Highly Efficient Biosynthesis of Salidroside by a UDP-glucosyltransferase-catalyzed Cascade Reaction

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Abstract

Objective Salidroside is an important plant-derived aromatic compound with diverse biological properties. The main objective of this study was to synthesize salidroside from tyrosol using UDP-glucosyltransferase (UGT) with *in situ* regeneration of UDP-glucose (UDPG).

Results The UDP-glucosyltransferase 85A1 (UGT85A1) from *Arabidopsis thaliana*,which showed high activity and regioselectivity towards tyrosol, was selected for the production of salidroside. Then, an in vitro cascade reaction for *in situ* regeneration of UDPG was constructed by coupling UGT85A1 to sucrose synthase from *Glycine max* (*Gm*SuSy). The optimal UGT85A1-*Gm*SuSy activity ratio of 1:2 was determined to balance the efficiency of salidroside production and UDP-glucose regeneration. Different cascade reaction conditions for salidroside production were also determined. Under the optimized condition, salidroside was produced at a titer of 6.0 g/L with a corresponding molar conversion of 99.6% and a specific productivity of 199.1 mg/L/h in a continuous feeding reactor.

Conclusion This is the highest salidroside titer ever reported so far using biocatalytic approach.

Introduction

The phenolic glycoside salidroside is the major bioactive ingredient in various *Rhodiola* plants; it has attracted extensive research interest because of its powerful anti-hypoxic, anti-fatigue, anti-aging, and antioxidant activities (Biswal et al. 2018; Darbinyan et al. 2000; Zhang et al. 2021). Salidroside is in high demand owing to its wide applications in food, health products, cosmetics, and pharmaceuticals.

Currently, salidroside is mainly produced by direct extraction from *Rhodiola* or by chemical synthesis, which has many disadvantages (Grech-Baran et al. 2015; Liu et al. 2017). Metabolic engineering and synthetic biology have enabled heterologous production of salidroside in microorganisms (Jiang et al. 2018; Liu et al. 2022). However, many problems still need to be resolved before industrial production can be achieved, for example, (i) the purification of salidroside from a complex fermentation broth is generally difficult and expensive, (ii) the recombinant strains are not sufficiently stable, and (iii) both the yield and productivity of salidroside are still low.

Compared to the above methods, biocatalytic synthesis provides an alternative way to realize a greater market value for salidroside because of the simplicity, convenience, as well as low cost of product recovery (Li et al. 2020; Li et al. 2022). Industrially, UDP-glucosyltransferases (UGT; EC 2.4.x.y), members of the glucosyltransferase family, which catalyze the glucosylation of tyrosol at its alcoholic hydroxyl group in the presence of UDP-glucose, are the preferred biocatalysts for the production of salidroside. Although great progress has been made in recent years, there is still room for improvement considering the industrial production value. Previous studies indicated that the low glucosylation activity of UDP-glucosyltransferases and the supply of large amounts of the costly glucosyl donor UDP-glucose (UDPG) were the major bottlenecks for the industrial production of salidroside (Fan et al. 2017; Xue et al. 2016).

In this study, UGTs from different species were cloned and investigated for their efficiency in salidroside synthesis and a cascade reaction was constructed to regenerate UDP-glucose by coupling UGT85A1 from *Arabidopsis thaliana* to *Glycine max* sucrose synthase (*Gm*SuSy). Optimal cascade conditions for salidroside production were determined. Under such optimal conditions, the substrate-feeding mode for salidroside production was further investigated.

Materials And Methods

Strains, plasmids, and chemicals

Escherichia coli strains DH5α and BL21 (DE3) were used for plasmid construction and recombinant enzyme production. pET28a was purchased from Novagen (Darmstadt, Germany) and used as the expression vector. UDP and UDP-glucose were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Icariside D2 and salidroside were purchased from Energy Chemical (Shanghai,China) and used as the standards. Restriction enzymes and PrimeSTAR@HS DNA Polymerase were purchased from Takara Bio Inc. (Dalian, China). The one step cloning kit was purchased from Vazyme Biotech (Nanjing, China).

Gene cloning and construction of recombinants

The *BN*jiC gene (NCBI accession: WP_003182014.1) was amplified from the genome of *Bacillus licheniformis* ATCC14580. UGT85A1 (NCBI accession: Q9SK82.1), *Rt*UGT33 (NCBI accession: AUI41147.1), *Rt*UGT17 (NCBI accession: AUI41131.1), UGT73B6 (NCBI accession: AAS55083.1), UGT72B14 (NCBI accession: AQD20582.1), and *Gm*SuSy (NCBI accession: NP_001237525.1) genes were synthesized to incorporate *E. coli* codons by Sangon Biotech (Shanghai, China). All genes were subcloned into the expression vector pET28a at the *Noc* I and *Hin*d III sites according to the instructions of the one step cloning kit to create plasmids pET28a-*BN*jiC, pET28a-UGT85A1, pET28a-*Rt*UGT33, pET28a-*Rt*UGT17, pET28a-UGT73B6, pET28a-UGT72B14 and pET28a-*Gm*SuSy, respectively. All the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells. The plasmids, recombinant *E. coli* strains and primers used in this study are listed in Tables S1 and S2.

Preparation of recombinant enzymes.

Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was used for culturing recombinant *E. coli* strains. When the absorbance (OD_{600}) of the cultures reached 0.6–0.8, 0.1 mM IPTG was added and the recombinant *E. coli* strains were further incubated at 18 °C, and 200 rpm for 24 h. The cells were then harvested by centrifugation at 10,000 rpm, 4 °C for 10 min. The supernatants were extracted by ultrasonication (50% amplitude, 3 sec pulse and 3 sec pause cycles for 15 min) and centrifugation at 10,000 rpm for 20 min.

Enzyme activity assay.

Glycosyltransferase activity was measured in a 200 μL reaction mixture containing 50 mM Tris−HCl (pH 8.0), 1 mM tyrosol as a substrate, 2 mM UDP-galactose, and the appropriate amounts of glycosyltransferase. The reaction mixture was incubated for 30 min at 30°C and terminated by the addition of 400 μL methanol. One unit of enzyme activity was defined as the amount of enzyme required to synthesize 1 μmol of salidroside per min.

The *Gm*SuSy activity was measured in a reaction mixture containing 50 mM Tris−HCl (pH 7.0), 10 mM UDP, appropriate amounts of *Gm*SuSy, and 50 mM sucrose,. (Ma et al. 2014). The mixture was incubated for 30 min at 30°C. The resultant reducing sugar content was determined by adding DNS reagent (3,5-dinitrosalicylic acid) and immediately boilling for 10 min. The reduced product (3-amino-5-nitrosalicylic acid) was detected at 540 nm. One unit of enzyme activity was defined as the amount of *Gm*SuSy required to release1 μmol of fructose per min.

Optimizing the conditions for the cascade reaction.

The standard cascade reaction mixture (200 μL) contained 50 mM Tris−HCl (pH 8.0), 1 mM tyrosol, 0.2 mM UDP, 50 mM sucrose, 50 U/mL UGT85A1, and 50 U/mL *Gm*SuSy. The reaction was performed at 30 °C, and 1,000 rpm in a constant temperature metal bath. The effects of different ratios of UGT85A1 to *Gm*SuSy on the coupled reaction were investigated at different addition amounts (U/mL): 50:0, 50:25, 50:50, 50:100, and 50:150. The optimal pH for the cascade reaction was determined using 50 mM Na_2HPO_4–NaH_2PO_4 (pH 6.5–7.5) and 50 mM Tris−HCl buffer (pH 7.5–8.5). The effect of the concentrations of UDP (0–0.5 mM), sucrose (0–400 mM), and tyrosol (1–9 mM) on the cascade reaction was also investigated. The reaction was terminated by adding methanol to twice the reaction volume. The reaction mixture was assayed using high-performance liquid chromatography (HPLC).

Synthesis of salidroside by the UGT85A1-*Gm*SuSy cascade reaction under fed-batch and continuous modes.

Under the fed-batch mode, the reaction mixture (100 mL) contained 50 mM Tris−HCl (pH 7.5), 3 mM tyrosol, 0.3 mM UDP, 100 mM sucrose, 50 U/mL UGT85A1, and 100 U/mL *Gm*SuSy. The reaction was stirred at 30 °C. Tyrosol (3 mM) was periodically added to the reaction mixture based on the consumption of this substrate. Under the continuous mode, the substrate concentration was maintained below 1 mM throughout the reaction by controlling the substrate flow speed. During the reaction, 50 μL of the reactant was periodically collected, terminated, and analyzed using HPLC.

HPLC and NMR analysis

The column was maintained at 30 °C with a 20% methanol flow rate of 1.0 mL/min, and absorbance was measured at 275 nm. All assays were performed in triplicates. Salidroside and icariside D2 were purified using semipreparative HPLC. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE IIII 600 spectrometer using DMSO-d6 as the solvent (Fig. S4 and Fig. S5).

Results

Screening UGTs for salidroside production

The glycosylation of tyrosol at the alcoholic hydroxyl group requires a highly efficient glucosyltransferase (Fig. 1a). It has been reported that *BN*jiC from *B. licheniformis* (Liu et al. 2018), UGT85A1 from *Arabidopsis thaliana* (Liu et al. 2018), UGT73B6 and UGT72B14 from *Rhodiola sachalinensis* (He et al. 2017; Xue et al. 2016), and RrUGT33 and RrUGT17 from *R. rosea* (Torrens-Spence et al. 2018) can catalyze the glycosylation of tyrosol. To improve heterologous expression in *E. coli*, the UGT genes from plants were codon-optimized and sub-cloned into pET-28a (+). The recombinant expression vectors were transformed into *E. coli* BL21(DE3) and induced at 18 ℃ for 24 h. After ultrasonic breakage, the samples were centrifuged and the supernatants and precipitates were collected. According to SDS-PAGE analysis, four out of six UGTs (*BN*jiC, UGT85A1, UGT73B6, and *Rr*UGT17) were well expressed, while UGT72B14 and *Rr*UGT33 were mostly distributed in the precipitates (Fig. S1). The glucosylation activity of the supernatant from each UGT sample was then analyzed. Comparatively, *BN*jiC, UGT85A1 and RrUGT17 showed higher activities than the other UGTs, with conversion ratios of 43.9%, 28.8% and 22.6%, respectively (Fig. 1b). Notably, the six UGTs demonstrated distinct regioselectivity towards tyrosol (Fig. 2b). Specifically, UGT85A1 and RrUGT33 glucosylated the alcoholic hydroxyl group of tyrosol generating salidroside as the sole product. In contrast, other UGTs showed low regioselectivity, producing significant amounts of icariside D2 (Fig. 1b). In view of its high activity and regioselectivity, UGT85A1 was further used for the production of salidroside.

Construction of a UGT85A1-*Gm*SuSy cascade reaction

In general, tyrosol glycosylation requires UDP-glucose (UDPG), an expensive nucleotide sugar, as donor molecule. However, *Gm*SuSy is a glycosyltransferase that can efficiently convert inexpensive sucrose into UDPG and fructose in the presence of UDP (Gutmann et al. 2014). Therefore, the coupling of *Gm*SuSy and UGT85A1 in one-pot could lead to *in situ* regeneration of UDPG in the presence of catalytic amounts of UDP. Consequently, a cascade biocatalysis for salidroside production was designed using UGT85A1 and *Gm*SuSy (Fig. 2a). The enzyme ratio on salidroside synthesis by the reconstituted cascade reaction was confirmed by directly detecting the conversion of tyrosol (Fig. 2b). First, a 0.5-fold increase in *Gm*SuSy activity relative to that of UGT85A1 was included. The cascade biocatalysis system could convert tyrosol into salidroside, with a significantly improved conversion of 28.7%. The amount of *Gm*SuSy was then increased to further facilitate the cascade reaction. With 2-fold *Gm*SuSy activity, the conversion rate reached 36.7%, which was 4.8-fold higher than in the absence of *Gm*SuSy (7.6%). Further

increases in *Gm*SuSy activity of over 2-fold were not beneficial. These results indicated that the presence of *Gm*SuSy was necessary for recycling UDPG, and that the optimal enzyme activity ratio of UGT85A1:*Gm*SuSy should be 1:2 in this cascade biocatalytic system.

Optimizing the parameters of the cascade reaction

Although the optimal pH for UGT85A1 was 8.0, it is worth noting that *Gm*SuSy activity was inhibited under alkaline conditions (Fig. S2). Therefore, to investigate the optimal pH for the cascade system, reactions were conducted at different pH values, and the highest conversion of tyrosol was obtained at a pH of 7.5.

Given that UDPG was the key factor in the UGT85A1-*Gm*SuSy reaction mixture, the effects of different concentrations of UDP on salidroside production were investigated (Fig. 3b). The conversion of tyrosol was rapidly increased as the concentration of UDP increased from 0.1 to 0.3 mM and a slight decrease was observed at UDP concentrations above 0.3 mM. Therefore, it was determined that 0.3 mM UDP was the optimal concentration for the coupled reaction system. In addition, the effect of different concentrations of sucrose, the cosubstrate, on salidroside production was also investigated (Fig. 3c). The conversion of tyrosol significantly increased when the sucrose concentration was increased from 0 to 100 mM. In contrast, when the sucrose concentration was above 200 mM, the conversion rate decreased rapidly. This may have been caused by enzyme activity inhibition in the reaction mixture at a relatively high concentration of sucrose.

The effect of tyrosol concentration on the UGT85A1-*Gm*SuSy cascade reaction was determined after optimizing the above parameters. As shown in Fig. 3d, the conversion rate reached > 99% with 1–5 mM tyrosol as the substrate, while significantly decreased with tyrosol concentrations above 7 mM, even with extended reaction times. It appeared that the cascade was inhibited by high concentrations of the substrate. The enzymatic activities of UGT85A1 and *Gm*SuSy at different tyrosol concentrations were then investigated (Fig. 4). The results showed that the activity of both enzymes, especially that of UGT85A1, decreased significantly with an increase in tyrosol concentration, indicating substrate inhibition, which can be addressed if the substrate is constantly consumed by the cascade reaction and fed in either a fed-batch or continuous mode.

One-pot synthesis of salidroside by the substrate fed-batch reaction

To improve the final concentration of salidroside and avoid serious substrate inhibition, a fed-batch cascade reaction was conducted by the periodic addition of 3 mM tyrosol. As shown in Fig. 5a, kinetic analyses of salidroside production and tyrosol consumption over time were performed. The specific productivity during the first batch cultivation period was 220.5 mg/L/h. Productivity gradually decreased,

as the reaction proceeded, to 147.6 mg/L/h, 102.9 mg/L/h, and 55.9 mg/L/h during the second, third, and fourth batch cultivation periods, respectively. Finally, 3.6 g/L (11.9 mM) salidroside was produced, with a corresponding molar conversion of 99.2% and a specific productivity of 93.9 mg/L/h.

To explore the possibility of further improving the performance of the UGT85A1-*Gm*SuSy cascade reaction, the substrate was fed into a continuous model and maintained at concentrations below 1 mM by controlling the flow rate of the pump. As shown in Fig. 5b, the production of salidroside reached 6.0 g (19.9 mM) with a corresponding molar conversion of 99.6% and a specific productivity of 199.1 mg/L/h. Compared with the fed-batch mode, the continuous mode provided 66.7% higher concentration and 112.0% higher productivity of salidroside. To our knowledge, this is the highest titer reported to date using a biocatalytic method. This data laid the foundation for mass production and simplified downstream purification.

Structural identification of products

Salidroside: ^{1}H NMR (600 MHz DMSO-d6) 9.15 (s, 1H), 7.04 (s, 1H), 7.03 (s, 1H), 4.97-4.96 (d, *J* = 6.0 Hz, 1 H), 4.92-4.91 (d, *J* = 6.0 Hz, 1 H), 4.88-4.87 (d, *J* = 6.0 Hz, 1 H), 4.48-4.46 (t, 1H), 4.17-4.15 (d, *J* = 12.0 Hz, 1 H), 3.89-3.85 (m, 1H), 3.67-3.64 (m, 1H), 3.58-3.54 (m, 1H), 3.45-3.40 (m, 1H), 2.77-2.69 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d6): 156.08, 130.23, 129.05, 115.48, 103.31, 77.36, 77.25, 73.91, 70.55, 70.38, 61.55, 35.30 ppm (Fig. S4). Icariside D2: ^{1}H NMR (600 MHz, DMSO-d6): δ 7.12-7.10 (m, 2H), 6.94–6.91 (m, 2H), 4.79 (d, 1H), 3.69–3.67 (m, 1H), 3.56-3.53 (t, *J* = 6.0 Hz, 2 H), 3.46-3.43 (m, 2H), 3.27-3.13 (m, 4H), 2.66-2.64 (t, 2H). ^{13}C NMR (151 MHz, DMSO-d6) δ 156.23, 133.21, 130.13, 116.52, 101.06, 77.47, 77.11, 73.72, 70.21, 62.88, 61.18, 38.2 ppm (Fig. S5).

Conclusion

The glycosyltransferase UGT85A1 from *B. licheniformis* was identified as an efficient biocatalyst for the salidroside production. A UDPG recycling system was established for efficient synthesis of salidroside by coupling UGT85A1 with the sucrose synthase *Gm*SuSy from *G. max*. With bioprocess engineering efforts, salidroside was produced at a final titer of 3.6 g/L mg/L with a corresponding molar conversion of 99.2%, and a specific productivity of 93.9 mg/L/h in a fed-batch mode and 6.0 g/L with a corresponding molar conversion of 99.6%, and a specific productivity of 199.1 mg/L/h in a continuous mode. This study suggests that the in vitro UGT85A1-*Gm*SUS biocatalytic cascade reaction could be exploited as an efficient approach for potential industrial-scale production of salidroside in an economically effective manner.

Declarations

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

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Figures

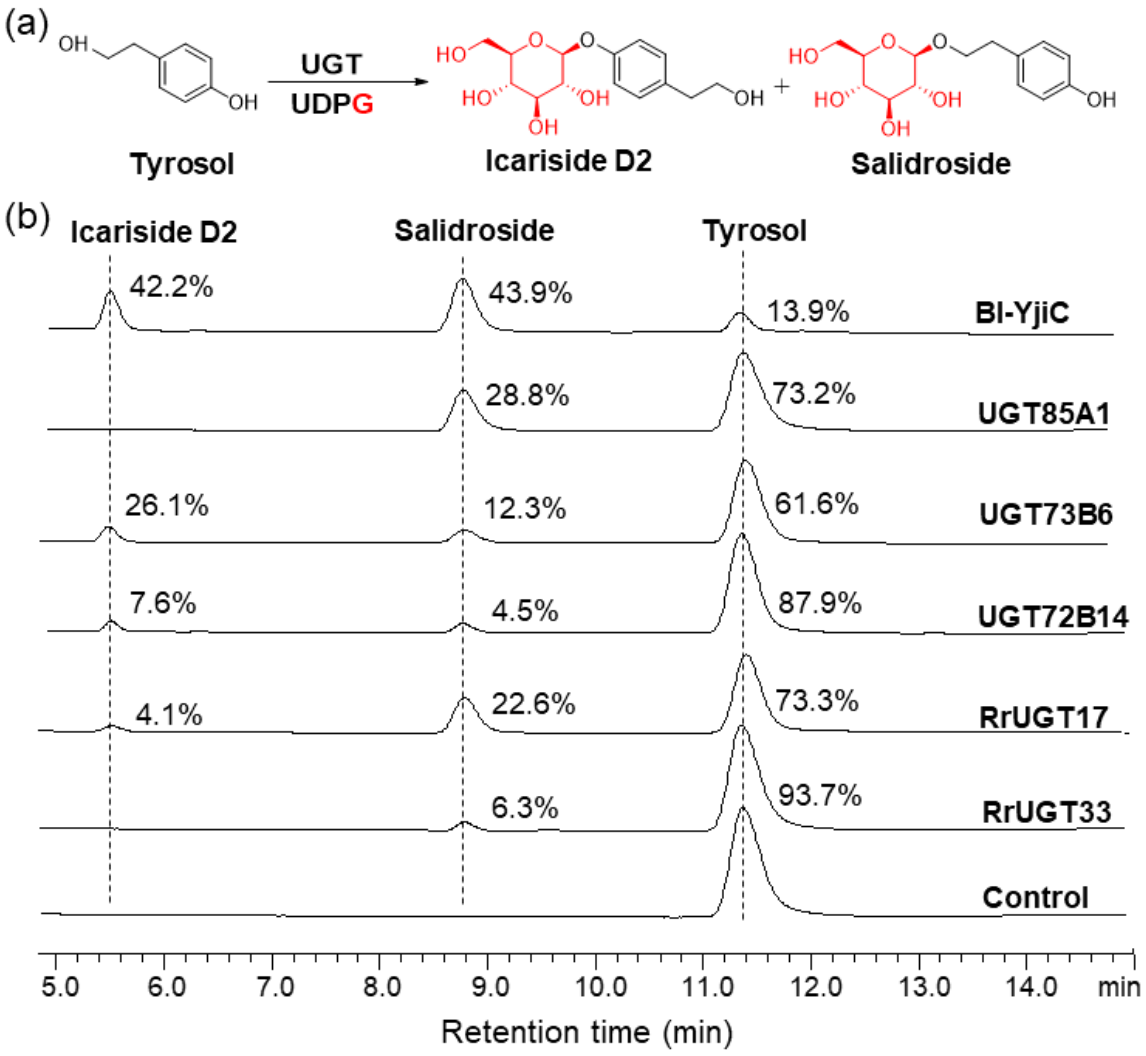

Figure 1

Screening of candidate UGTs for salidroside production. (a) Reaction schemes of tyrosol glucosylation; (b) HPLC analysis results of the glucosylation reactions catalyzed by six UGTs.

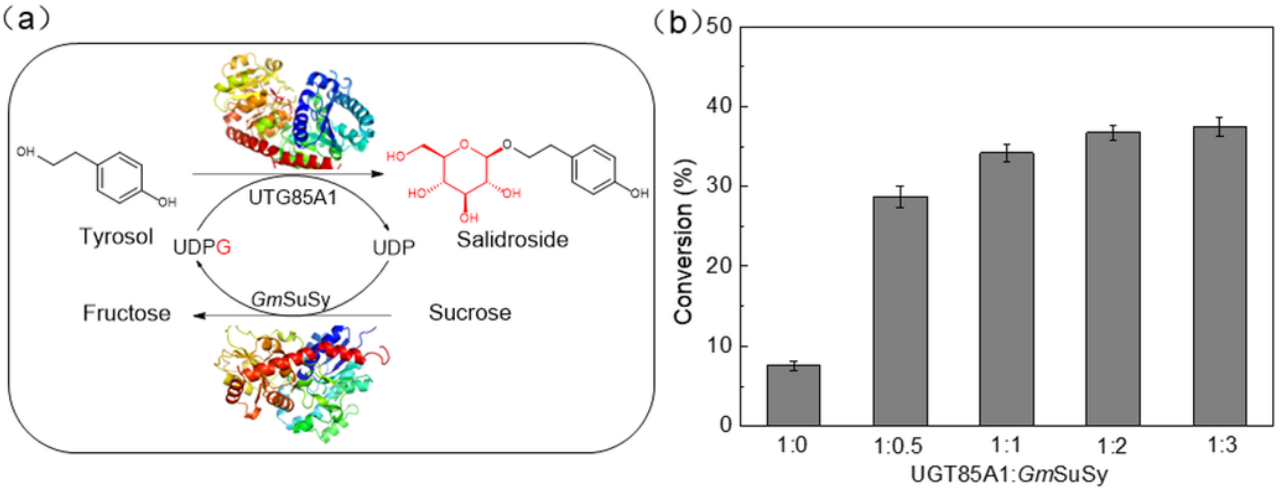

Figure 2

Biocatalytic cascade reaction for salidroside production. (a) UGT85A1 was coupled with *Gm*SuSy for *in situ* UDPG regeneration; (b) Effect of the UGT85A1:*Gm*SuSy ratio on salidroside production.

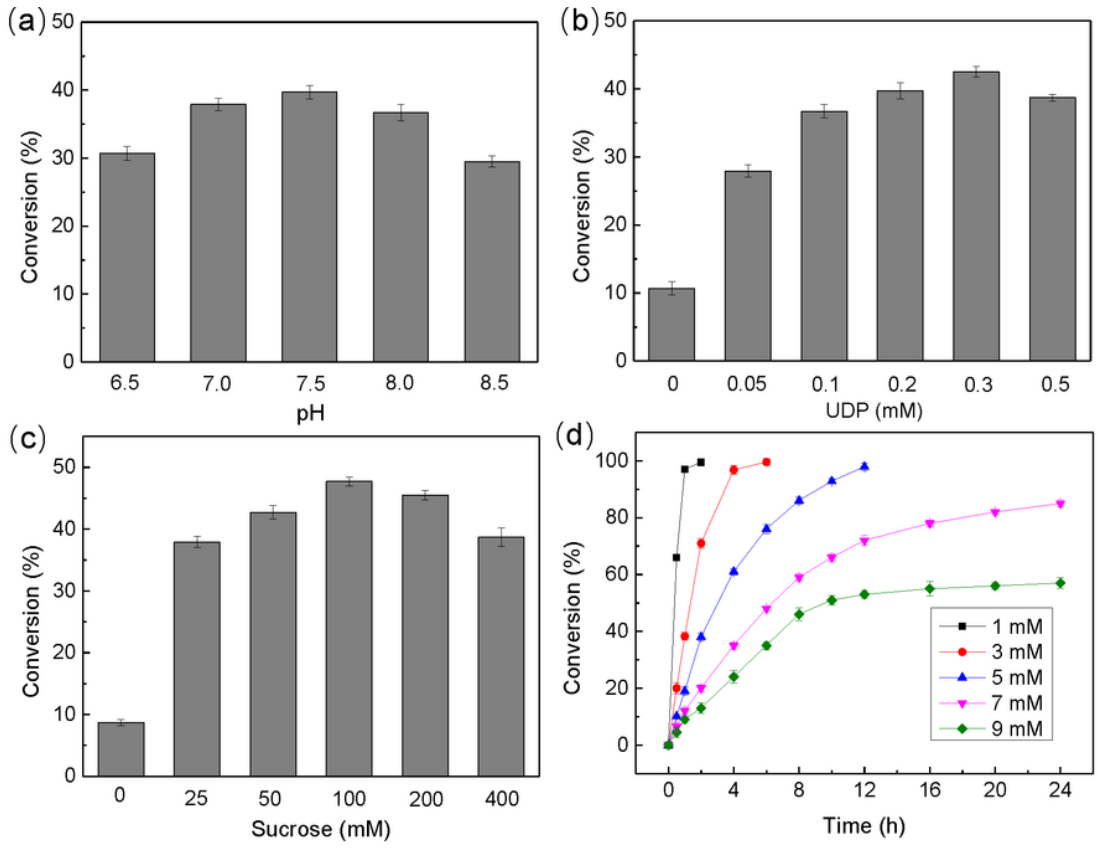

Figure 3

Optimization of the conditions for the cascade reaction catalyzed by UGT85A1 and *Gm*SuSy. Effect of (a) pH, (b) UDP concentration, (c) sucrose concentration, and (d) substrate concentration on the cascade

reaction.

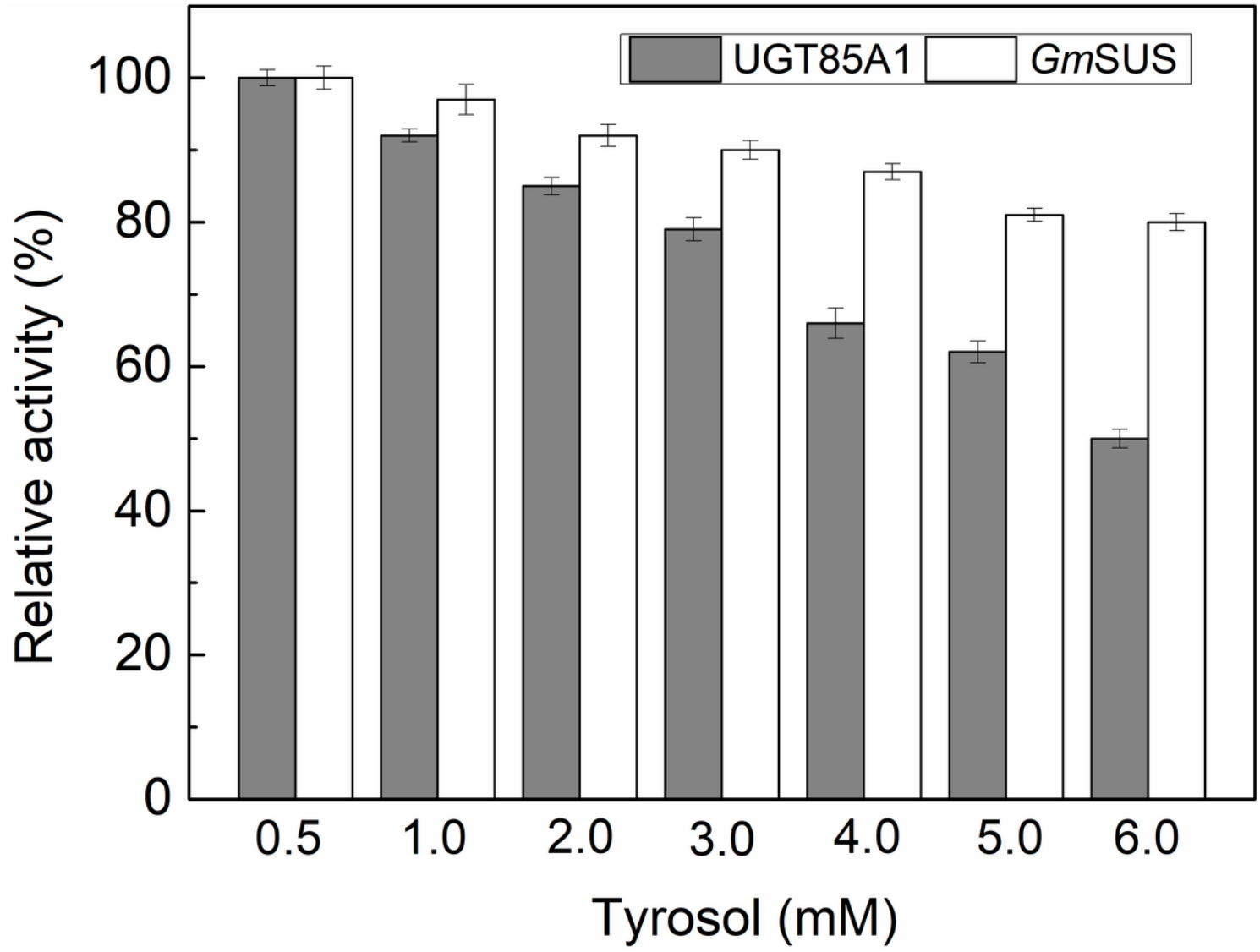

Figure 4

Relative activity of UGT85A1 and *Gm*SUS under different substrate concentration.

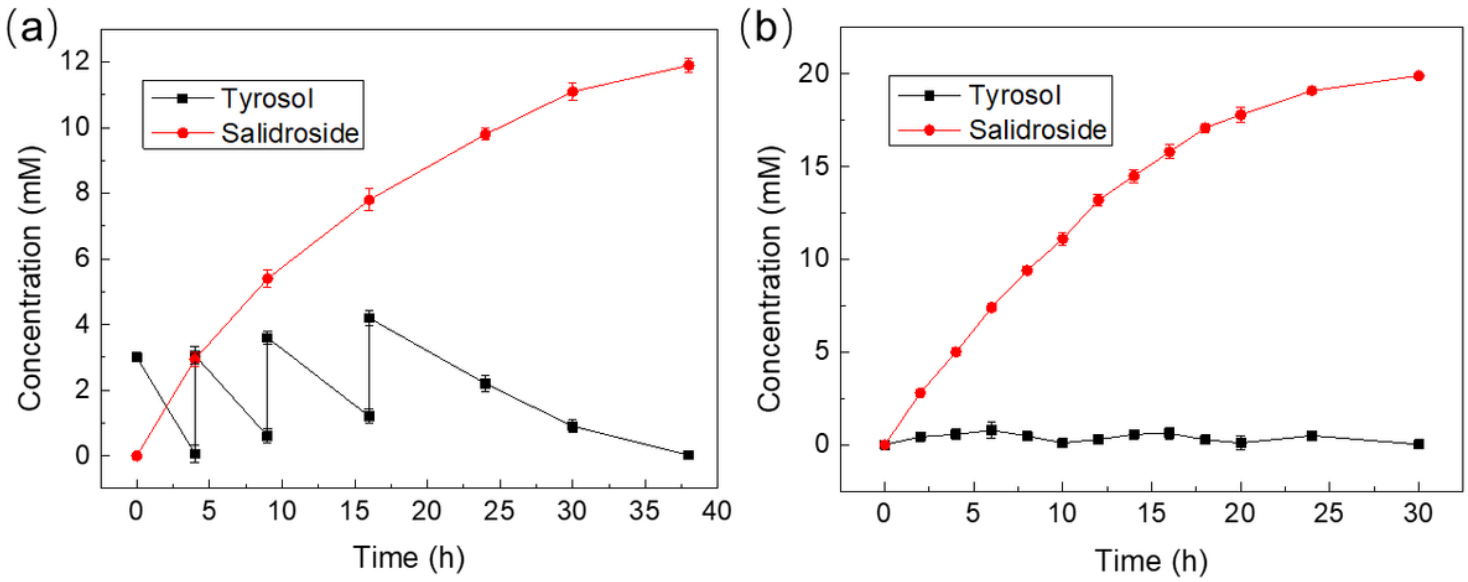

Figure 5

Synthesis of salidroside by the UGT85A1-*Gm*SuSycascade reaction in (a) fed-batch and (b) continuous modes.

Supplementary Files

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