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Efficient Biosynthesis of Salidroside via Artificial *in Vivo* enhanced UDP-Glucose System Using Cheap Sucrose as Substrate

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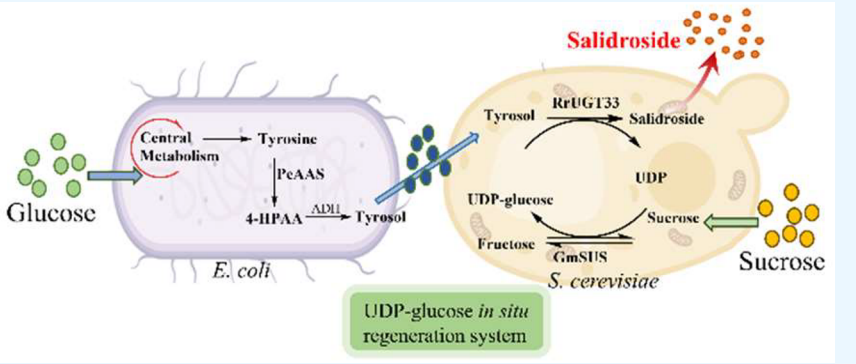

ABSTRACT: Salidroside, a valuable phenylethanoid glycoside, is obtained from plants belonging to the *Rhodiola* genus, known for its diverse biological properties. At present, salidroside is still far from large-scale industrial production due to its lower titer and higher process cost. In this study, we have for the first time increased salidroside production by enhancing UDP-glucose supply *in situ*. We constructed an *in vivo* UDP-glucose regeneration system that works in conjunction with UDP-glucose transferase from *Rhodiola* innovatively to improve UDP-glucose availability. And a coculture was formed in order to enable *de novo* salidroside synthesis. Confronted with the influence of tyrosol on strain growth, an adaptive laboratory evolution strategy was implemented to enhance the strain's tolerance. Similarly, salidroside production was optimized through refinement of the fermentation medium, the inoculation ratio of the two microbes, and the inoculation size. The final salidroside titer reached 3.8 g/L. This was the highest titer achieved at the shake flask level in the existing reports. And this marked the first successful synthesis of salidroside in an *in situ* enhanced UDP-glucose system using sucrose. The cost was reduced by 93% due to the use of inexpensive substrates. This accomplishment laid a robust foundation for further investigations into the synthesis of other notable glycosides and natural compounds.

1. INTRODUCTION

Glycosides, widely distributed and exhibiting significant diversity in plants, represent a prevalent and crucial class of compounds in nature. The utilization of plant secondary metabolites as aglycones for glycoside formation via glycosylation frequently results in substantial modifications to solubility, biological effects, or stability.[1] Salidroside is a polyphenolic compound that is soluble in water derived from *Rhodiola* species with significant biological activities and health-enhancing effects, including antifatigue, antistress, antiradiation, anti-inflammatory, and the ability to prevent cardiovascular, neurodegenerative, and liver diseases.[2−4] *Rhodiola* plants are acknowledged as the primary reservoirs of salidroside.[2] However, the increasing commercial demand for salidroside from natural plants is challenging to meet due to the stringent growth requirements, extended growth cycles, and low salidroside concentration.[5] To tackle this issue, a range of biotechnological methods and microbial engineering techniques has been swiftly advanced to boost the production of salidroside.

Numerous successful tactics involving operational genes have been employed within the field of microbial metabolic engineering to elevate the ultimate titers of tyrosol and salidroside. These include pathway reconstruction through the incorporation of foreign genes, augmentation of metabolic flux by overexpressing or coexpressing pivotal genes, or suppression of metabolic flux.[6−10] By conducting these studies, the

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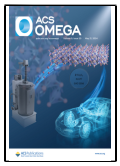

production levels of tyrosol using sugar (such as glucose or a mixture of xylose and glucose) as the carbon source have been significantly enhanced, with titers increasing from 69.08 mg/L to 1.47 g/L.[11,12] Tyrosol titers in *Saccharomyces cerevisiae* were increased to 927.7 mg/L in the shake flask and 8.37 g/L in the bioreactor.[7,8] UGT is the Critical enzyme in the downstream pathway for the transformation from tyrosol to salidroside. Much work has been done to mine efficient glycosyltransferases because suitable UGT genes are not available in either *Escherichia coli* or *Saccharomyces cerevisiae*.[8,13] Torrens-Spence et al. conducted a functional analysis of 34 UGT genes to unveil the entire the synthetic route of salidroside biosynthesis in *Rhodiola*.[14] Consequently, it was discovered that UGTs (RrUGT 17, 29, 32, and 33) exhibited site-specific T8GT activity, converting tyrosol to salidroside, where RrUGT33 demonstrated the most pronounced activity. To date, the highest titers of tyrosol and salidroside were produced in a 5 L bioreactor by collaborative engineering strategy in *S. cerevisiae* metabolic engineering, which were about 10 g/L and 26 g/L, respectively.[15] In addition to regulating genes with specific functions, fusion engineering of microbial coculture is also a technology with great potential. Through the utilization of a coculture strategy, salidroside production increased from 56.9 mg/L to 670.6 mg/L in shake flasks and further escalated to 6.03 g/L in a 5 L bioreactor.[11] Existing studies have focused on increasing salidroside production by increasing tyrosol production and mining efficient UGT enzymes. However, blindly pursuing a high tyrosol titer will produce toxic effects on the strain, negatively affect its growth and metabolism, and ultimately not be conducive to the production of the final product, salidroside. Moreover, it is difficult to fundamentally improve the titer of salidroside by mining only the efficient UGT enzyme without the sufficient sugar donor UDP-glucose.

Within the cytoplasm of the engineered *E. coli*, the inadequate UDP-glucose supply constrained the efficiency of glycosylation. In addition, the low production of glycosylated products was also caused by the absence of biosynthetic pathways for other nucleoside sugars or a significantly inadequate supply. Various approaches have been employed to increase the intracellular UDP-glucose reservoir, including the elimination of competitive genes such as *pgi*, *zwf*, or *ushA*,[16,17] and the upregulation of crucial genes in the UDP-glucose production pathway. Nevertheless, not every gene excessive expression tactic yields favorable outcomes in the buildup of the desired products. For instance, a prior research demonstrated that the concurrent overexpression of *PGM1* and *UGP1* could elevate the UDP-glucose accumulation.[18] But the overexpression of these two genes did not boost the production of salidroside in *S. cerevisiae*.[19] Another effective strategy was the utilization of the exogenous UDP-glucose supply pathway for recruitment.[20] For example, Pei et al. introduced cellobiose phosphorolysis into *E. coli* and reconstructed a novel UDP-glucose synthesis pathway to increase UDP-glucose supply module and improved the bioconversion efficiency of luteolin to isoorientin.[20] Moreover, UDP, a product resulting from enzymatic glycosylation, acts as a strong inhibitor of UGT activity. Consequently, the rate of the glycosylation reaction gradually decreases with the accumulation of UDP in the reaction mixture. Sucrose synthase (SUS), as a sucrose lyase, could promote the reversible conversion of sucrose and UDP to UDP-glucose and fructose.[21] Enzymatic approaches enabling the on-site replenishment of UDP-sugars have garnered significant interest due to their ability to lower the expense associated with UDP-sugar substrates and prevent UDP-induced product inhibition.[22,23] To date, research efforts on salidroside have focused on UGT enzyme expression or optimization of precursor tyrosol production, while little effort has been devoted to the effective production and regeneration of UDP-glucose as a substrate for the glycosylation process. In contrast, an efficient UDP-glucose supply has a very important impact on improving the yield of salidroside.

Traditional microbial biosynthesis depends on the control of a single species of microorganism, a process that poses difficulties, especially when dealing with lengthy and intricate foreign synthetic pathways that heavily strain the metabolic capacity of the host organism.[24] In contrast, through the strategy of dividing the complete synthesis pathway into two strains and cocultivating them (referred to as the division of labor), the metabolic load on each strain can be significantly alleviated.[25−27] This approach provides a simple method for balancing different metabolic modules and has proven effective in the production of numerous natural compounds,[28,29] including paclitaxel and tanshinone precursors,[30] flavanoids,[31,32] and resveratrol.[33] Due to their ease of genetic engineering and swift rate of growth, *E. coli* and *S. cerevisiae* have been widely utilized as standard prokaryotic and eukaryotic hosts for genetic engineering purposes.[34] The choice of host cells was a crucial prerequisite in the modification of microorganisms' metabolism for the synthesis of natural plant compounds.[35] *Saccharomyces cerevisiae* emerges as a eukaryotic organism notably more conducive than prokaryotes for the expression of natural product pathways derived from plants.[36] Attempts have also been undertaken to utilize cross-species cocultures to harness the biosynthetic capabilities of the incorporated microbes for the purpose of biosynthesis. For instance, Yuan et al. utilized *S. cerevisiae-E. coli* cocultures to achieve biosynthesis of resveratrol from glucose.[37] Liu et al. designed *S. cerevisiae-E. coli* cocultures for biosynthesis of hydroxytyrosol.[38] Instead, scant scholarly investigations have been disseminated presently on the subject of salidroside production utilizing the coculture system, and the coculture of *E. coli* and *S. cerevisiae* for salidroside biosynthesis is almost blank.

In cell factories, when the growth of the strain was affected by the substrate, intermediate, or target product, adaptive laboratory evolution (ALE) strategies were commonly employed to mitigate the adverse impact. The ALE method proved to be a simple yet effective approach for enhancing desired phenotypic characteristics in industrial microbial strains.[39] Throughout ALE, microorganisms were exposed to particular stress conditions to induce mutations that enhance their adaptability.[40,41] It has been widely used to generate phenotypes such as increased growth rate,[42] improved substrate utilization,[43] and improved tolerance.[44] Sun et al. used increased ventilation (high oxygen supply) as a selective stress to increase DHA production in the genus Merocytochytrid. DHA titers increased by 30%.[45] In our study, high concentrations of tyrosol had toxic effects on the strains and affected the growth metabolism of the cocultured strains. This strategy could be used to domesticate the strains and improve their tolerance, thereby improving the cooperative ability of the strains.

Here, we described the coexpression of a *SUS* gene from *Glycine max* as *GmSUS*[46] and *UGT* genes from *Rhodiola* as *RrUGT33*[15] in *S. cerevisiae* cells for enhancing the *in vivo*

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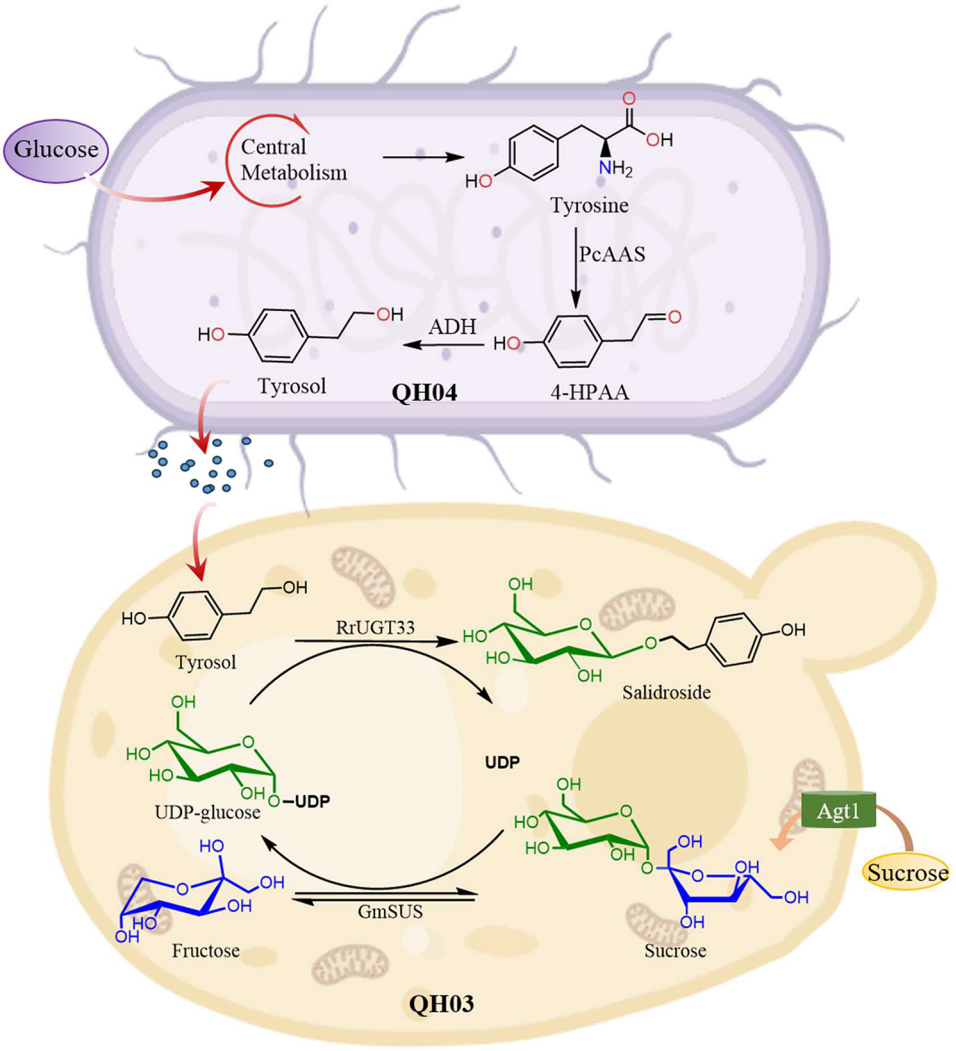

Figure 1. Diagram showcasing the synthetic coculture of *S. cerevisiae* and *E. coli* designed for the production of salidroside using a blend of glucose and sucrose. The AAS enzyme was introduced from the high tyrosine-producing pathway to form *E. coli* QH04 for tyrosyl synthesis. Sucrose synthase GmSUS from *Glycine max* and glycosyltransferase RrUGT33 from *Rhodiola* were introduced into endogenous UDP-glucose-producing *S. cerevisiae* to form strain QH03, which could achieve *in situ* UDP-glucose circulation and salidroside synthesis. QH03 and QH04 strains ingested sucrose and glucose, respectively, and cocultured stably. PcAAS: acetaldehyde synthases enzymes, 4-HPAA: 4-hydroxyphenylacetaldehyde, ADH: alcohol dehydrogenase, UDP: uridine diphosphate, UDP-glucose: uridine 5, 9-diphosphoglucose, RrUGT33: UDP-glucosyltransferase from *Rhodiola*, GmSUS: sucrose synthase from *Glycine max*, Agt1: Alpha-Glucoside Transporter.

synthesis of small molecular tyrosol glucosides. The findings validated that coexpressing SUS enables the on-site regeneration of UDP-sugars, leading to enhanced salidroside production. To synthesize salidroside from cheap feedstock, the acetaldehyde synthetase *AAS* was introduced into the high-leve tyrosine-producing *E. coli* to provide aglycol tyrosyl for the synthesis of salidroside, and the *E. coli*/*S. cerevisiae* coculture system was established (Figure 1). At the same time, to improve the coculture system's stability and titer, the initial inoculation ratio of strains, IPTG induction time and fermentation medium composition were optimized. Moreover, to improve the tolerance of host bacteria to high concentrations of intermediate tyrosol, we also used a laboratory adaptive evolution strategy. In summary, this work described an efficient biosynthesis of salidroside from inexpensive substrate.

2. MATERIALS AND METHODS

2.1. Strains, Plasmids, and Primers. The strains and plasmids used were all detailed in the Supporting Information (Table S1). *E. coli* BL21 (DE3) was employed as the host to produce tyrosol; *S. cerevisiae* BY4743 was used as the host for salidroside production. For gene editing, *E. coli* DH5α (Novagen, USA) was utilized. *PcAAS* (accession number MH710582.1), *GmSUS* (accession number NM001250596.2), and *RrUGT33* (accession number MF674558.1) were codon optimized and chemically synthesized. The nucleotide sequences of the synthesized genes *PcAAS*, *GmSUS*, and *RrUGT33* were listed in Table S2. Primers used in this study are listed in Table S3. *GmSUS* and *RrUGT33* were cloned into the pTEF426 expression vector to yield GmSUS-pTEF426 and RrUGT33-pTEF426, respectively. The recombinant plasmid ptDH3-GmSUS-ptDH3-RrUGT33-pTEF426 carrying *GmSUS* and *RrUGT33* was constructed using the pTDH3 promoter and named PDWZ0320 (Table S1). The plasmid, PDWZ0321,

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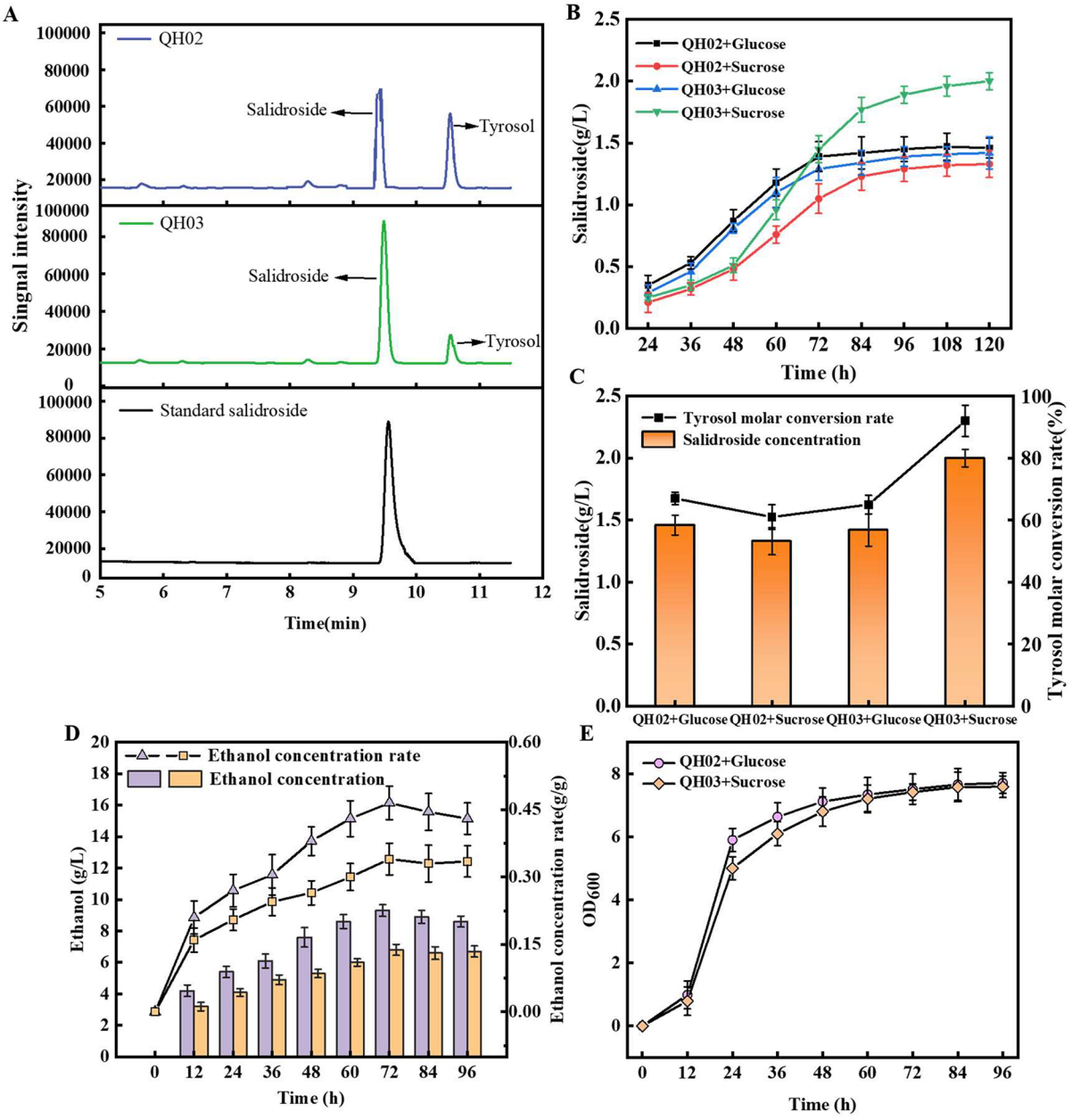

Figure 2. Strain QH02 and QH03 used tyrosol as a substrate to produce salidroside. (A) The HPLC of fermentation broth after 120 h culture. Recombinant QH02 (expressing RrUGT33 alone in *S. cerevisiae*) and QH03(coexpressing GmSUS and RrUGT33 in *S. cerevisiae*) were incubated with tyrosol and sucrose, respectively. (B) Plot of the titer variation of strains QH02 and QH03 using tyrosol as substrate to produce salidroside by fermentation with sucrose and glucose. (C) The final titer of salidroside and molar conversion of tyrosol produced by QH02 and QH03 with different carbon sources. (D) Variation curves of the concentration and conversion rate of ethanol. Purple represents the alcohol and alcohol conversion produced by fermentation of the QH02 strain with glucose. Orange represents the alcohol and alcohol conversion produced by fermentation of the QH03 strain with sucrose. (E) Variation curves of OD600 nm. Purple circles represent the growth of strain QH02 in glucose, and orange squares represent the growth of QH03 in sucrose. The trials were conducted three times, with the error bars indicating the standard deviations.

carrying *PcAAS* was constructed and cloned into pET28a to form PDWZ0321 (Table S1). Then the plasmid GmSUS-pTEF426, RrUGT33-pTEF426, and PDWZ0320 was introduced into *S. cerevisiae* BY4743 to form the novel recombinant QH01, QH02, QH03, respectively (Table S1). The plasmid PDWZ0321 was introduced into *E. coli* BL21 (DE3) to form the novel recombinant QH04. The design of all gene deletion (*hxt1*−*hxt7* and *gal2*) strains was constructed using the method of Reifenberger et al.[47] and further confirmed by PCR.

2.2. Culture Media. Synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids and 2 g/L amino acid drop-out mix). In the coculture system, media containing 50 μg/mL kanamycin and 100 μg/mL ampicillin were utilized as supplements. Medium1:1/2 SC-Ura and 1/2 M9, sucrose: glucose = 1:2 (5 g/L sucrose and 10 g/L glucose); medium 2:1/2 SC-Ura, and 1/2 M9, sucrose: glucose = 1:1 (10 g/L sucrose and 10 g/L glucose); medium 3:1/2 SC-Ura and 1/2 M9, sucrose: glucose = 2:1(10 g/L sucrose and 5 g/L glucose); medium 4:1/2 SC-Ura, and 1/2 M9, sucrose:

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glucose = 3:1 (15 g/L sucrose and 5 g/L glucose); medium 5:1/2 SC-Ura, and 1/2 M9, sucrose: glucose = 4:1 (16 g/L sucrose and 4 g/L glucose). The synthetic fermented medium optimization contained 5 g/L yeast extraction, 10 g/L maltose; the composition of inorganic salts was referred to previous studies.[36]

2.3. Cultivation Condition. To initiate the cultivation of yeast single-strain cultures, monoclones of the engineered BY4743 strain were inoculated into 5 mL of SC-ura medium. The inoculated cultures were then incubated overnight at 30 °C with agitation at 200 rpm to facilitate the growth of the seed cultures. Subsequently, 2% (v/v) of the seed cultures were transferred to 50 mL of fresh SC-ura medium and incubated under identical conditions for 96 h. The medium was supplemented with 2 g/L tyrosol when it was needed to provide the substrate. The engineered *E. coli* BL21 (DE3) monoclonal clones were inoculated in 5 mL M9 medium containing 50 μg/mL kanamycin and 100 μg/mL ampicillin. The inoculation was carried out at 30 °C and 200 rpm overnight. A volume of one mL from overnight cultures was inoculated into 50 mL of M9 medium at 30 °C and agitated at 200 rpm for 4−6 h. Subsequently, 1 mM isopropyl-β-d-thiogalactoside (IPTG) was introduced to trigger gene expression, and the mixture was maintained at 30 °C and 200 rpm for an additional 24 h. The method used to prepare engineered bacterial seeds in the *E. coli*/*S. cerevisiae* coculture system was the same as in monoculture. To investigate the effect of media composition on the titer of salidroside, 2 mL of mixed seed cultures were collected, and QH03/QH04 strains were added at a ratio of 1:1 into 50 mL of five synthetic fermentation media (medium 1−5) supplemented with 50 μg/mL kanamycin and 100 μg/mL ampicillin. The mixture was stirred at 200 rpm for 4 h at 30 °C. Subsequently, 1 mM IPTG was introduced, and the mixture was further cultured at 30 °C with agitation at 200 rpm for 96 h. In order to study the effects of the strain ratio on salidroside productivity, different ratios of QH03 strain to QH04 strain (1:2, 1:1, 2:1, and 3:1) were examined using identical protocols. The fermentation medium chosen was medium 4, IPTG was supplemented 4 h postinoculation in the seed culture, and the fermentation continued for 96 h. To explore the effects of different IPTG addition times (2, 4, 6, and 8 h), the following protocol was established: fermentation was conducted using medium 4, the ratio of QH03 strain to QH04 strain was 2:1, and the fermentation period lasted for 72 h. To explore the effects of different fermentation durations (24, 36, 48, 72, and 96 h), the following protocol was established: utilizing medium 4 as the fermentation medium; maintaining a QH03 strain to QH04 strain ratio of 2:1; and introducing IPTG after 6 h.

2.4. Analytical Methods. To conduct measurements, cells in 1.0 mL of culture were collected through centrifugation at 12000 rpm for 10 min, followed by filtration of the supernatants using a microporous membrane (0.22 μm) and subsequent transfer to sample vials for high-performance liquid chromatography (HPLC). The mobile phase conditions and elution procedures for HPLC were referred to previous studies.[15] In order to measure the quantity of salidroside, standard calibration curves were created by dissolving known concentrations of the standard compounds in a culture medium. Ethanol concentrations in the supernatant of the centrifuged fermentation broth were analyzed as previously described.[48] Glucose content detection methods are described in supplementary methods of Supporting Information. R^2 value for the standard curve was >0.999. All experiments were conducted in triplicate and repeated a minimum of two times. The titers were presented as the average value with a standard deviation.

3. RESULTS AND DISCUSSION

3.1. Efficient Synthesis of Salidroside from Tyrosol by *in Situ* UDP-Glucose Regeneration. UDP-glucose was utilized as the activated glycosyl donor for attaching a glucose molecule to tyrosol at the ethanol hydroxyl group during the synthesis process of salidroside. In order to assess the utilization of UDP-glucose in the *S. cerevisiae* BY4743 cell, the recombinant QH02 (expressing *RrUGT33* in *S. cerevisiae*) was incubated with 2 g/L tyrosol and 20 g/L glucose for the production of salidroside. As shown in Figure 2B, when *RrUGT33* was expressed in strain QH02, the salidroside titer fermentation hardly increased and remained at 1.45 g/L. The insufficient availability of UDP-glucose in the *S. cerevisiae* cell could be the reason for this. GmSUS-pTEF426, RrUGT33-pTEF426 and PDWZ0320 were transformed into *S. cerevisiae* BY4743, and the successfully transformed strains were selected on Ura-deficient medium. Staining gels showed that the molecular sizes of *GmSUS* and *RrUGT33* proteins were approximately 92 kDa and 56 kDa, respectively (Figure S1). The findings showed that *GmSUS* and *RrUGT33* were effectively coexpressed in *S. cerevisiae* BY4743 cells. To verify the action of *GmSUS*, the recombinant QH02 (singly expressed *RrUGT33* in *S. cerevisiae*) and the recombinant QH03 (coexpressed *GmSUS* and *RrUGT33* in *S. cerevisiae*) were coincubated with tyrosol and sucrose, respectively (Figure 2A). The results showed that strain QH03 containing *GmSUS* obtained a higher salidroside titer (Figure 2B).

In the present study, both QH02 and QH03 were able to form salidroside when incubated with tyrosol and glucose or tyrosol and sucrose, respectively (Figure 2B). A small amount of glucose could be detected in the fermentation broths of QH02 and QH03 with tyrosol and sucrose, respectively. This implyed that sucrose did undergo hydrolysis to glucose and fructose before entering the cell.[49] Compared with glucose, the fermentation rate of sucrose was slightly behind, probably because of the hydrolysis process required to utilize the carbon source (Figure 2B). In addition, the salidroside production of strain QH02 gradually slowed after 72 h of fermentation, while the salidroside production of strain QH03 (incubation with sucrose) continued to increase, suggesting that strain QH03 had more sugar donors at the later stage of fermentation. When sucrose was used as the sole carbon source, the final salidroside titer was 1.33 g/L for strain QH02 and 2 g/L for strain QH03. As expected, *GmSUS* was able to enhance the supply of UDP-glucose, thereby increasing salidroside production.

Finally, the highest content of salidroside was obtained when QH03 was incubated with tyrosol and sucrose. The molar conversion rate of tyrosol reached 92%, which was about 30% higher than that of QH02 (Figure 2C). In addition to this, the fermentation of strain QH03 with sucrose produced less alcohol than did the fermentation of strain QH02 with glucose as the carbon source. The conversion rate of alcohol was as low as about 0.3 g/g (Figure 2D). This is QH03 still in the late fermentation with a high productivity of a factor. Strain QH03 grew well in the fermentation of sucrose (Figure 2E), and only a small amount of fructose was detected in the fermentation

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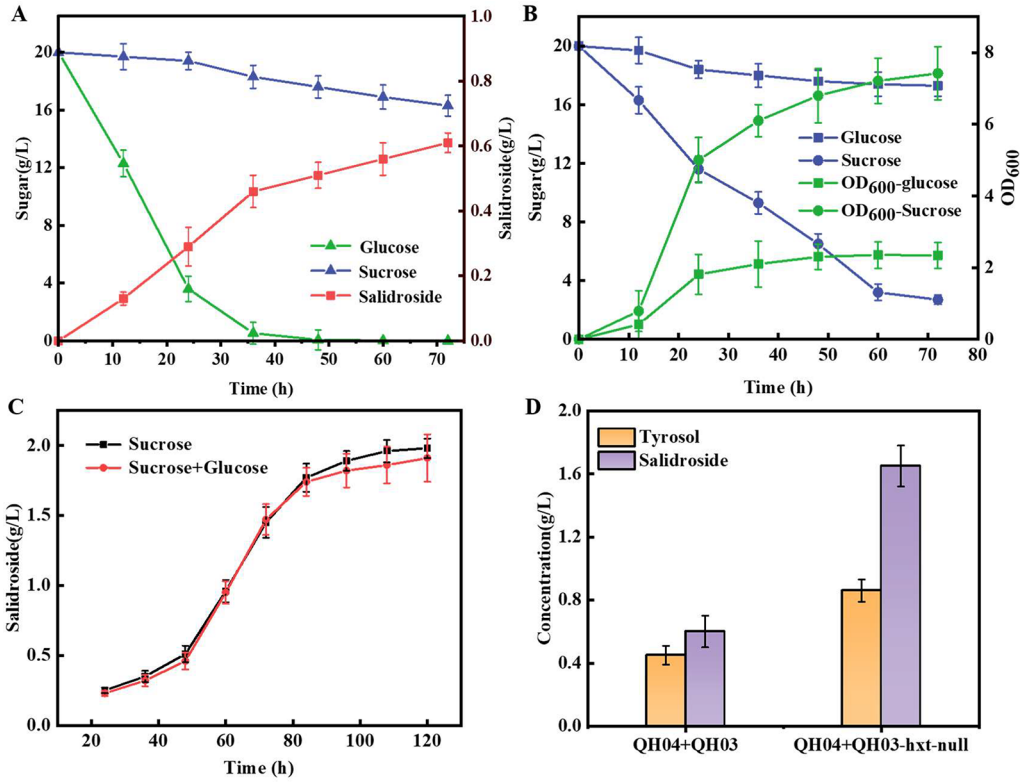

Figure 3. Coculture engineering of the QH03 strain with the QH04 strain. (A) QH03 and QH04 strains were fermented in a sucrose and glucose mixture, sugar consumption comparison, and salidroside production. (B) Comparison of sugar consumption and growth of strain QH03-hxt-null in single glucose and sucrose, respectively. (C) Salidroside titer of QH03-hxt-null on the sole sucrose and mixture of glucose and sucrose. (D) Production of tyrosol and salidroside produced by coculture before and after modification of QH03 strain. The trials were conducted three times, with the error bars indicating the standard deviations.

broth, indicating that the fructose decomposed by sucrose was sufficient to provide carbon source for its own metabolism.

The results indicated that *GmSUS* had the capability to increase the level of UDP-glucose, which was a sugar donor essential for the enzymatic glycosylation reaction. This also reconfirmed the idea that sucrose could be ingested directly by *S. cerevisiae*,[50−54] allowing *in situ* recycling of UDP-glucose to be achieved within the cell. Hence, it was crucial for the microbial synthesis of glycosides to optimize the production of UDP-sugars in the host organisms through a range of regulations and enhancements. The high accumulation of UDP-glucose in microbial hosts was not always guaranteed by the overexpression of endogenous pathway genes, as this overexpression frequently induces a negative cellular response, resulting in repression of gene expression or enzyme deactivation.[55] Glycosyltransferase converted UDP-glucose and aglycons into glycosides and UDP. The activity of glycosyltransferase would be inhibited by UDP, thereby impacting the production of glycosides. The *in situ* recycling strategy of intracellular UDP used in this study could effectively increase the storage of UDP-glucose *in vivo* without affecting the production of glycosides, which has great application prospects for the production of glycoside compounds.

3.2. Construction of a Coculture System Was Used for *De Novo* Synthesis of Salidroside. High biosynthetic capability was required for the efficient *de novo* production of salidroside on a microbial platform. Within *Rhodiola* plants, the crucial intermediate tyrosol was obtained from tyrosine through the AAS-ADH pathway. Recently, the styrene degradation pathway of *Pseudomonas putida* has been utilized for the production of tyrosol.[56] In this pathway, 4-coumarate, derived from tyrosine by the action of tyrosine ammonia-lyase (TAL), undergoes a series of enzymatic reactions catalyzed by ferulate decarboxylase 1 (FDC1), styrene monooxygenase (SMO), and styrene oxide isomerase (SOI) to transform it into the precursor 4HPAA of tyrosol, known as the SMO/SOI pathway. To produce tyrosol through microbial means, *E. coli* required the incorporation of an external pathway. As an illustration, the exogenous TDC-TYO pathway,[57] and the exogenous Ehrlich pathway engineered by *aro10* were implemented.[58] The Ehrlich pathway had a substantial loss of precursors, and the TDC-TYO pathway was highly dependent on the activity of the enzyme. To solve this limitation, some researchers have introduced an exogenous AAS pathway to directly convert tyrosine into 4-HPAA. Studies on plant aromatic amino acid decarboxylases (AAADs) have shown that some AAADs are bifunctional enzymes that can catalyze both decarboxylation and oxidation.[59−61] This AAADs family is called aromatic aldehyde synthase (AASs). AAS converts tyrosine directly to 4-HPAA.[62] Moreover, Chung et al. screened three AAS genes, and *PcAAS* from *Petroselinum crispum* showed the highest catalytic efficiency.[63]

In this study, for the microbial synthesis of tyrosol, we introduced the *PcAAS* from *Petroselinum crispum* into high tyrosine-producing *E. coli* LTy5213 to get QH04 strains and attain a one-step tyrosine to 4-HPAA conversion in the intended host, which produces tyrosol in response to endogenous reductase Figure 1. After supplementation with 20 g/L glucose, their broth supernatants were examined using

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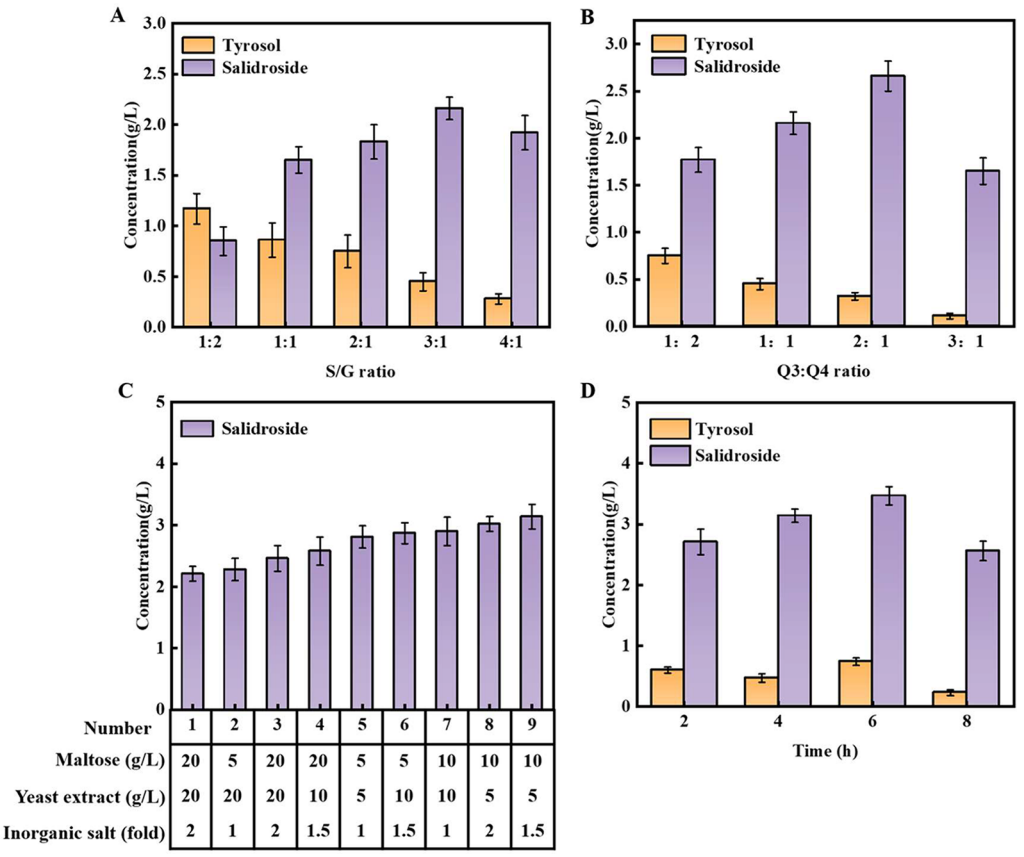

Figure 4. Salidroside production through metabolic balancing of QH03-hxt-null and QH04 strains in a synergistic coculture. (A) Enhancing salidroside production by adjusting the sucrose-to-glucose ratio, starting with an initial S/G ratio of 1/1. (B) Improving salidroside production by adjusting the inoculation ratio of the QH03-hxt-null strain and QH04 strain, using an S/G ratio of 3/1. (C) Optimization of salidroside production by altering the content of maltose, inorganic salt mixture, and yeast extract with the S/G ratio of 3/1 and initial Q3/Q4 ratio of 2/1. (D) Optimization of salidroside production by altering the induction time of IPTG. The trials were conducted three times, with the error bars indicating the standard deviations.

HPLC. The outcomes demonstrated that PcAAS expression in strains QH04 triggered the tyrosol's biosynthesis (Figure S2).

A coculture system involving *S. cerevisiae* and *E. coli* was developed for the *de novo* synthesis of salidroside. In this system, the QH04 strain was used to produce tyrosol, while the QH03 strain (an engineered BY4743 yeast coexpressing GmSUS and RrUGT33) was responsible for converting tyrosol into salidroside (Figure 1). In this study, salidroside was successfully synthesized *de novo* by coculturing tyrosol producing strain QH04 with engineered yeast QH03 and adding sucrose and glucose respectively at a final concentration of 20 g/L in the medium mixed 1:1 with M9 medium and SC-URA medium (Figures S3 and S4). We found that the glucose content in the fermented liquid was extremely low, and sucrose abound (Figure 3A). Therefore, it was inferred that the strains in the coculture system preferentially consumed glucose, which resulted in carbon source competition, resulting in a low salidroside titer of 0.6 g/L. We modified the QH03 strain based on previous studies. Batista et al. found that,[64] a yeast strain deficient in key hexose transporters (*hxt1*−*hxt7* and *gal2*) was impotent of growing on or fermenting glucose. The hxt-null strain remained capable of fermenting sucrose through direct absorption of sucrose into the cells. We constructed strain QH03-hxt-null by deleting *hxt1*−*hxt7* and *gal2* in strain QH03, and expected to have minimal uptake of glucose. As shown in Figure 3B, when the QH03-hxt-null strain was provided with glucose as the only carbon source, it exhibited slow consumption, and no additional consumption was noted after 24 h. In other words, strain QH03-hxt-null grew very poorly on only glucose (Figure 3B). In comparison to glucose alone, strain QH03-hxt-null utilized sucrose more rapidly and grew better (Figure 3B). Additionally, we examined how different carbon sources impact the production of salidroside. This was shown in Figure 3C, where strain QH03-hxt-null produced 1.91 g/L of salidroside in the surcose and glucose mixture, comparable with that in the sole surcose. When strains QH04 and QH03-hxt-null were incubated again with a mixed carbon source (glucose: sucrose = 1:1), both tyrosol and salidroside production increased significantly, and salidroside production increased from 0.6 to 1.65 g/L, as shown in Figure 3D.

In contrast to the previous QH03 strain, which produced salidroside (2.0 g/L) using glucose as a carbon source, deleting both *hxt1-hxt7* and *gal2* of QH03 had no obvious effect on the production of salidroside. It has been shown that this hxt-null strain did not have much of a negative effect on metabolism because of the direct intake of sucrose into the cells. When two strains were cocultivated on the same carbon source, competition for growth would lead to the incompatibility and instability of the consortium.[65] Implementing the strategy of engineering the coculture strains to utilize distinct carbon sources, such as glucose or sucrose, aimed to decrease growth

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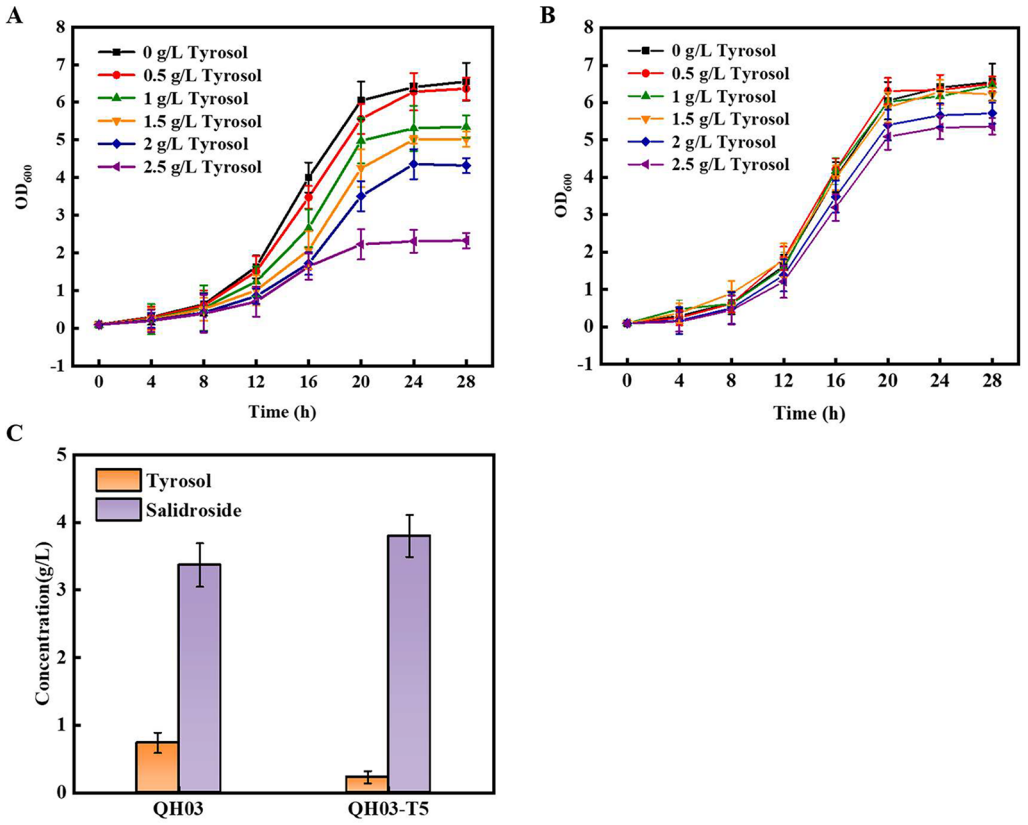

Figure 5. Test of cytotoxicity of tyrosol to *S. cerevisiae*. (A) Effect of tyrosol at different concentrations on the growth of QH03-htx-null. (B) Tolerance of QH03-T5 strain to tyrosol after domestication. (C) Comparison of salidroside titer of QH03 strain and QH03-T5 strain. The trials were conducted three times, with the error bars indicating the standard deviations.

competition and enhance growth compatibility. The production of tyrosol and salidroside in the modified coculture system was increased, which again proved the importance of strain compatibility in coculture.

3.3. To Optimize the Coculture System for *De Novo* Salidroside Production. In order to increase the titer of *de novo* synthesis of salidroside, we optimized the coculture conditions. Based on previous research, The performance of the microbial consortia was greatly impacted by the formulation medium's composition.[66−68] When engineering the QH03-hxt-null and QH04 strains in the symbiotic coculture to utilize glucose and sucrose with varying efficiencies, our initial focus was on adjusting the metabolic capabilities of the two strains by manipulating the sucrose-to-glucose ratio (referred to as the S/G ratio). A sugar blend with varying sucrose and glucose proportions was examined using an inoculation ratio of 1:1 for the QH03-hxt-null and QH04 strains. When the S/G ratio was raised in the product profile (Figure 4A), there was a decrease in tyrosol accumulation, while salidroside production increased rapidly until the S/G ratio reached 3/1, resulting in the attainment of a high salidroside titer of 2.16 g/L. Increasing the S/G ratio to 4/1 not only reduced the accumulation of tyrosol but also hindered the production of salidroside.

Next, we tried to adjust the initial inoculation ratio between the QH03-hxt-null and QH04 strains in the coculture system. Various ratios of QH03-hxt-null and QH04 strains (referred to as the Q3/Q4 ratio, ranging from 1/2 to 3/1) were introduced into the coculture system at a S/G ratio of 3/1 (15 g/L sucrose and 5 g/L glucose) and cultivated together. As depicted in Figure 4B, when the initial Q3/Q4 ratio was 2/1 in the coculture, the production of salidroside reached 2.66 g/L with minimal accumulation of tyrosol, representing 123% salidroside and 71% tyrosol compared to those with a Q3/Q4 ratio of 1/1. A significant hindrance in the production of salidroside was observed with further expansion of the initial subpopulation of the QH03-hxt-null strain. In *S. cerevisiae*, direct sucrose intake was linked to maltose metabolism.[64] Indeed, higher sucrose fermentation rates have been observed by them when the S. cerevisiae-hxt-null strain was pregrown on maltose. Therefore, maltose was used as an additive component of the medium to improve sucrose uptake by strain QH03-hxt-null. To further optimize the medium, the inoculation ratio and carbon source ratio were determined to be 2:1 and 3:1, respectively. The effects of maltose, an inorganic salt mixture, and yeast extract on titer were explored using orthogonal studies. The ninth condition, which consisted of 10 g/L maltose, 5 g/L yeast extract, and 1.5 times the inorganic salt content, yielded the highest salidroside production at 3.14 g/L after 120 h, marking a 20% improvement compared to the original culture condition (Figure 4C). The significant impact of maltose and inorganic salts on salidroside production was clearly evident. As the quantity of yeast extract demonstrated an inverse relationship, the concentration of yeast extract was maintained at 5 g/L, maltose at 10 g/L, and inorganic salt at 1.5 times the original level.

Furthermore, the timing for initiating IPTG induction was fine-tuned to stimulate the expression of the *PcAAS* gene, facilitating the effective conversion of tyrosine into tyrosol. To enhance tyrosol bioproduction, various timings for initiating

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IPTG induction (ranging from 1 to 8 h) were examined to determine the optimal time that promotes the synchronized growth and biosynthetic capabilities of the QH03-hxt-null strain−QH04 strain consortia. The coculture platform, as depicted in Figure 4D, produced the highest salidroside (3.47 g/L) after 120 h of fermentation when IPTG induction was initiated at 6 h. The production of salidroside increased by 10% compared to 3.14 g/L when the initial induction time was 4 h. Initiating IPTG induction either too soon or too late could result in heightened metabolic strain or unintended expression, potentially impeding tyrosol production.

Consistently, a primary goal in microbial coculture engineering has been to improve the regulation of population dynamics and behavior. This was due to the highly dynamic nature of the interactions between strains and the considerable difficulty of maintaining balanced growth and metabolic function.[69] The proportion of strain composition, the nutrient composition of the medium, and the carbon source are the key factors affecting the stability of the coculture system. Two strains sharing and consuming the same carbon source can lead to unexpected competitive interactions and unstable cocultures during the industrial fermentation of natural products. Nutritional differences in cocultures can reduce or eliminate competitive exclusion and make coexistence possible. In this study, strain QH03 was modified so that *E. coli* and *S. cerevisiae* would not compete with glucose. Finally, the production of salidroside was increased from 0.6 to 1.65 g/L, and the production was increased by 64%. The carbon source ratio (S/G) was adjusted to increase the production by 31%. The adjustment of the strain ratio (Q3/Q4) increased the production by 23%; the adjustment of the medium composition increased the production by 20%; and the regulation of IPTG increased the production by 10%. Therefore, in the coculture system, the most critical factor affecting the production of salidroside was the competition of carbon sources. These findings demonstrate that the synergistic interaction between QH03-hxt-null and QH04 strains was compatible, resulting in a robust coculture enabling high salidroside titer.

3.4. Adaptive Laboratory Evolution Strategy to Improve the Tolerance of Host Bacteria. If the desired product or intermediate is toxic to the host cell, an imbalance in the metabolism of the cell factory may lead to inefficient microbial synthesis. Considering the growth rate of *E. coli* was faster than that of *S. cerevisiae*, the tyrosol produced by *E. coli* would accumulate during yeast growth, which would affect the growth of yeast. To evaluate the cytotoxic effects of tyrosol on *S. cerevisiae*, different quantities of tyrosol were introduced into the QH03-hxt-null strain cell cultures, resulting in final concentrations ranging from 0 to 2.5 g/L. As depicted in Figure 5A, a significant reduction in QH03 cell growth was noted at concentrations exceeding 1 g/L. The OD_{600} value at 2.5 g/L of tyrosol showed a 60% decline compared to the control (0 g/L of tyrosol concentration), suggesting that the growth of QH03 cells was impeded by the elevated tyrosol concentration.

The ALE strategy proved to be effective in enhancing the tolerance of host bacteria.[70,71] We employed a stepwise ALE strategy to develop novel yeast strains that can grow normally at high concentrations of tyrosol. In the first stage, the cells were subjected to ten serial passages in growth medium containing 0.5 g/L tyrosol as the substrate. When the optical density at 600 nm (OD_{600}) reached 4, which corresponds to the middle and late stages of logarithmic growth, it was selected as the transfer point for subsequent rounds of adaptive evolution. At this point, the yeast culture, with an OD_{600} nm of 4, was transferred to a new culture flask with a tyrosol concentration of 1 g/L. In the second stage, the tyrosol concentration was further increased to 1.5 g/L after 20 passages in 1 g/L tyrosol. This process was repeated with successive increases of 0.5 g/L tyrosol until the adapted yeast strain, QH03-T5, reached a maximum OD_{600nm} of 5 at a concentration of 2.5 g/L (Figure 5B). Cocultivation of the domesticated QH03-T5 strain with QH04, using sucrose and glucose as substrates, led to about a 10% increase in salidroside titer (3.8 g/L) after 120 h compared to the initial condition, as illustrated in Figure 5C. Cocultivation of the QH03 strain with the QH04 strain reduced the cost by 93% compared with the QH02 strain (Table 1).

Table 1. Comparison of the Cost with Different Strains[a]

Strains	Substrates	Cost (dollar per kilogram salidroside)	Tyrosol utilization (%)	Salidroside titer (g/L)	Cost saving (%)
QH02	Glucose, Tyrosol	449	67%	1.46	0
QH03	Sucrose, Tyrosol	328	92%	2.0	27
QH03 and QH04	Sucrose, Glucose	30	-	3.8	93

[a]Strain QH02 was fermented with 20 g/L glucose and 2 g/L tyrosol for 120 h to obtain the cost of 1 kg salidroside for comparison. The QH03 strain was incubated with 20 g/L glucose and 2 g/L tyrosol for 120 h. The QH03 and QH04 coculture systems were fermented with 15 g/L sucrose and 5 g/L glucose as substrates for 120 h.

In this study, after ALE, the maximum optical density (OD_{600}) value of yeast was significantly increased at a concentration of 2.5 g/L tyrosol, leading to about a 10% increase in the final salidroside titer. These findings indicated that employing an ALE microbial community strategy involving mutually beneficial cross-feeding could enhance the production of desired compounds.

4. CONCLUSION

In this study, a multistrategy approach was employed to improve the production of salidroside in microorganisms. First, the supply of UDP-glucose was enhanced by coupling the UDP-glucose regeneration system with UDP-glucosyltransferase from *Rhodiola* (RrUGT33) to establish an *in situ* UDP-glucose recycling system for the glucosylation of tyrosol using inexpensive sucrose as the substrate. The molar conversion of tyrosol reached 92%, resulting in salidroside production reaching 2.0 g/L at the shake flask level. Additionally, a coculture system for salidroside production was developed by leveraging the interaction between *E. coli* and *S. cerevisiae*, with sucrose and glucose serving as carbon sources. The distribution of the artificial biosynthetic route of salidroside occurred between strains QH03 and QH04. QH03 carried the *GmSUS* and *RrUGT33* genes responsible for UDP-glucose and salidroside biosynthesis, while QH04 contained the *PcAAS* genes responsible for tyrosol biosynthesis. To optimize salidroside production, an orthogonal design was employed to develop synthetic media in this study. The inoculum size and proportion were also taken into consideration, leading to an increase in product concentration to 3.47 g/L. Furthermore, the tolerance of host bacteria to the intermediate tyrosol was

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enhanced through laboratory adaptive evolution, resulting in higher salidroside production, which reached 3.8 g/L at the shake flask level. Notably, this study successfully implemented the recycling of *in situ* UDP-glucose *in vivo* to compensate for the inadequate supply of UDP-glucose. Moreover, it was the first time that sucrose was utilized as a carbon source for the production of a high-value product, salidroside. Further research is required to investigate the efficient utilization of sucrose for salidroside production. These findings suggested that the combination of microbial coculture and *in situ* UDP-glucose enhancement holds significant potential for future salidroside production. Additionally, it provided valuable insights into the production of other glycosidated products.

■ ASSOCIATED CONTENT

Data Availability Statement

All data produced or examined in this study are incorporated within this published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02060.

Supporting tables (Tables S1−S3), supplementary method, and supporting figures (Figures S1−S4) (PDF)

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Author Contributions

D.W. conceived and supervised the project. Xj.Z. and Xx.Z. designed and performed the main experiments. Data was evaluated by all authors. Xj.Z. wrote the manuscript, D.W., Z.Q., and R.L. revised the manuscript. All authors have reviewed and endorsed the final manuscript.

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Notes

The authors declare no competing financial interest.

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