composition (per liter)
Luria-Bertani (LB)	10 g tryptone, 5 g yeast extract, 10 g NaCl (Adjust pH to 7.0)
Terrific Broth (TB)	12 g tryptone, 24 g yeast extract, 4 mL glycerol; 17 mM KH_2PO_4, 72 mM K_2HPO_4 (Sterilize phosphate buffer separately; combine after cooling)
2×YT	16 g tryptone, 10 g yeast extract, 5 g NaCl (Adjust pH to 7.0)
Super Broth (SB)	32 g tryptone, 20 g yeast extract, 5 g NaCl
Super Optimal Broth (SOB)	20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ (Adjust pH to 7.0)
SOC	SOB + 20 mM glucose (Add glucose after sterilization via filter (0.22 μm))

All media were prepared with deionized water and sterilized by autoclaving unless otherwise specified. SOC medium was prepared fresh and used within 24 h for optimal performance. All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Tabe S5. Kinetic parameters of sucrose synthase (SuSy) for sucrose cleavage.

Origin	Isoforms	Temperature	pH	K_m (mM)	K_{cat} (s^{-1})[f]	Reference
Plant SuSys						
Arabidopsis thaliana	SuSy1	25℃	6.0	31.58	7.3	[2]
		37℃	7.0	53	907	[3]
	SuSy3	25℃	6.0	108.2	4.6	[2]
		37℃	7.0	48	1470	[3]
Pisum sativum	SuSy1	30℃	7.5	32.1	n.a.	[4]
	SuSy2	30℃	7.5	42.7	n.a.	[4]
	SuSy3	30℃	7.5	67.5	n.a.	[4]
Pyrus pyrifolia	SuSy1	37℃	7.0	28.2	n.a.	[5]
Phaseolus aureus	n.a.	25℃	7.5	17	n.a.	[6]
Ipomoea batatas	n.a.	37℃	7.0	31	n.a.	[7]
Glycine max	n.a.	37℃	7.5	31.3	20.0	[8]
Hordeum vulgare	n.a.	37℃	7.0	30	435	[9]
Prunus persica	n.a.	25℃	7.0	62.5	n.a.	[10]

Lycopersicon	n.a.	25℃	6.5	53	n.a.	[11]
Manihot utilissima	n.a.	25℃	6.5	10	n.a.	[12]
Oryza sativa	n.a.	37℃	5.0	30	n.a.	[13]
Solanum tuberosum	n.a.	37℃	6.0	55	n.a.	[13]
Zea mays	n.a.	30℃	6.5	40	n.a.	[14]
Beta vulgaris	n.a.	35℃	6.5	100	n.a.	[15]
Stevia rebaudiana	n.a	55℃	7.0	95.61	36.4	[16]
Bacterial SuSys						
Acidithiobacillus caldus	n.a.	60℃	7.0	7.8	82.0	[17]
Nitrosomonas europaea	n.a.	60℃	7.0	321	96.7	[17]
Thermosynechoccus elongatus	n.a.	60℃	7.0	1.3	3.5	[18]
Anabaena sp.	n.a.	60℃	6.5	303	n.a.	[19]
Micractinium conductrix	n.a.	60℃	7.0	0.14	17.3	[20]

Note: n.a., not available. SuSy1-3 represent isoforms of sucrose syntha

Table S6. Recent enzymatic and whole-cell systems for the biosynthesis of Rebaudioside M.

System type	Host/format	Enzyme(s)/strategy	Substrate(s)	Reb-M titer (g/L)/yield	Reference
***In vitro* multi-enzyme cascade**	Crude enzyme	UGT76G1-M88V coupled with *Gm*SuSy	Reb-D	92.40% yield in 36 h	21
	Crude enzyme	UGT76G1(T284S/M88L/L200A) coupled with *Gm*SuSy	Reb-D	26.03 g/L Reb-M; 100% conversion of Reb-D	22
	Crude enzymes	UGT94B1 (I146G/P174V) and UGT76G1-M3	Stevioside/Reb-A	38.8 g/L Reb-M; 85.5% yield	23
	Purified enzymes	UGT76G1 (T284S/M88L/L200A) coupled with *At*SuSy	Reb-D	23.37 g/L Reb-M from 22.5 g/L Reb-D; 90.50%	1
	Purified enzymes	UGT76G1 (S195Q) coupled with *Mc*SuSy	Reb-E	12.8 g/L Reb-M from 20 g/L Reb-E; 64% yield in 32 h	24
	Purified enzymes	Co-immobilized; EUGT11 and UGT76G1	Reb-A	4.82 g/L Reb-M; 72.2% yield	25
	Purified enzymes	UGT76G1 (I30M/K53A/R141P/G349P)	Reb-D	29.24 g/L Reb-M; 90.6% yield	26
whole-cell	*Pichia pastoris*	Fusion enzyme; UGT76G1 and UGT91C1	Reb-A	0.31 g/L Reb-M; 95% yield in 96 h	27
	Saccharomyces cerevisiae	Co-expressing UGT91D2, UGT76G1, UGP1, SIR2	Stevioside	12.5 g/L Reb-M; 77.9% yield in 108 h	28
	E. coli	Co-expressing UGT76G1 (T284S/M88L/ L200A) and *Gm*SuSy; Multi-Copy and RBS Engineering; Fed-Batch; Byproduct valorization	Reb-D	30.56 g/L Reb-M; 95.9%	This study

Note: MCSuSy: sucrose synthase from *Micractinium conductrix*; EUGT11: glycosyltransferase from Oryza sativa; *UGP1*:phosphoglucomutase; SIR2: silencing

information regulator 2

Figures

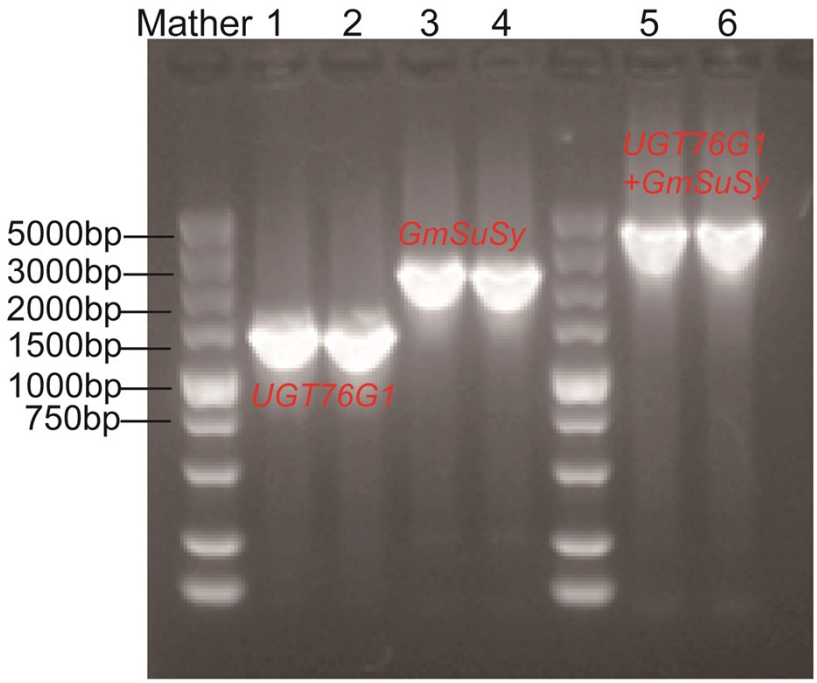

Fig. S1. Colony PCR verification of the engineered strain BL0. Lanes 1-2 show a single band between 1,300 and 1,500 bp, consistent with UGT76G1 (expected 1,410 bp). Lanes 3-4 show a single band between 2,000 and 3,000 bp, consistent with GmSuSy (expected 2,649 bp). Lanes 5-6 show two bands between 1,300 and 1,500 bp (UGT76G1) and between 2,000 and 3,000 bp (GmSuSy), indicative of single-plasmid co-expression clones.

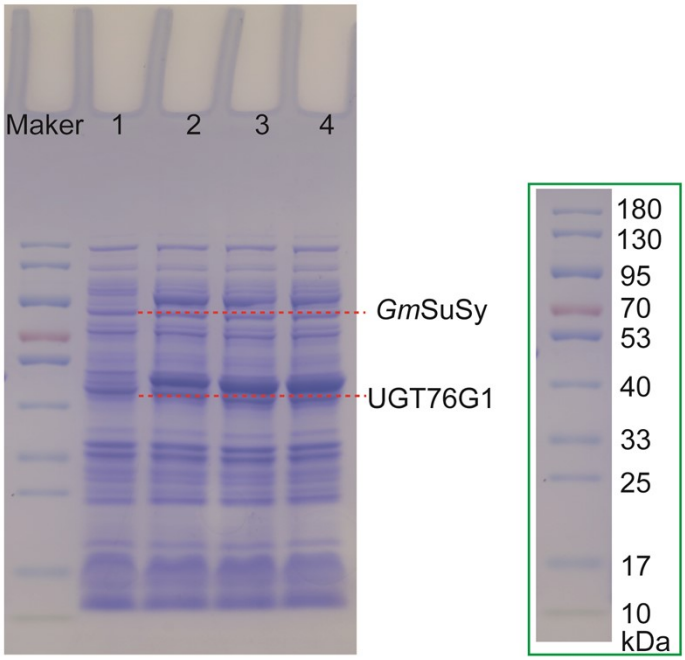

Fig. S2. Uncropped, full-length SDS-PAGE gels corresponding to Fig. 2d, showing the expression of UGT76G1 and GmSuSy in recombinant strains. Lane M, protein marker; lane 1, BL (uninduced control); lane 2, BL0 (1:1 copy ratio); lane 3, BL1 (2:1); lane 4, BL2 (3:1).

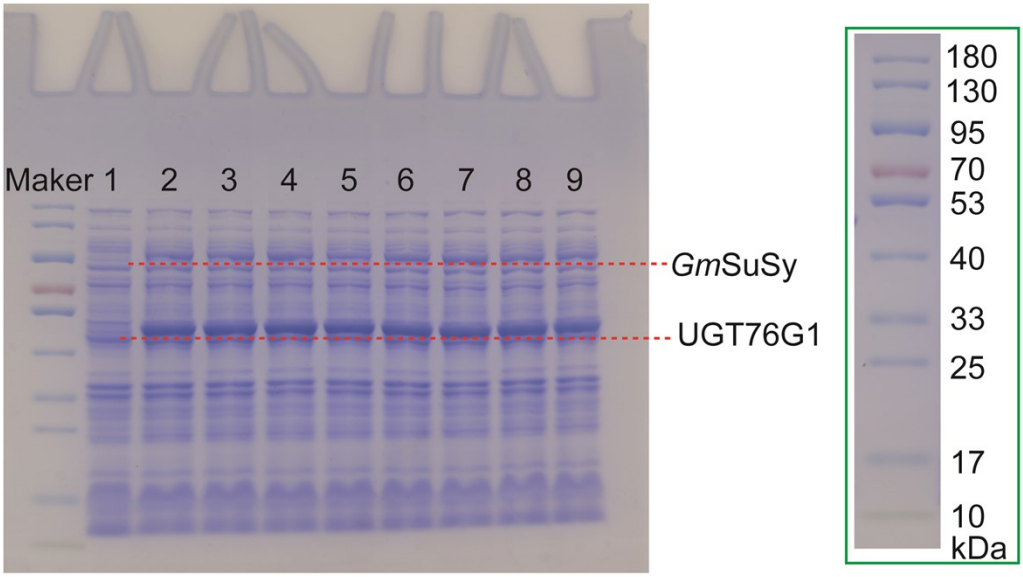

Fig. S3. Uncropped, full-length SDS-PAGE gels corresponding to Fig. 3c, showing expression of UGT76G1 and GmSuSy in recombinant strains BL2-BL9 with different ribosome-binding site (RBS) strengths. Lane M, protein marker; lane 1, BL (uninduced control); lanes 2-9, BL2-BL9

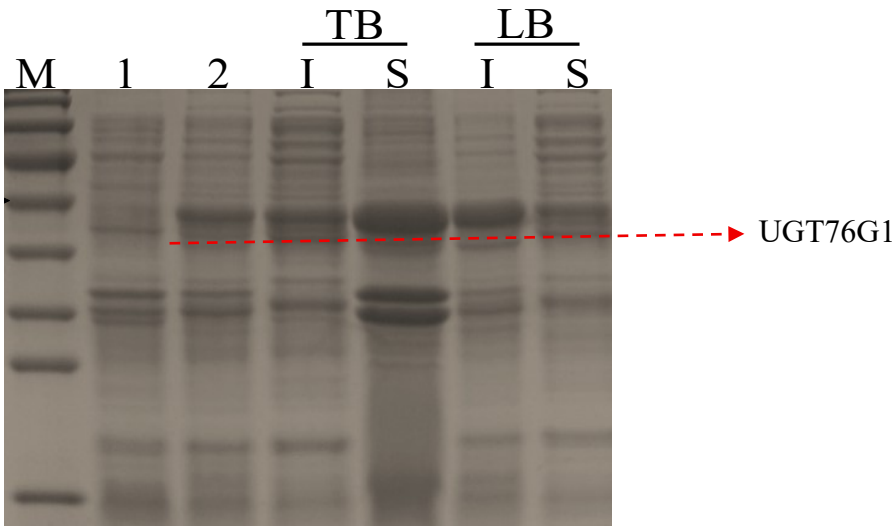

Fig. S4. SDS-PAGE analysis of soluble and insoluble fractions of UGT76G1 expressed in *E. coli* BL21(DE3)/pRSF-UGT76G1 cultured in TB and LB media. Lane M: protein marker; Lane 1: blank (negative control); Lane 2: positive control; Lanes S and I (TB): soluble and insoluble fractions from cells grown in TB medium; Lanes S and I (LB): soluble and insoluble fractions from cells grown in LB medium.

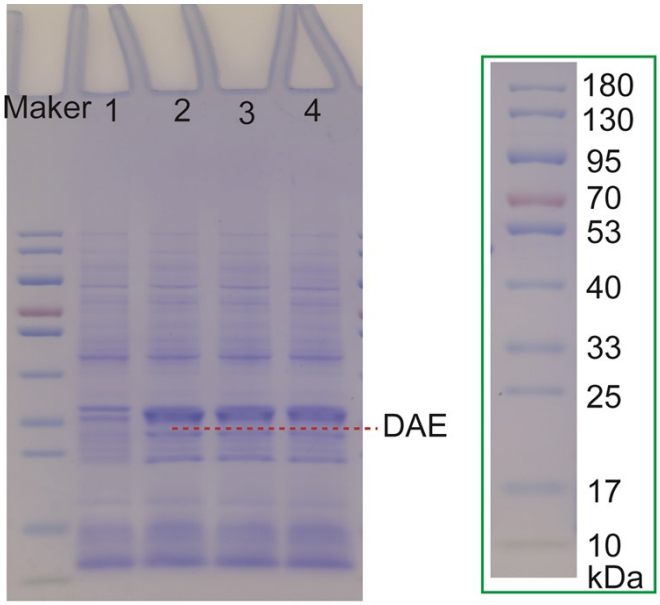

Fig. S5. SDS-PAGE analysis of recombinant D-allulose 3-epimerase (DAE) expressed in E. coli BL21(DE3). Lane 1, BL21(DE3) host (uninduced control); lanes 2-4, IPTG-induced cell lysates showing a prominent band at 33-40 kDa, consistent with DAE (expected 34.2 kDa).

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