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Research article

Biosynthesis of β-Arbutin via In Situ UDPG regeneration and glycosyltransferase engineering

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Abstract: β-Arbutin is a widely used skin-lightening agent in the cosmetology industry. However, the high cost of uridine diphosphate glucose (UDPG) and the low activity of glycosyltransferase have limited its practical biosynthesis. In this study, we constructed a UDPG regeneration system using sucrose synthase (SuSy) to reduce UDPG consumption and enhanced glycosyltransferase efficiency through site-directed mutagenesis. The single-point mutant A343V increased the hydroquinone (HQ) conversion efficiency by 1.25-fold. Under optimized conditions, the β-arbutin yield reached 98%, and the in situ UDPG regeneration cycles reached 39 times. This work demonstrates that the bi-enzymatic cascade reaction with UDPG regeneration and enzyme engineering provides an efficient and environmentally friendly method for β-arbutin biosynthesis.

Keywords: β-arbutin; biosynthesis; UDPG regeneration; glycosyltransferase; mutation

1. Introduction

β-arbutin, which is produced in wheat, pear, and bearberry plants, is widely used as a powerful skin-lightening agent in the cosmetology industry due to its strong inhibition of tyrosinase activity [1–3]. It is also used in the pharmaceutical industry due to its antioxidant, anti-inflammatory,

antibacterial, and antitussive properties [4–6]. With the improvement of people's living standards, the market demand for β-arbutin is increasing rapidly. However, β-arbutin production generally depends on direct extraction from plants or chemical synthesis [7]. The recovery efficiency and the purity of β-arbutin are low due to the low β-arbutin content that can be extracted from plants. Seasons and climate also strongly influence the growth and production of plants [8]. Therefore, substantial quantities of β-arbutin cannot be acquired by extraction from plants. Chemical synthesis of β-arbutin is usually achieved using Koenigs–Knorr glycosylation [9]. In this process, toxic reagents are used, and the production of isomers decreases the safety and purification of β-arbutin [10,11]. Instead, environmentally friendly bioprocessing, which has milder reaction conditions, high stereoselectivity, and low waste, has become the most promising production process [12]. Compared with fermentation, the enzymatic catalytic process is simple, does not form by-products, and enables easy separation of products, therefore having attracted much attention [8,13].

β-arbutin is synthesized from hydroquinone (HQ) by uridine sugar glycosyltransferase (UGT) using UDPG as the glycosyl donor. The costly and unstable nature of UDPG is the main limitation to the glycosylation process [14,15]. A higher UDPG concentration leads to a higher product yield but also an increase in UDP content, the by-products of which inhibit UGT activity [16]. Therefore, an efficient UDPG in situ regeneration system is urgently needed. In the glucose metabolism pathway, sucrose synthase (SuSy) catalyzes sucrose and uridine 5'-diphosphate (UDP) to produce fructose and UDPG [17–19]. SuSy decreases UDPG usage and alleviates UDP inhibition; therefore, combining UGT and SuSy is an attractive strategy for enhancing the glycosylation of natural products [16,20–23]. Glycosyltransferases play a critical role in glycoside synthesis. Studies have identified arbutin synthase (AS) from *Rauvolfia serpentine* as a catalyst for β-arbutin synthesis in *Escherichia coli*, *Yarrowia lipolytica*, *Komagataella phaffii*, *Corynebacterium glutamicum*, and *Pseudomonas chlororaphis* [12,24–27]. However, the glycosyltransferase activity is typically low. Directed evolution, in particular, has led to glycosyltransferases with improved activity and expanded substrate specificity [23].

Here, arbutin synthase (AS) from *Rauvolfia serpentine* and sucrose synthase (SuSy) from *Glycine max* were heterologously expressed in *E. coli*. After optimizing the protein expression conditions, a one-pot cascade reaction with UDPG regeneration for β-arbutin synthesis was established by combining AS and SuSy using HQ and UDPG as the aglycone and sugar donors, respectively (Figure 1). In addition, rational site-directed mutagenesis based on sequence conservation analysis and molecular docking improved the catalytic activity of AS toward HQ and UDPG. After optimizing the enzymatic reaction conditions and with 39 UDPG regeneration cycles, the substrate conversion was higher than 98%. This study provides a strategy for β-arbutin biosynthesis by integrating enhanced UDPG production with enzyme engineering.

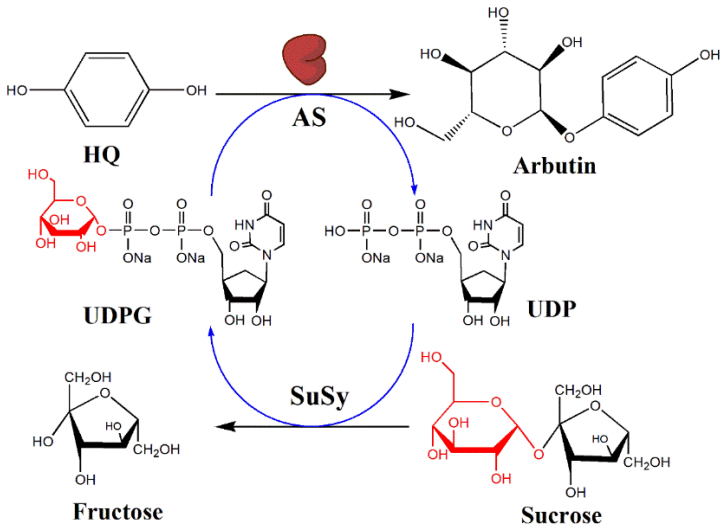


Figure 1. Strategy for the construction of a bi-enzymatic cascade reaction for β-arbutin biosynthesis with in situ UDPG regeneration. HQ, hydroquinone; UDPG, uridine diphosphate glucose; UDP, uridine diphosphate; AS, arbutin synthase; SuSy, sucrose synthase, which regenerates UDPG from sucrose and UDP with fructose as the by-product.

2. Materials and methods

2.1. Materials

E. coli DH5α and BL21 (DE3) were used for the plasmid amplification and expression of recombinant enzymes. The coding sequences of *AS* from *Rauvolfia serpentina* (GenBank accession no. AJ310148.1) and *SuSy* from *Glycine max* (GenBank accession no. NP_001341844.1) were synthesized with *E. coli* codon optimization by Synbio Technologies, Inc. (Suzhou, China). The *AS* gene was inserted into pRSFDuet-1 via the NdeI/XhoI restriction sites, and the *SuSy* gene was inserted into pET-28a via the NcoI/XhoI restriction sites, yielding the recombinant plasmids pRSFDuet-1-AS and pET-28a-SuSy, respectively. HQ, sucrose, and UDPG were purchased from Shanghai Aladdin Biotechnology Co., Ltd (Shanghai, China). All other chemicals were of analytical grade.

2.2. Preparation of recombinant enzymes [28]

The recombinant plasmids were transformed into *E. coli* BL21 (DE3) for AS and SuSy production. The recombinant strain was then incubated in LB medium at 37 °C and 220 rpm until the absorbance (OD_{600}) reached 0.6–0.8. At that point, IPTG was added to the medium to a final concentration of 0.2 mM, and the recombinant strains were further incubated at 30 °C for approximately 12–15 h. Then, the bacterial cells were suspended in pH 6.5, 200 mM PBS buffer, lysed by sonication (10 min at 0 °C with alternating on-off cycles of 3 s each), and then centrifuged at 10,000 rpm for 20 min at 4 °C. Protein expression was detected by SDS-PAGE. The cell lysate was centrifuged, and the supernatant containing the soluble recombinant proteins was loaded onto a Ni-NTA affinity column pre-equilibrated with binding buffer. After washing to remove nonspecifically bound proteins, the His6-tagged AS and SuSy were eluted with an imidazole gradient, and the fractions containing the target

proteins were pooled and desalted. The purified enzymes were analyzed by SDS-PAGE (Figure 2) and stored at −80 °C until later use.

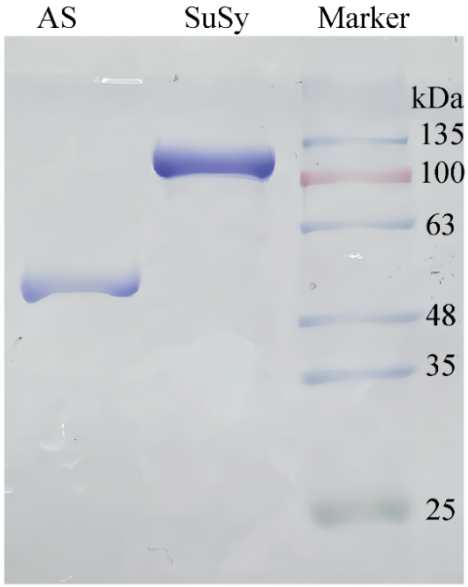


Figure 2. SDS-PAGE of purified AS and SuSy. The target bands of AS (52 kDa) and SuSy (92 kDa) are indicated by arrows. M, protein molecular weight marker.

2.3. Effect of reaction time on HQ conversion

The cascade reaction was performed in a 10 mL one-pot system containing AS or A343V (20 μg/mL), SuSy (80 μg/mL), HQ (2 mM), UDPG (0.05 mM), and sucrose (8 mM) in 200 mM pH 6.5 buffer and stirred at 40 °C and 600 rpm for 1, 2, 5, 8, 10, 15, 18, and 21 h. A catalytic amount of UDPG (0.05 mM, 2.5% molar equivalent of HQ) was added to prime the reaction, because UDPG is continuously regenerated in situ by SuSy from sucrose and the UDP released during glycosylation.

After each reaction, the enzyme was deactivated by boiling for 10 min, and the mixture was centrifuged at 12,000 rpm at 4 ℃ for 10 min. The supernatant was then filtered using a 0.22 μm filter membrane and detected by HPLC. Each reaction was repeated three times, and three parallel reactions were set for each reaction.

2.4. Directed evolution of AS

Site-directed mutagenesis of AS was conducted via PCR using the pRSFDuet-1-AS plasmid as the template, using primer sets of A343V-FP (GTCTTGGgttCCGCAGACTGAAATTCTTAGCC) and A343V-RP (TCTGCGGaacCCAAGACGGGACCAAAAGAC). The ligated products were transformed into *E. coli* DH5α chemical competent cells, and the transformants were grown for 12 h at 37 ℃ on LB agar. Subsequently, the positive transformant was pooled and cultured overnight, and the plasmid was prepared. The successful introduction of the A343V mutation was verified by Sanger DNA sequencing of the plasmid, confirming that the desired point mutation was introduced before protein expression.

2.5. Analysis

2.5.1. Protein quantification

Protein was quantified using Bradford's method. 1 mL of enzyme solution was mixed with 5 mL of Bradford reagent and incubated at room temperature for 15 min; then, the absorbance was measured at 595 nm. The protein content was estimated using a calibration curve with BSA as a standard [29]. One unit (U) of AS activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of β-arbutin per minute under the standard assay conditions, and one unit (U) of SuSy activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of UDPG per minute from sucrose and UDP under the standard assay conditions. Enzyme activities were determined in triplicate and expressed as U/mg protein.

2.5.2. HPLC analysis

The samples were analyzed by high-performance liquid chromatography (HPLC) using a Thermo Fisher C-18 column (dimensions: 4.6 mm × 150 mm, dp: 5 μm) maintained at 30 ℃. Detection was performed using an ultraviolet detector (UV), and methanol/water (5:95) was used as the mobile phase at a flow rate of 1.0 mL/min for 20 min. HQ and β-arbutin were detected at 220 nm, with typical retention times of 7.98 and 5.38 min, respectively (Figure S1). HQ conversion was defined as $(1-C_{HQ,t}/C_{HQ,0})\times 100\%$, with $C_{HQ,0}$ and $C_{HQ,t}$ denoting the HQ concentrations at reaction initiation and time t.

2.5.3. Kinetic analysis

Kinetic parameters of AS and the A343V variant were determined by measuring the initial reaction rates at varying concentrations of HQ (1–10 mM) at a saturating UDPG concentration, and of UDPG (1–10 mM) at a saturating HQ concentration, in 200 mM pH 6.5 buffer at 40 °C. Reactions were terminated after 30 min. All measurements were performed in triplicate. Data are presented as the mean ± SD. The kinetic constants (Km and kcat) were obtained by fitting the initial-rate data to the Michaelis–Menten equation using nonlinear regression in GraphPad Prism 10.1.

3. Results and discussion

3.1. Optimization of enzymatic reaction conditions

Arbutin synthase (AS) from *Rauvolfia serpentine* and sucrose synthase (SuSy) from *Glycine max* were heterologously expressed in *Escherichia coli* and then purified. The bands at 52 and 92 kDa in Figure 2 demonstrate that the target proteins were successfully translated.

Reaction temperature affects both the activation energy of the reaction and the stability of the enzyme [30]. Therefore, it is necessary to balance these two factors and select the proper reaction temperature. As shown in Figure 3A and C, the highest conversion was obtained at 35 °C for both AS and SuSy. With increasing temperature, enzyme conversion will gradually increase, speeding up the enzymatic reaction. However, when the temperature is higher than 35 °C, the conversion decreases.

This is due to the negative correlation between the enzyme conformation and the activation energy of the reaction. Thus, in follow-up studies, 35 °C was selected as the optimal catalytic temperature for AS and SuSy.

In addition to temperature, the pH of the solution affects enzyme conformation and activity. We therefore examined the effects of a range of pH values from 5.5 to 8.0 (Figure 3B and D). The highest conversion was defined as 100% relative conversion. The highest conversion was recorded at pH 7.5 for AS and pH 6.0 for SuSy. SuSy and AS conversion remained higher than 90% and 70% when the pH value was 6.5; however, SuSy conversion only reached 40% when the pH value was 7.5. Therefore, pH 6.5 was selected for constructing the AS and SuSy cascade reaction system.

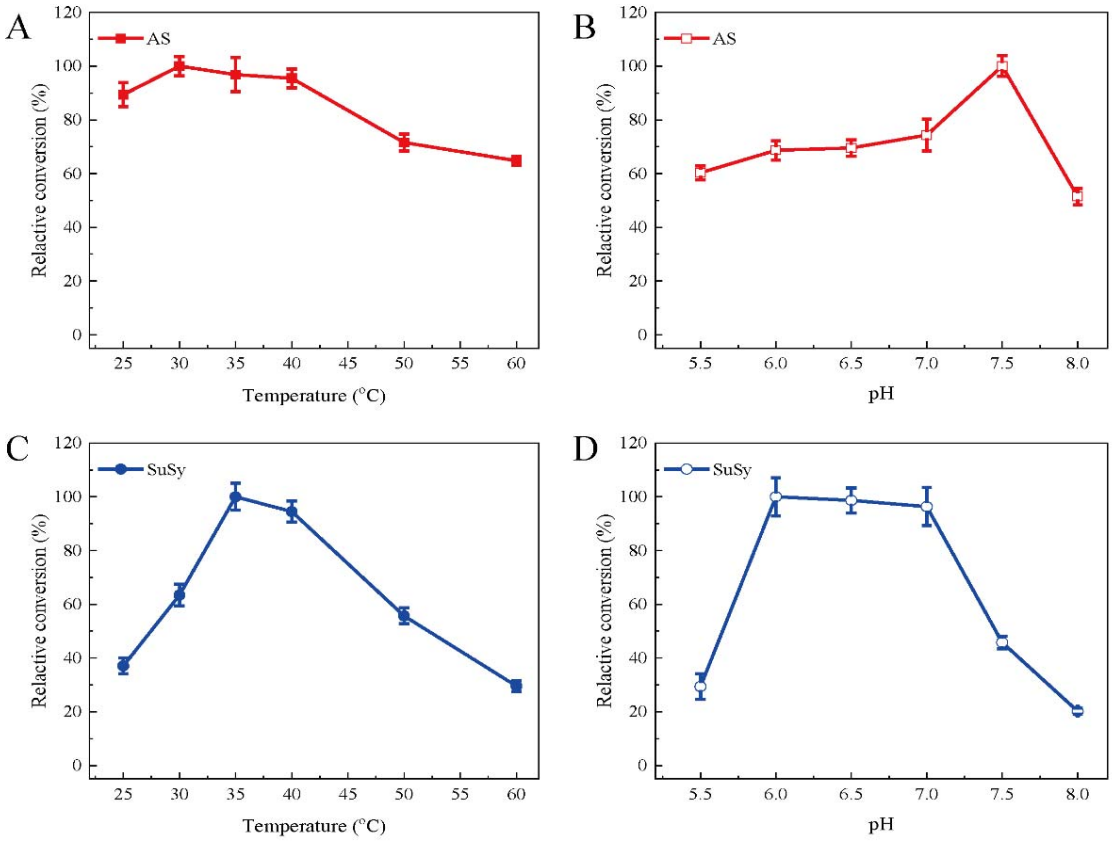


Figure 3. (A, C) Effect of reaction temperature (°C) on the relative conversion catalyzed by AS and SuSy; (B, D) Effect of reaction pH on the relative conversion catalyzed by AS and SuSy. The highest conversion in each panel was defined as 100%.

3.2 Effect of reaction conditions on HQ conversion

UGT-catalyzed glycosylation of HQ requires costly UDPG as a glucosyl donor. To produce β-arbutin in an efficient and economical way, an artificial bi-enzymatic cascade reaction of AS–SuSy was designed and optimized. The enzyme ratio was an important factor in synthesizing a high yield of β-arbutin (Figure 4A). In the cascade reaction, the AS concentration was fixed, and the HQ conversion increased as the AS:SuSy ratio increased to 1:4, reaching a 2-fold greater conversion than that obtained

with a 1:1 ratio of AS:SuSy. When further increasing the AS:SuSy ratio to 1:5, the HQ conversion only increased slightly. Thus, an AS:SuSy mass ratio of 1:4 was used for further experiments.

The amount of enzyme loaded was also a crucial factor in the synthesis of β-arbutin. As shown in Figure 4B, the HQ conversion increased as the amount of AS loaded increased from 1% to 8% (w/v), with the highest HQ conversion obtained at 8%. Further increasing the amount of AS loaded did not increase the HQ conversion. However, when the amount of AS loaded increased past 2%, the HQ conversion increased only modestly. Therefore, to optimize both the amount of enzyme loaded and the resultant HQ conversion, 2% was selected for β-arbutin cascade synthesis.

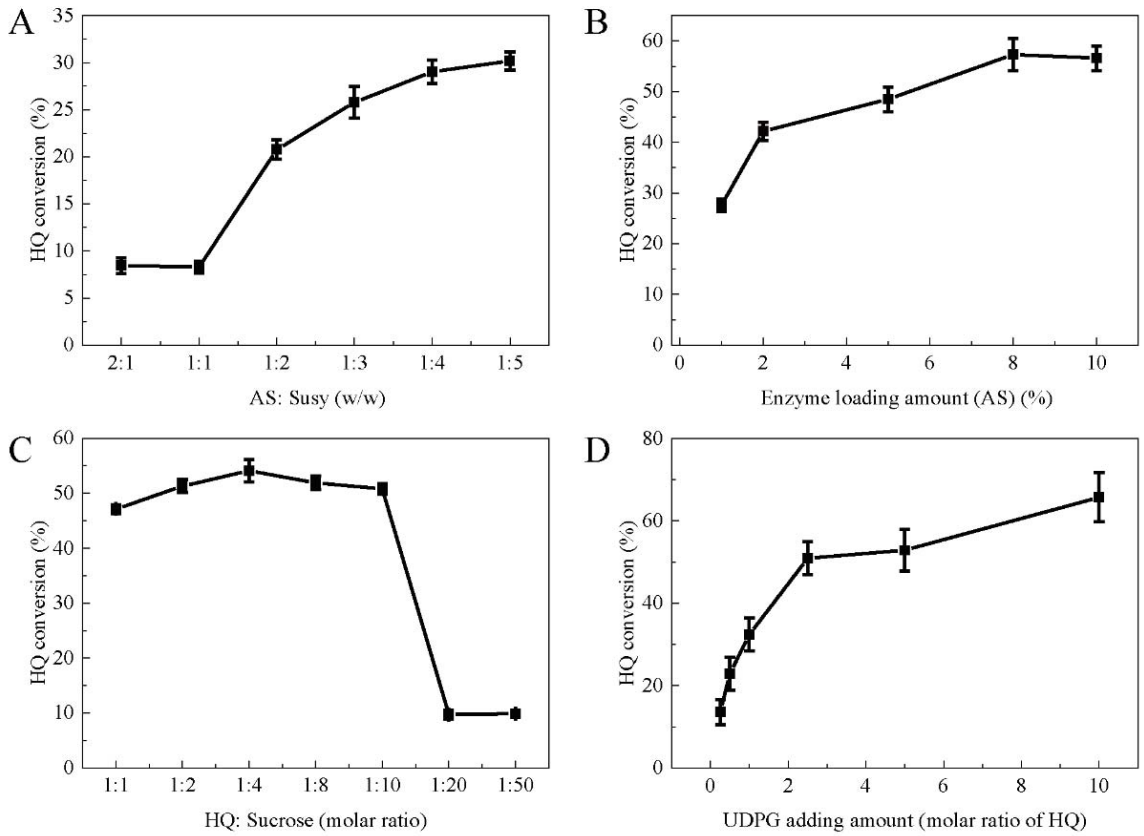


Figure 4. Effect of the reaction conditions on HQ conversion. (A) Effect of the AS:SuSy mass ratio on HQ conversion. (B) Effect of the amount of enzyme loaded on HQ conversion. (C) Effect of the HQ:sucrose molar ratio on HQ conversion. (D) Effect of the initial UDPG:HQ molar ratio on HQ conversion.

The substrate ratio affects the mass transfer of a system [31]. We therefore explored the effect of the HQ:sucrose ratio on HQ conversion. Figure 4C shows that the HQ conversion slowly increased as the HQ:sucrose ratio decreased from 1:1 to 1:4; the conversion sharply decreased when the ratio varied from 1:10 to 1:20. These data demonstrate that higher sucrose concentration affects mass transfer in the system. Therefore, an HQ:sucrose ratio of 1:4 is optimal for β-arbutin synthesis.

The cascade reaction process produces the by-product UDP. SuSy not only catalyzes the regeneration of expensive UDPG but also overcomes the inhibition of AS caused by UDP

accumulation [32]. However, the initial amount of UDPG affects the initial reaction rate. As can be seen in Figure 4D, the effect of the initial amount of UDPG on HQ conversion was initially optimized in the cascade reaction. The HQ conversion rapidly increased when the molar concentration of UDPG ranged from 0.25% to 10% of HQ, illustrating that the UDPG regeneration system is efficient and that the initial amount of UDPG is still important for β-arbutin synthesis. Considering the cost of UDPG, a 2.5% molar content of HQ of the UDPG addition amount was selected for further cascade reaction experiments.

3.3 Site-directed mutagenesis of glycosyltransferase

Enzymes play a fundamental role in biosynthesis [28]. At present, significant progress has been made in enzyme engineering using sequence conservation analysis [33]. To enhance β-arbutin synthesis yield, we performed site-directed mutagenesis on the glycosyltransferase AS. The conserved PSPG box motif is essential for recognizing and binding sugar donors [34], and mutations in the donor-binding site can significantly alter catalytic efficiency. As shown in Figure 5A, docking results indicated that residue A343 of AS interacts directly with the nucleoside group of UDPG, suggesting its key role in catalysis. Therefore, we reasoned that mutating this residue could tune the donor-binding pocket and enhance the catalytic efficiency of AS. Sequence alignment further identified Ala343 as a target for mutagenesis to enhance enzyme activity (Figure 5B). We generated the A343V variant and evaluated its impact on glucosylation. The A343V variant was generated and evaluated for glucosylation; it enhanced HQ conversion 1.25-fold under the same conditions (Figure 5C).

The bi-enzymatic cascade reaction for β-arbutin biosynthesis was carried out using 2 mM HQ, 8 mM sucrose, 20 μg/mL AS or A343V, 80 μg/mL SuSy, and 0.05 mM UDPG in 200 mM pH 6.5 buffer at 40 °C and 600 rpm for 21 h. During the glycosylation process, samples were taken for HPLC analysis to calculate the HQ conversion. As can be seen in Figure 5D, the conversion rate and the equilibrium of A343V were higher than those of AS. The conversion of HQ to β-arbutin reached equilibrium with 98% and 85% conversion, demonstrating that A343V was more suitable for β-arbutin biosynthesis, highlighting that the site-directed mutation of AS improved the catalytic efficiency and demonstrating the compatibility between the UDPG regeneration system and the glycosylation catalyzed by the glycosyltransferase. The number of UDPG regeneration cycles was calculated as the molar amount of β-arbutin produced divided by the initial molar amount of UDPG added (cycles = $[HQ]_0$ × conversion/$[UDPG]_0$, where $[HQ]_0$ = 2 mM and $[UDPG]_0$ = 0.05 mM). Accordingly, the number of UDPG regeneration cycles was 34 (2 × 85%/0.05) and 39 (2 × 98%/0.05) for AS and A343V, respectively. Our data demonstrate that combining glycosyltransferase and sucrose synthase in a bi-enzymatic cascade reaction for β-arbutin synthesis regenerates UDPG in the presence of a catalytic amount of UDPG and overcomes thermodynamic restrictions on β-arbutin formation. Similar results were reported in our previous research [23]. These results confirmed that high conversion and in situ UDPG recycling were retained in a one-pot reaction. Further enhancement of glycosyltransferase activity can maximize UDPG utilization and improve β-arbutin yield. Compared to other UDP-glycosyltransferases coupled to different approaches for regenerating UDPG for the glycosylation of various natural products [18,35], the present method shows obvious advantages in terms of productivity. Compared with recent whole-cell β-arbutin production systems based on *Escherichia coli* [24], *Yarrowia lipolytica* [25], *Komagataella phaffii* [26], and *Corynebacterium glutamicum* [27], which require cell cultivation, substrate uptake, and extensive metabolic engineering to suppress

byproduct formation, the present cell-free AS–SuSy cascade achieves near-quantitative HQ conversion (98%) in a simple one-pot format with high stereoselectivity and easy downstream separation, highlighting its practical advantage for cosmetic-related biomanufacturing.

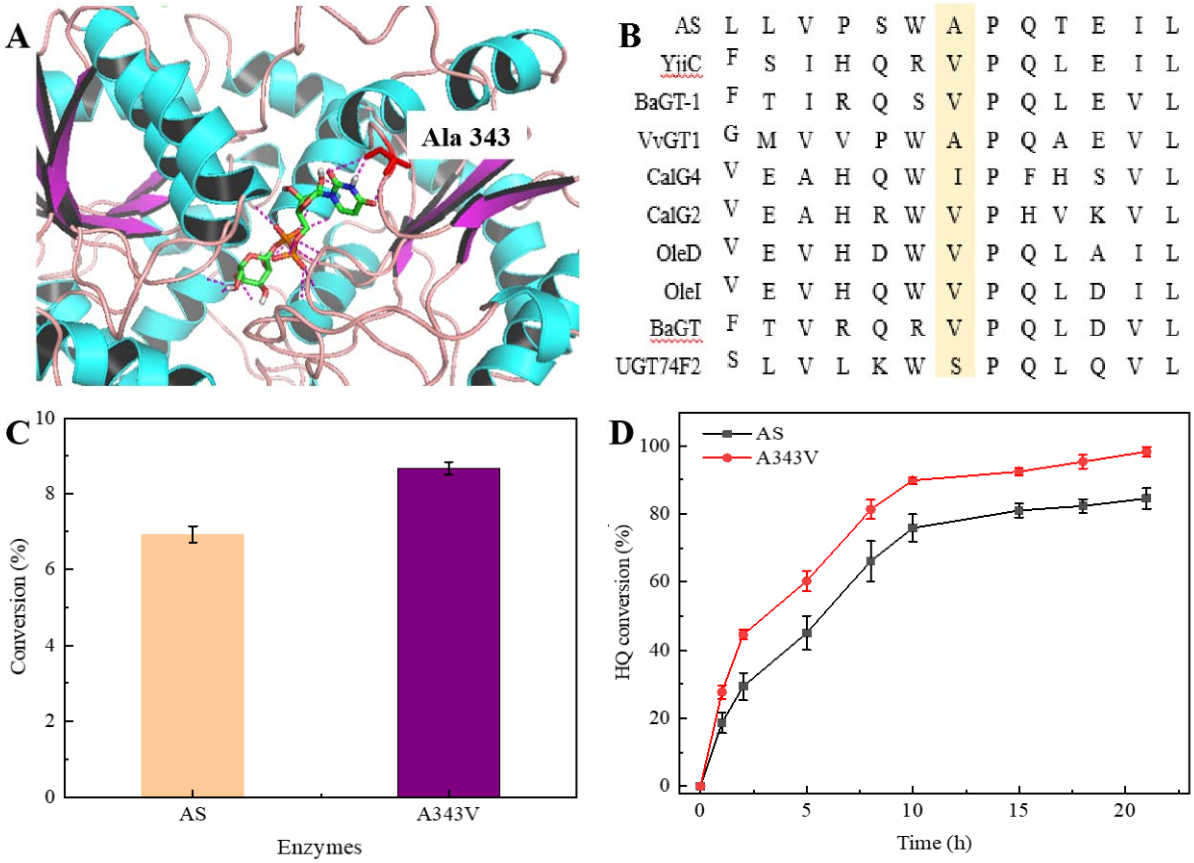


Figure 5. Site-directed mutation of glycosyltransferase. (A) Molecular docking of AS and UDPG. (B) Amino acid sequence alignment of various glycosyltransferases of the PSPG conserved domain. The aligned amino acid sequences are YjiC (WP_003182014.1) from *Bacillus licheniformis*, BsGT-1 (WP_072174202.1) from *Bacillus subtilis*, VvGT1 (AAB81683.1) from *Vitis vinifera*, CalG2(3RSC_A) and CalG4 (3IA7_A) from *Micromonospora echinospora*, OleI (AAC12648.1) and OleD (ABA42119.2) from *Streptomyces antibioticus*, BaGT (ADP31706.1) from *Bacillus atrophaeus*, and UGT74F2 (OAP07463.1) from *Arabidopsis thaliana*. (C) HQ conversion of different types of enzymes. (D) Effect of reaction time on HQ conversion.

Kinetic analysis revealed substantial improvements in the A343V variant (Table 1); the Michaelis–Menten plots used to determine the kinetic constants and all kinetic assays were performed in triplicate, with the data presented as the mean ± SD (Figure S2). Its *Km* for HQ and UDPG decreased from 1.2 to 0.71 μM and from 6.91 to 0.82 μM, respectively, indicating greater substrate affinity, especially for UDPG. The kcat value for HQ and UDPG slowly decreased. The catalytic efficiency (*kcat/Km*) of A343V for HQ and UDPG was increased by 1.2-fold and 1.8-fold, respectively (Table 1). These results highlight the A343V variant as a highly efficient enzyme for β-arbutin biosynthesis.

Table 1. Kinetic constants of AS and the A343V variant toward HQ and UDPG.

Enzyme	Substrate	*Km* (μM)	*kcat* (s^{-1})	*kcat/Km* ($s^{-1}\cdot\mu M^{-1}$)
AS	HQ	1.20 ± 0.24	2.28 ±0.44	1.87 ±0.05
AS	UDPG	6.91 ± 0.93	7.34 ± 0.59	1.06 ± 0.08
A343V	HQ	0.71 ± 0.02	1.64 ± 0.01	2.31 ± 0.31
A343V	UDPG	0.82 ± 0.01	1.58 ± 0.01	1.94 ± 0.03

4. Conclusions

In this study, we successfully developed an efficient bi-enzymatic cascade reaction for the biosynthesis of β-arbutin by constructing a UDPG regeneration system using sucrose synthase (SuSy) and enhancing glycosyltransferase activity through site-directed mutagenesis. The single-point mutant A343V significantly improved the catalytic efficiency of AS, resulting in an increase in β-arbutin synthesis. We achieved an HQ conversion of 98% with 39 in situ UDPG regeneration cycles under optimized conditions. This work demonstrates that integrating UDPG regeneration with enzyme engineering provides a cost-effective and environmentally friendly method for β-arbutin production.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Jinyuan Yan: Project administration, Xi'neng Hou: Data curation, Minghai Han: Data curation, Yanying Zhu: Data curation, Mingjing He: Data curation and Caixia Cui: Project administration, Writing – review & editing, Supervision. All authors read and approved the final version of the manuscript.

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