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Production of Rebaudioside A from Stevioside Catalyzed by the Engineered *Saccharomyces cerevisiae*

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Abstract Rebaudioside A has superior taste quality among the steviol glycosides extracted from *Stevia rebaudiana* leaves. Given its high purity as a general-purpose sweetener, rebaudioside A has received significant attention and has been widely applied in food and beverages in recent decades. Stevioside is one of the major steviol glycosides and can be converted to rebaudioside A by the uridine-diphosphate dependent glucosyltransferase UGT76G1 in *S. rebaudiana*. To explore the applicability of and limits in producing rebaudioside A from stevioside through whole-cell biocatalysis, the engineered *Saccharomyces cerevisiae* expressing UGT76G1, using a newly constructed constitutive expression vector, was used as the whole-cell biocatalyst. Citrate was added to the reaction mixture to allow metabolic regulation when glucose was fed to provide the activated sugar donor UDP-glucose for glycosylation of stevioside in vivo. In an evaluation of the whole-cell reaction parameters involving cell permeability, temperature, pH, citrate and Mg^{2+} concentrations, and glucose feeding, production of 1160.5 mg/L rebaudioside A from 2 g/L stevioside was achieved after 48 h without supplementation of extracellular UDP-glucose.

Keywords Rebaudioside A · *Saccharomyces cerevisiae* · Stevioside · UDP-glucosyltransferase · Whole-cell biocatalyst

Introduction

Rebaudioside A is the sweetest diterpene glycoside extracted and purified from the leaves of *Stevia rebaudiana* Bertoni, and is commercially significant as the natural sweetener used in the food and beverage industry [1, 2]. The United States Food and Drug Administration granted

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"generally recognized as safe" (GRAS) regulatory acceptance of rebaudioside A in 2008. In recent years, various methods were developed for the separation of rebaudioside A from *Stevia* extracts, including high-speed counter-current chromatography, preparative high-performance liquid chromatography (HPLC), and adsorptive separation [3–6]. However, stevioside, the other predominant steviol glycoside, remains in the extract residues. Stevioside is 250–300 times sweeter than sucrose, but produces a bitter aftertaste, constraining its use for human consumption and application in food and pharmaceutical products [1, 7, 8]. Therefore, conversion of stevioside into rebaudioside A, which is sweeter than stevioside and presents a clean, sweet taste with no significant undesirable taste characteristics [9], would result in a more cost-effective rebaudioside A preparation process.

In *S. rebaudiana*, steviol glycosides share part of their biosynthetic pathways with gibberellins. Kaurenoic acid, an intermediate from gibberellic acid biosynthesis, is hydroxylated to form steviol, which is then sequentially glucosylated by a series of uridine-diphosphate dependent glucosyltransferases (UDP-glucosyltransferases, UGTs) to produce a variety of steviol glycosides [10, 11]. Three UGTs (UGT85C2, UGT74G1, and UGT76G1) involved in biosynthesis of major sweet glucosides have been isolated from the *S. rebaudiana* expressed sequence tag collection [12], with all of these UGTs localizing to the cytosol. UGT85C2 catalyzes the glycosylation reaction of steviol to steviolmonoside, while UGT74G1 primarily catalyzes glycosylation of steviolbioside to stevioside. Rebaudioside A is the end product of the pathway for steviol glycoside biosynthesis in *S. rebaudiana* and can be obtained through a one-step glucosylation of stevioside by UGT76G1, with UDP-glucose used as the activated sugar donor [11]. Recently, the functional role of a UGT gene from *S. rebaudiana* (*UGTSr*) showing similarities to UGT76G1 was revealed in the generation of rebaudioside A [13]. Recombinant technologies have made it possible to obtain adequate concentrations of enzymes; however, enzymatic synthesis of rebaudioside A in vitro is not scalable due to the expense of adding or recycling the UDP-glucose sugar donor [14]. Therefore, using whole cells as catalysts where nucleotide sugars are generated in vivo is preferred [15].

Saccharomyces cerevisiae is a promising whole-cell biocatalyst host for the production of valuable glycosides [16]. *S. cerevisiae* is a GRAS organism, which is advantageous for the production of glycosides as dietary supplements and pharmaceuticals. Additionally, yeast contains large cytoplasmic pools of UDP-glucose capable of being redirected for production purposes [17]. Here, the *S. rebaudiana* UDP-glucosyltransferase UGT76G1 was expressed in *S. cerevisiae* in order to develop a whole-cell catalyst for conversion of stevioside to rebaudioside A. Glucose was used as the carbon source for in vivo generation of UDP-glucose, and citrate, which is a phosphofructokinase inhibitor [18], was added in order to limit carbon flux generated during glycolysis in the process of UDP-glucose synthesis. The effect of reaction parameters on stevioside conversion were also investigated, including cell permeability, temperature, pH, citrate and Mg^{2+} concentrations, and glucose feeding.

Materials and Methods

Plasmid Construction

For constitutive expression of UGT76G1, a new plasmid, pTP (Fig. 1), was generated by replacing the *GAL1*/*GAL10* promoter in pESC-LEU (Stratagene, San Diego, CA, USA) with the *PGK1*/*TEF1* promoter. Two promoters (p*TEF1* and p*PGK1*) were amplified by

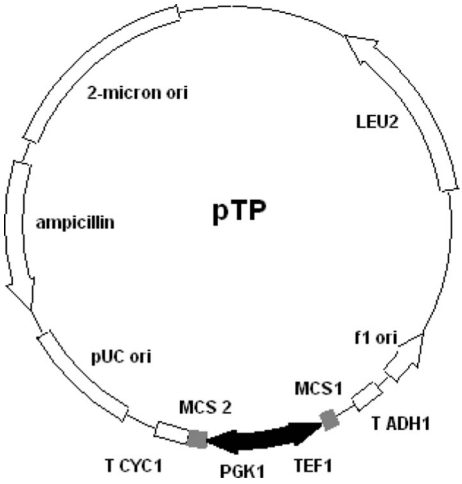

Fig. 1 The pTP vector contains a *PGK1-TEF1* bidirectional promoter. LEU2, yeast *LEU2* selection marker open reading frame; f1 ori, f1 origin of ss-DNA replication; T ADH1, yeast *ADH1* terminator; MCS1, multiple cloning site 1; TEF1, yeast *TEF1* promoter; PGK1, yeast *PGK1* promoter; MCS2, multiple cloning site 2; T CYC1, yeast *CYC1* terminator; pUC ori, pUC origin of replication; ampicillin, ampicillin resistance (*bla*) open reading frame; 2-micron ori, 2 μm yeast origin of replication

polymerase chain reaction (PCR) from the genome of *S. cerevisiae* YPH499 (*MATa ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-Δ63 his3-Δ200 leu2-Δ1*; Stratagene). To construct divergent promoters in TEF1-PGK1, the *TEF1* and *PGK1* promoters were fused by fusion PCR [19] with PrimeSTAR HS DNA polymerase (TaKaRa, Dalian, China) in two steps. In the first step, the promoters, p*TEF1* and p*PGK1*, were each amplified using the primers Tef-fw/Tef-NotI and Pgk-fw/Pgk-BamHI, as shown in Table 1. The second step was conducted using the mixed PCR products from the first step as templates and the primers Tef-NotI/Pgk-BamHI. The resulting TEF1-PGK1 cassette was digested by *Bam*HI and *Not*I, and cloned into pESC-LEU.

The codon-optimized gene (LC037193), derived from *S. rebaudiana* UGT76G1 mRNA (AY345974), was synthesized by GenScript (Nanjing, China) and then cloned into the yeast expression vector pTP between the *PGK1* promoter and the *CYC1* terminator, resulting in the pUGT plasmid. The primers Ugt-SalI and Ugt-XhoI (Table 1) with the *Sal*I and *Xho*I restriction endonuclease sites (Table 1, underlined) were used for pUGT construction.

S. cerevisiae Transformation

The pUGT and pTP plasmids were transformed into *S. cerevisiae* YPH499 (Stratagene) using the Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA) according to

Table 1 Oligonucleotide primers used in this study

Primer name	Sequence (5′–3′)
Pgk-fw	GGAAGTACCTTCAAAGAATGG
Pgk-BamHI	GTTGTTGGATCCTTGTTTTATATTTGTTGTAAAAAGTAG
Tef-fw	CCATTCTTTGAAGGTACTTCCGGCCGGCCGCACACACCATAGCTTCAAA
Tef-NotI	GTTGTTGCGGCCGCTTGTAATTAAAACTTAGATTAGATTGC
Ugt-SalI	CTATAGGGCCCGGGCGTCGACATGGAAAATAAGACTGAAACTACTG
Ugt-XhoI	GCGGTACCAAGCTTACTCGAGTTATAATGATGAAATATAAGAAACC

manufacturer instructions. Transformants were selected on SD (-Leu) agar plates (6.7 g/L yeast-nitrogen base without amino acids, plus 20 g/L of glucose and 1.3 g/L amino acid mixture lacking leucine) incubated at 30 °C for 3 days. All plasmids and strains used in this study are listed in Table 2.

Expression of Glucosyltransferase in *S. cerevisiae*

An appropriate aliquot of yeast transformants picked from SD (-Leu) agar plates were inoculated into 100 mL of SD (-Leu) medium in a 500-mL flask and shaken at 200 rpm and 30 °C for 24 h, or afterwards, a 100-mL culture was inoculated into 3 L SD (-Leu) medium in a 5-L fermentor (East Bioengineering Equipment Technology Co., Ltd., Zhenjiang, China) and shaken at 500 rpm and 30 °C for 5 h, then at 700 rpm for another 15 h. The presence of growing cells indicated successful expression of recombinant glucosyltransferase in *S. cerevisiae*.

Glucosyltransferase Activity Assay

The cells were harvested and washed twice in 100 mM potassium phosphate buffer (pH 7.2), and the lysate containing soluble proteins was prepared using the Yeast Total Protein Extraction Kit (Sangon Biotech, Shanghai, China) according to manufacturer instructions. The supernatant was obtained by centrifugation at 12,830×*g* at 4 °C for 5 min and used as the crude extract. Total protein concentrations were measured by the Bradford assay using bovine serum albumin as a standard [20]. The assay mixture contained 1 mg of total protein from the crude cell extract, 1 mM stevioside, 2 mM UDP-glucose, and 3 mM $MgCl_2$ in 100 mM potassium phosphate buffer (pH 7.2) for a total volume of 3 mL [13]. Reactions were performed at 30 °C for 1 h, after which the samples were prepared for HPLC analysis. One unit (U) of glucosyltransferase activity was defined as the amount of enzyme that produced 1 μmol of rebaudioside A per minute under the described conditions.

Table 2 Plasmids and strains used in this study

Plasmids	Relevant details	Source
pESC-LEU	*GAL1* and *GAL10* promoters in opposing orientation, with *CYC1* and *ADH1* terminators, respectively; 2 μm, *LEU2* marker, Amp^R	Stratagene
pTP	*PGK1* and *TEF1* promoters in opposing orientation, with *CYC1* and *ADH1* terminators, respectively; 2 μm, *LEU2* marker, Amp^R	This study
pUGT	pTP with UGT76G1-encoding gene	This study
Strains	**Genetic details**	**Source**
YPH499	*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*	Stratagene
YPH(pTP)	*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1* pTP (*LEU2*)	This study
YPH(pUGT)	*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1* pUGT (*LEU2*)	This study

Whole-Cell Biocatalyst Assay in Shaking Flasks

The recombinant yeast strain YPH (pUGT) cultured at 30 °C for 24 h in shaking flasks, or for a total of 20 h in the fermentor, were harvested by centrifugation at 8228×*g* for 6 min, or at 4629×*g* for 10 min, respectively, and the supernatants discarded. The pellets were washed twice with 100 mM potassium phosphate buffer (pH 7.2) in preparation for the assay or stored at 4 °C for later use.

Washed cells (OD_{600} = ~1500) were resuspended in 20 mL 100 mM potassium phosphate buffer (pH 7.2) containing 2 g/L stevioside, 40 g/L glucose, 0.1–1 % reagent for enhancing cell penetration, 15 g/L sodium citrate, and 6 g/L $MgCl_2$, and incubated at 30 °C with shaking at 200 rpm for 48 h. Stevioside (95 %) used in the reactions was obtained from Aoxing Stevia (Jining, China). The reaction parameters such as temperature, pH, and the concentrations of citrate, Mg^{2+}, or glucose used for feeding varied separately, while the others except the investigated parameter were kept constant. Samples (1 mL) were collected every few hours. Cells were removed by centrifugation at 13,523×*g* at room temperature for 1 min, and the resulting supernatants were heated at 95 °C for 10 min and then clarified by centrifugation at 13,523×*g* for 10 min at room temperature.

HPLC Analysis

Samples of the reaction mixture were extracted three times with equal volumes of water-saturated 1-butanol and dried using a nitrogen purge device (QYN100-1; Joyn Electronic, Shanghai, China). The pooled butanol fractions were dried and re-dissolved in 300 μL water-saturated 1-butanol.

For analysis of rebaudioside A concentration, HPLC was performed on the Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) using a Galaksil BF-NH_2 column (4.6 × 250 mm, 5 μm, 120 Å) maintained at 40 °C with UV detection at 210 nm. The flow rate was 1 mL/min using 20 % H_2O, with CH_3CN (pH adjusted to 4.2 with acetic acid) as the mobile phase. The injection volume was 10 μL. Quantification was done by the external standard method using rebaudioside A standard (99 %) obtained from Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The line arrange for rebaudioside A concentration was 40–1000 mg/L. The average recoveries of rebaudioside A were 100.6 % (n = 6, RSD = 1.68 %). The rebaudioside A yield was calculated as rebaudioside A yield (%) = C(RA)/C(St), where C(St) represents the initial stevioside molar concentration and C(RA) represents the detected rebaudioside A molar concentration during the reaction.

Results and Discussion

Construction of the Expression Vector with Constitutive Promoters

S. cerevisiae has been widely used as a host organism for efficiently expressing heterologous proteins. The pESC vector series (Stratagene) is a widely used *S. cerevisiae* expression vector, with *GAL1* and *GAL10* promoters oriented opposite to each other, and enabling simultaneous expression of two different genes from a single vector. This system is induced by galactose and repressed by glucose, which is the preferred carbon

source for yeast. Therefore, the target protein regulated by the *GAL1* or *GAL10* promoter was highly expressed only after glucose was exhausted in the presence of galactose, or following transfer to galactose-rich medium without glucose. Expression levels using seven different promoters during growth in the presence of glucose were evaluated and compared with two galactose-induced promoters, p*TEF1* and p*PGK1*, which displayed consistent activity patterns at different glucose concentrations as reported by Partow et al. (2010). p*TEF1* is derived from the *TEF1* gene encoding transcriptional elongation factor EF-1α [21], and p*PGK1* is a promoter associated with a key glycolytic gene, *PGK1*, encoding phosphoglycerate kinase [22, 23]. In this study, a new divergent expression cassette called pTP (Fig. 1) was constructed by replacing the *GAL1*/*GAL10* promoters in pESC-LEU with a *PGK1*-*TEF1* bidirectional promoter cassette. pTP retained all the features from the pESC-LEU plasmid, except that gene expression was mediated by the new constitutive promoters.

The gene encoding the *S. rebaudiana* UDP-glucosyltransferase UGT76G1 was then inserted under the control of the *PGK1* promoter and constitutively overexpressed in *S. cerevisiae* YPH499.

UDP-Glucosyltransferase In Vitro Assay

In order to confirm the functionality of the enzyme expressed in *S. cerevisiae*, YPH (pUGT) cells grown in SD (-Leu) medium for 12, 16, and 24 h were sampled for the in vitro assay. Crude protein extracts from YPH (pUGT) yeast strains expressing UGT76G1 under the control of the *PGK1* promoter displayed glucosyltransferase activity by formation of rebaudioside A when stevioside was used as the sugar acceptor and UDP-glucose as the donor. As indicated in Table 3, the maximum enzyme activity in the crude extract obtained at 16 h of cultivation was 2.737 ± 0.206 mU/mg. However, the UDP-glucosyltransferase activity decreased to 2.132 ± 0.028 mU/mg after 24 h of cultivation, with the highest biomass achieved after 24 h measured at OD_{600} 10.1 (OD_{600} = 8.4 at 16 h). No glucosyltransferase activity was detected in the YPH (pTP) cells grown for 24 h.

Based on in vitro assay results, the specific UDP-glucosyltransferase activity did not reach the maximum level when high biomass was acquired. The *TEF1* promoter would likely be more suitable for glucosyltransferase expression, given that the enzyme activity increases as the cells grow [19]. Additionally, >30 % of the decrease in activity was observed after the cells were stored at 4 °C for 1 week.

Table 3 Glucosyltransferase in vitro assay

Yeast strain	Cultivation time (h)	Specific activity (mU/mg total protein)
YPH(pUGT)	12	2.464 ± 0.007
	16	2.737 ± 0.206
	24	2.132 ± 0.028
YPH(pTP)	24	N.D.

The activities are the mean ± standard deviation (n = 3)

N.D. not detected

Whole-Cell Biocatalytic Assay

Besides the challenge of providing active eukaryotic glycosyltransferases, sugar-donor (nucleotide sugars) provisions for glycosyltransferase reactions are another issue. The number of enzymes involved in the in vitro synthesis of a nucleotide sugar makes an enzymatic approach less desirable for large-scale applications. Consequently, whole-cell catalysts were adopted by virtue of their facile cofactor provisions.

In order to further characterize YPH (pUGT) as a whole-cell biocatalyst for production of rebaudioside A, 2 g/L stevioside was added as the substrate to the 20 mL reaction with OD_{600} = ~75 washed YPH (pUGT) cells, which were cultured for approximately 20 h in SD (-Leu) medium to express the glucosyltransferase. Glucose (40 g/L) was added as the exogenous carbon source, which would be metabolized by *S. cerevisiae* in order to generate UDP-glucose. However, the catalytic activities of intracellular glucosyltransferase may be low in whole-cell biocatalysis due to the poor permeability of the cell in relation to the substrate and/or product as they diffuse to and from the reaction solution. In an effort to optimize whole-cell catalysis by *S. cerevisiae* YPH (pUGT), we compared the effectiveness of the chemical treatment methods by toluene (0.5 %, 1 %) [24] and triton X-100 (1 %) [25] to assess permeabilization of *S. cerevisiae* cells. From the results shown in Fig. 2, growth of *S. cerevisiae* YPH (pUGT) in the presence of 1 % toluene, 0.5 % toluene, and 1 % triton X-100, and as assessed by monitoring OD_{600}, were 24, 20, and 12 % lower than control, respectively, while the accumulation of rebaudioside A was 77.6-, 28.3-, and 3-fold higher than that observed in the control reaction mixture after 48 h, respectively. Perforating the natural barrier by efficient cell permeabilization using 1 % toluene resulted in the whole-cell biocatalyst with the highest product concentration (1160.5 mg/L, with a rebaudioside A yield of 48 %).

Using 1 % toluene to improve cell permeabilization, whole-cell catalysis was conducted under different temperatures, including 20, 25, 30, and 37 °C for 24 and 48 h. As shown in Fig. 3, the concentration of rebaudioside A detected at 30 °C (415.6 mg/L) was higher than that observed at the other temperatures after 24 h. However, rebaudioside A reached the highest concentration (652.4 mg/L) after 48 h at 25 °C, which was 2.5-fold higher than that observed at 37 °C (262.4 mg/L). While enzyme activity is generally increased at temperatures below the optimum temperature for enzyme activity, higher temperatures also promote metabolism of reserve carbohydrates in *S. cerevisiae*, such as synthesis of glycogen and

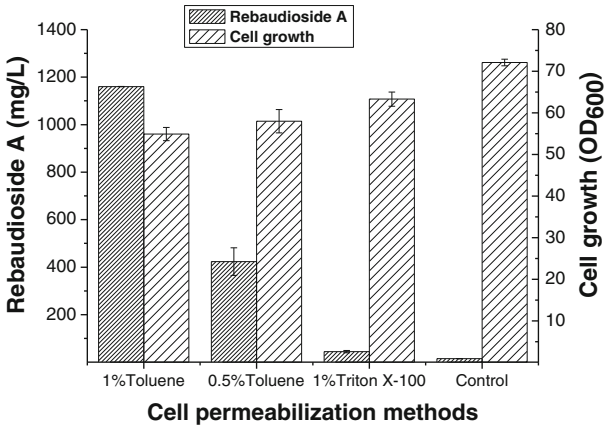

Fig. 2 Cell permeabilization effect on whole-cell biocatalysis using YPH (pUGT) yeast cells. Samples were collected from the reaction mixtures at 48 h

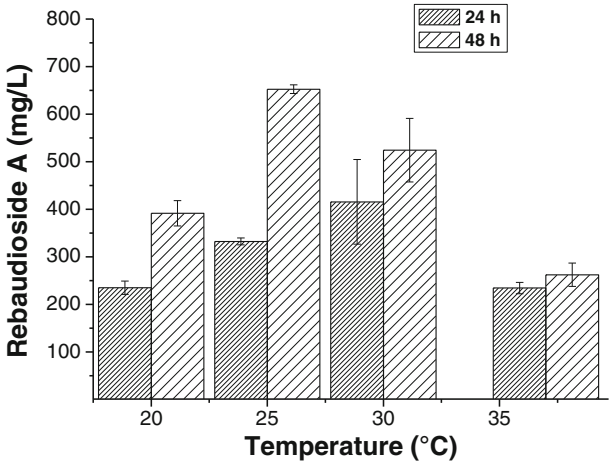

Fig. 3 The effect of temperature on whole-cell biocatalysis using YPH (pUGT) yeast cells

trehalose [26], which would likely compete with glucosyltransferase for UDP-glucose [27]. Therefore, this result indicated that it was UDP-glucose rather than the glucosyltransferase enzyme that may have been the limiting factor in rebaudioside A production at temperatures above 25 °C.

During the transfer of glucose from UDP-glucose to the acceptor substrate stevioside, a proton is released [28], increasing the acidity of the reaction solution and affecting enzyme activity. We investigated the effect of pH on rebaudioside A production by whole-cell catalysis at 30 °C in 100 mM potassium phosphate buffer at pH ranges from 6.8 to 8.0 (Fig. 4). As shown in Fig. 4, increased rebaudioside A concentrations were obtained in the more alkaline buffer, with rebaudioside A concentration reaching 1000.7 mg/L, and a corresponding yield of 42 % after 48 h at pH 8.0.

Phosphofructokinase, one of the key rate-limiting enzymes in the Embden–Meyerhof–Parnas pathway, catalyzes adenosine triphosphate and fructose 6-phosphate to generate adenosine diphosphate and fructose 2,6-bisphosphate (Fig. 5). Citrate has a strong inhibitory effect on phosphofructokinase [18]. As shown in Fig. 6, increased accumulation of rebaudioside A

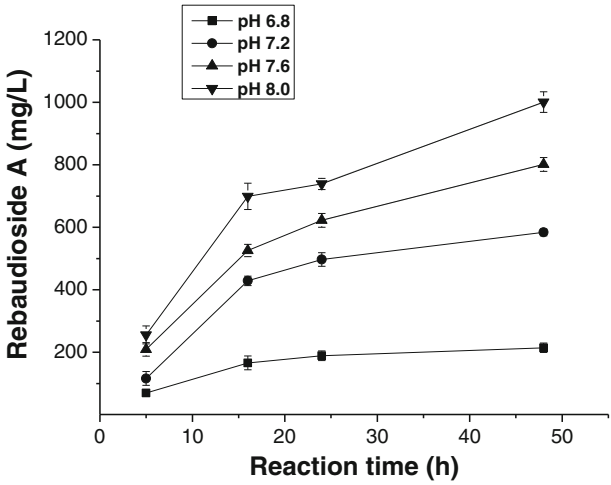

Fig. 4 The effect of pH on whole-cell biocatalysis using YPH (pUGT) yeast cells

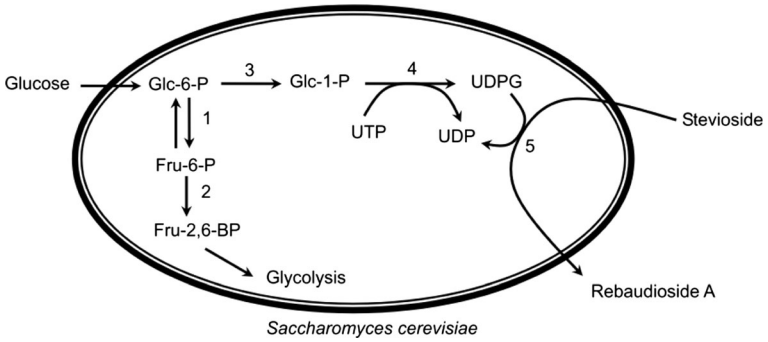

Fig. 5 Endogenous UDP-glucose synthesis pathway involved in the conversion of stevioside into rebaudioside A in the recombinant *S. cerevisiae* strain expressing UGT76G1 glucosyltransferase. Products/intermediates: Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; Fru-2,6-BP, fructose 2,6-bisphosphate; Glc-1-P, glucose 1-phosphate; UDPG, UDP-glucose. Enzymes: *1* phosphoglucose isomerase; *2* phosphofructokinase; *3* phosphoglucomutase; *4* UDP-glucose pyrophosphorylase; *5* UGT76G1

was observed in the presence of higher citrate concentrations in the reaction mixtures. The highest concentration of rebaudioside A produced was 441.6 mg/L after 36 h in the presence of 20 g/L of citrate. This result indicated that the addition of citrate regulated yeast metabolism by inhibiting phosphofructokinase activity, resulting in limited glycolysis and enhancement of carbon flux toward the pathway for UDP-glucose synthesis, which was favorable to the increased rebaudioside A production.

Mg^{2+} is required for the phosphofructokinase activity [18]. As shown in Fig. 7a, the highest rebaudioside A concentration observed was 974.7 mg/L after 48 h without Mg^{2+} in the reaction mixture. The decreased OD_{600} values were less for the samples without or with 2 g/L $MgCl_2$ than those for the samples with 6 or 10 g/L $MgCl_2$ in 48 h. Additionally, the effect of Mg^{2+} concentration on recombinant UDP-glucosyltransferase activity was investigated (Fig. 7b). UDP-glucosyltransferase activity was inhibited by Mg^{2+}, achieving only 62 % of enzyme activity in the presence of 5 mM (equivalent to 0.476 g/L) $MgCl_2$. Our results indicated that Mg^{2+} deprivation enhanced rebaudioside A production.

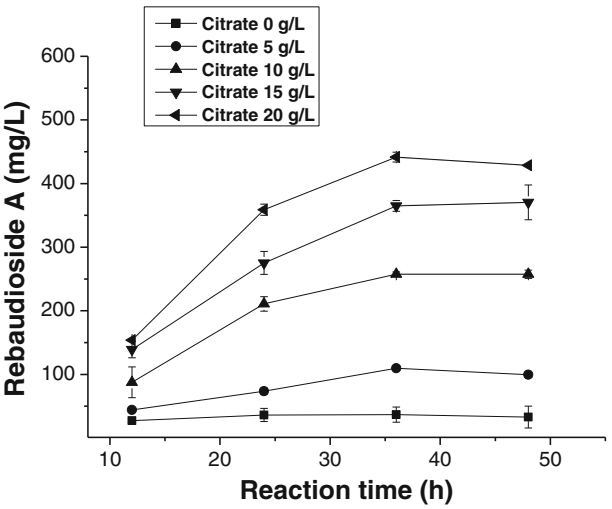

Fig. 6 The effect of citrate concentration on whole-cell biocatalysis using YPH (pUGT) yeast cells

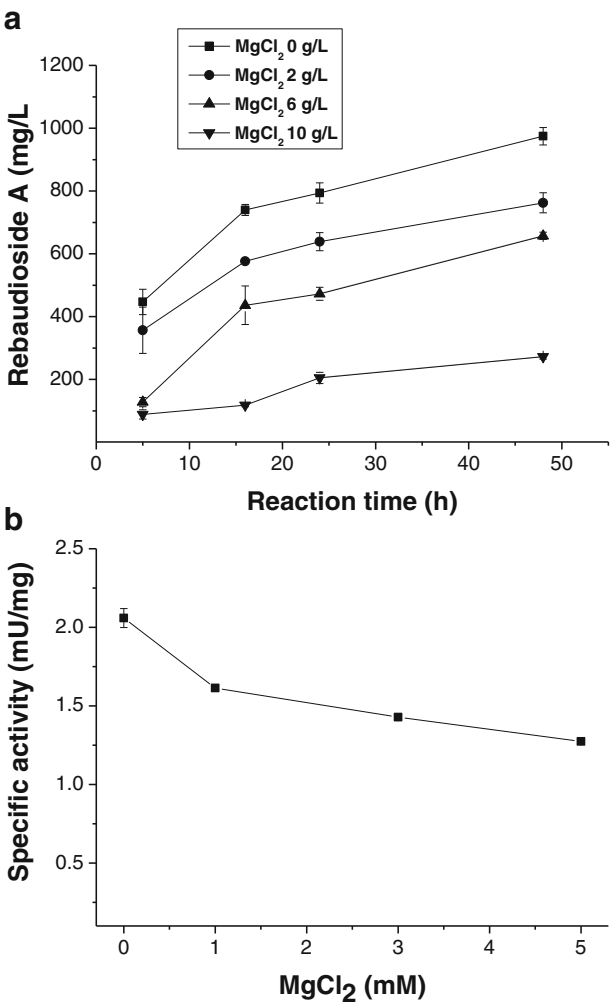

Fig. 7 Mg^{2+} effects on whole-cell biocatalysis using **a** YPH (pUGT) yeast cells and **b** recombinant UDP-glucosyltransferase activity

Glucose is the most commonly used carbon source for UDP-glucose synthesis in whole-cell biocatalysis [15]. In our study, when the glucose concentration in the reaction mixture was <20 g/L, the product concentrations decreased after 48 h. Additionally, the reaction mixture became acidic after 24 h, which triggered hydrolysis of steviol glycosides [29]. When 20 or 40 g/L glucose powder was supplemented at 24 h, and after additional 24-h incubation, production of rebaudioside A increased 1.8- and 2.7-fold, respectively, relative to that observed without glucose feeding (Fig. 8). During that time (from 24 to 48 h), the measured OD_{600} values were similar (0.16 % decreased) or slightly decreased (2.66 % lower) for the cells in the reaction mixture following addition of 20 or 40 g/L glucose, respectively, while OD_{600} values decreased 12.91 % for the control without glucose feeding. These results indicated that when UDP-glucose generated from the exogenous carbon source was not adequate for supporting glycosyltransferase activity, addition of supplemental glucose effectively recovered rebaudioside A production and cell viability.

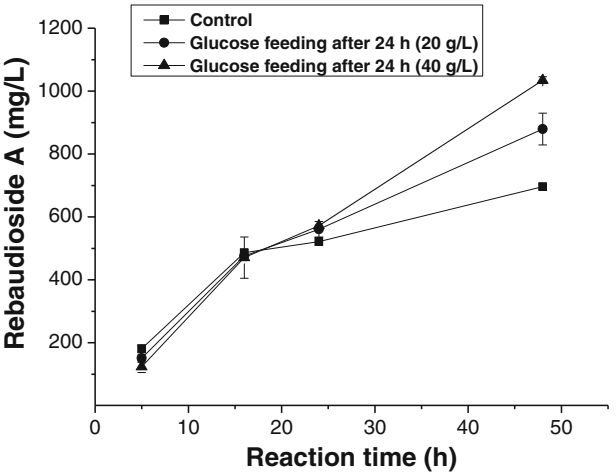

Fig. 8 The effect of glucose supplementation on whole-cell biocatalysis using YPH (pUGT) yeast cells

An adequate uptake of exogenous sugar can be an issue when the sugar is also necessary as a host-cell carbon and energy source. It is worth noting that citrate was demonstrated as an effective metabolic regulator, enabling UDP-glucose to be used for stevioside glycosylation (Fig. 6) but citrate-based regulation was not adequate for increased stevioside concentrations. Thus far, in our experiments, the substrate concentration has been limited to 2 g/L. The required nucleotide sugars are products of multiple interacting pathways, adding significant complexity to efforts in metabolic engineering. There have been successful examples of developing whole-cell biocatalysts with enhanced in vivo UDP-glucose synthesis [30–32]. Phosphoglucomutase and UDP-glucose pyrophosphorylase (Fig. 5) were important in diverting carbon flux toward UDP-glucose synthesis pathway [33, 34]. The same strategy used to co-express these two enzymes for adequate UDP-glucose provisions could potentially be applied to *S. cerevisiae* for rebaudioside A synthesis in order to enhance efficiency.

Conclusion

The *S. rebaudiana* UDP-glucosyltransferase UGT76G1 was constitutively overexpressed in *S. cerevisiae* YPH499, under the control of the *PGK1* promoter. The engineered *S. cerevisiae* cells expressing UGT76G1 were used as whole-cell biocatalyst in rebaudioside A production. By addition of citrate to regulate cell metabolism to increase availability of UDP-glucose, effective conversions of stevioside into rebaudioside A were achieved. The promoted UDP-glucose provisions would be beneficial to the engineered *S. cerevisiae* for enhanced efficient rebaudioside A synthesis.

Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no competing interests.

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