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Multienzyme cascade for synthesis of hydroxytyrosol via engineered *Escherichia coli*

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Hydroxytyrosol, a fine chemical, is widely utilized in food and pharmaceutical industries. In this study, we constructed a pathway to produce hydroxytyrosol by co-expressing tyrosin-phenol lyase (TPL), L-amino acid dehydrogenase (aadL), α-keto acid decarboxylase (KAD), aldehyde reductase (yahK) and glucose dehydrogenase (gdh). We changed combinations between plasmids with different copy numbers and target genes, resulting in 84% increase in hydroxytyrosol production. The yield of hydroxytyrosol was further increased 89.3% by optimizing the temperature and pH. Finally, 55.3 mM hydroxytyrosol was produced within 14 h by fed-batch biotransformation. This study provides a novel approach for hydroxytyrosol production.

Keywords Hydroxytyrosol, *Escherichia coli*, Cascade reaction, Whole cell biotransformation

Hydroxytyrosol is a potent antioxidant and mainly distributed in olive leaves and fruits. Hydroxytyrosol has a variety of effects in preventing diseases and improving human health, such as anti-proliferative, lipid regulation, anti-inflammatory and prevention of cardiovascular diseases[1–5].

Hydroxytyrosol can be obtained through the extraction of wastewater generated during the production of olive oil and is up to 98% in the waste water[6]. Extracting hydroxytyrosol from olive oil wastewater has many defects, mainly facing the difficulty of extraction, high cost and the complex extraction process. Hydroxytyrosol can be produced from catechol by chemical process[7]. However, chemical production of hydroxytyrosol has some disadvantages, such as high production cost, use of heavy metal ions that pollute the environment and harsh reaction conditions. Hydroxytyrosol can also be produced by photocatalysis[8]. However, photocatalysis has not been widely used in industrial production due to low conversion rate and concentration of the final product. At present, multienzyme cascade reactions have been employed for the synthesis of many compounds due to the mild reaction conditions, strong catalytic specificity and high efficiency[9]. Biotechnology shows great industrial application prospect with the enhancement of environmental protection awareness.

Many research groups have developed numerous biological methods for production of hydroxytyrosol. Metabolic engineering is an effective method to produce hydroxytyrosol. Li et al. proposed a method to produce hydroxytyrosol by co-expressing ketoacid decarboxylase, alcohol dehydrogenase, HpaB/HpaC and transamination. Assembly of the full pathway resulted in 0.64 g/L hydroxytyrosol[10]. Liu et al. optimized the biosynthesis of hydroxytyrosol by screening effective HpaB/HpaC combinations, biosynthesis flux recombination and cofactor engineering. Finally, the engineered strain produced 6.97 g/L hydroxytyrosol[11]. Wang et al. achieved the biosynthesis of hydroxytyrosol by introducing HpaB/HpaC from *Escherichia coli* and ARO10 and ADH6 from *Saccharomyces cerevisiae*. After the optimization of carbon source, pH and seed medium, the optimal engineered strain produced 9.87 g/L hydroxytyrosol[12].

To simplify the production steps and increase the yield of hydroxytyrosol, whole cell catalysis method has been widely developed. Pinero et al. built engineered strains using hydroxylase to convert tyrosol into hydroxytyrosol[13]. Wijata et al. reported a method for converting 3,4-dihydroxyphenylacetic acid to hydroxyltyrosol by overexpressing carboxylic acid reductase, phosphopantetheinyl transferase and aldehyde reductase[14]. Chen et al. established a method to produce hydroxytyrosol with tyrosine as substrate. To increase

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the yield of hydroxytyrosol, the catalytic activity of HpaB was improved through high-throughput screening. Finally, the yield of hydroxytyrosol was 5.6 mM within 36 h with a conversion yield of 93 mol%[15]. Yao et al. then increased tyramine oxidase activity by using in vivo-directed evolution. Finally, the yield of hydroxytyrosol was 13.5 mM within 36 h with a conversion yield of 95 mol%[16]. Li et al. constructed an engineered strain, which transformed L-3,4-dihydroxyphenylalanine (L-DOPA) to hydroxytyrosol. After optimizing the transformation conditions, the yield of hydroxytyrosol reached 36.3 mM within 10 h with a conversion yield of 89.4 mol%[17]. These methods are inefficient in producing hydroxytyrosol or the selected substrate is expensive. Therefore, there is still a need to develop more economical and efficient methods for production of hydroxytyrosol.

In the present study, we constructed engineered strains for production of hydroxytyrosol by introducing heterologous genes, including tyrosin-phenol lyase (*TPL*), amino acid dehydrogenase (*aadL*), α-keto acid decarboxylase (*KAD*), aldehyde reductase (*yahK*) and glucose dehydrogenase (*gdh*) (Fig. 1).

Materials and methods

Pathway and plasmid construction

The codon-optimized genes of glucose dehydrogenase (*gdh*) and L-amino acid deaminase (*aadL*) were obtained from previous studies[18]. Tyrosin-phenol lyase (*TPL*) (GenBank ID: AAL94078) was amplified from the genome of *Fusobacterium nucleatum* ATCC25586[19]. α-keto acid decarboxylase (*KAD*) (GenBank ID: KY441412) was amplified from the genome of *Proteus mirabilis* JN458[20]. Aldehyde reductase (*yahK*) (GenBank ID: AAC73428) was amplified from the genome of *E. coli* BL21 (DE3)[21].

All constructed plasmids were sequenced by Tianlin (Wuxi, China). All primers used for constructing plasmids are listed in Table S1. The plasmids and recombinant strains are listed in Table S2. The *KAD* was amplified from *Proteus mirabilis* JN458 using *KAD-Bam*HI (F) and *KAD-Sac*I (R) and cloned into pRSFDuet-1 between *Bam*HI and *Sac*I, the successfully constructed plasmid was named pRSF-*KAD*. The *yahK*, *TPL* and *aadL* was amplified using *yahK-Kpn*I (F) and *yahK-Xho*I (R), *TPL-Kpn*I (F) and *TPL-Xho*I (R), *aadL-Kpn*I (F) and *aadL-Xho*I (R). All fragments were cloned into pRSF-*KAD* between *Kpn*I and *Xho*I, the successfully constructed plasmids were named pRSF-*KAD-yahK*, pRSF-*KAD-TPL* and pRSF-*KAD-aadL*, respectively. Furthermore, *yahK* was amplified using *yahK-Sac*I (F) and *yahK-Not*I (R). The fragments were cloned into pRSF-*KAD-TPL* and pRSF-*KAD-aadL* between *Sac*I and *Not*I, respectively. The successfully constructed plasmids were named pRSF-*KAD-yahK-TPL* and pRSF-*KAD-yahK-aadL*, respectively.

Additionally, *TPL*, *yahK* and *aadL* was amplified using *TPL-Bam*HI (F) and *TPL-Sac*I (R), *yahK-Bam*HI (F) and *yahK-Sac*I (R), *aadL-Bam*HI (F) and *aadL-Sac*I (R). The fragments were cloned into pETDuet-1 between *Bam*HI and *Sac*I. The successfully constructed plasmids were named pET-*TPL*, pET-*yahK* and pET-*aadL*, respectively. The *aadL* was amplified using *aadL-Kpn*I (F) and *aadL-Xho*I (R). The fragments were cloned into pET-*TPL* and pET-*yahK* between *Kpn*I and *Xho*I, respectively. The successfully constructed plasmids were named pET-*TPL-aadL* and pET-*yahK-aadL*, respectively. The *yahK* was amplified using *yahK-Kpn*I (F) and *yahK-Xho*I (R). The fragments were cloned into pET-*TPL* between *Kpn*I and *Xho*I. The successfully constructed plasmid was named pET-*TPL-yahK*. The *gdh* was amplified using *gdh-Kpn*I (F) and *gdh-Xho*I (R). The fragments were cloned into pET-*aadL* and pET-*TPL* between *Kpn*I and *Xho*I, respectively. The successfully constructed plasmids were named pET-*aadL-gdh* and pET-*TPL-gdh*, respectively. The *gdh* was amplified using *gdh-Bam*HI (F) and *gdh-Sac*I (R). The fragments were cloned into pCDFDuet-1 and pACYCDuet-1 between *Bam*HI and *Sac*I, respectively. The successfully constructed plasmids were named pCDF-*gdh* and pACYC-*gdh*, respectively. The *TPL* was amplified using *TPL-Kpn*I (F) and *TPL-Xho*I (R). The fragments were cloned into pCDF-*gdh* between *Kpn*I and *Xho*I. The successfully constructed plasmid was named pCDF-*TPL-gdh*.

The resulting plasmids were transformed into *E.coli* BL21(DE3), the following strains were successfully constructed: strain 1 (pRSF-*KAD-yahK*/pET-*TPL-aadL*/pCDF-*gdh*), strain 2 (pRSF-*KAD-TPL*/pET-*yahK-aadL*/pCDF-*gdh*), strain 3 (pRSF-*KAD-aadL*/pET-*TPL-yahK*/pCDF-*gdh*), strain 4 (pRSF-*KAD-aadL*/pET-*TPL-yahK*/pACYC-*gdh*), strain 5 (pRSF-*KAD-aadL*/pET-*TPL-yahK-gdh*), strain 6 (pRSF-*KAD-aadL-TPL*/pET-*yahK-gdh*), strain 7 (pRSF-*KAD-aadL-yahK*/pET-*TPL-gdh*) and strain 8 (pRSF-*KAD-aadL-yahK*/pCDF-*TPL-gdh*).

Culture of *Escherichia coli* and preparation of cell extracts

The recombinant *Escherichia coli* was inoculated into 3 mL terrific broth (TB) medium at a volume fraction of 0.1% and incubated at 37 °C, 220 rpm, 12 h. Subsequently, the inoculum was transferred to fresh 50 mL TB medium at a volume fraction of 1%, and the culture was further incubated at 37 °C, 220 rpm until the OD_{600} reached 0.5, and then isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to achieve a final

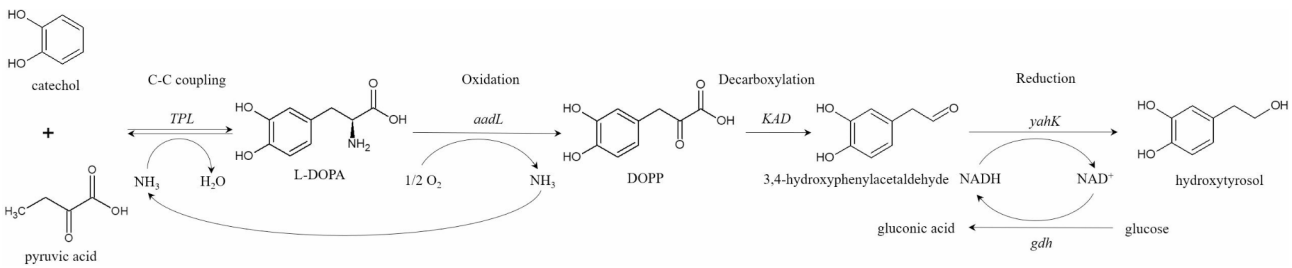

Fig. 1. schematic representation of the cascade reaction to produce hydroxytyrosol.

concentration of 0.4 mM. The strain was cultured at 20 °C, 220 rpm, 24 h. Cells were harvested by centrifugation (4 °C, 8000 g) and washed twice with phosphate buffer (50mM). Then phosphate buffer was added to resuspend the cells until the OD_{600} reached 30. The ultrasonic fragmentation was used to lyse cells. Cell debris was removed by centrifugation (4 °C, 8000 g, 2 min) and the supernatant containing the crude enzyme was collected.

Enzyme activity test

To determine the activity of TPL, the reaction mixture (50 mL) based on 1 mL TPL, 10 mL aadL, 10 mL KAD, 10 mL yahK, 10 mM NADH, 10 mM catechol, 10 mM pyruvic acid, 10 mM ammonium chloride. To determine the activity of aadL, the reaction mixture (50 mL) based on 1 mL aadL, 10 mL KAD, 10 mL yahK, 10 mM NADH, 10 mM L-DOPA. To determine the activity of KAD, the reaction mixture (50 mL) based on 10 mL yahK, 10 mM NADH, 10 mM DOPP. To determine the activity of yahK, the reaction mixture (50 mL) based on 10 mM NADH, 10 mM 3,4-hydroxyphenylacetaldehyde. The concentration of hydroxytyrosol was detected by high performance liquid chromatography (HPLC). To determine the activity of gdh, the reaction mixture (50 mL) based on 10 mM NAD^+, 10 mM glucose and NADH was detected at 340 nm. Increase of 1 μmol hydroxytyrosol (NADH)/min was 1 unit of total enzyme activity. The results were converted into enzyme activity units contained in 1 mL whole cell catalyst.

Whole-cell biotransformation for hydroxytyrosol production

Strains were washed with phosphate buffer and resuspended in an equivalent volume of phosphate buffer. Reaction system consisted of 50 g/L wet cells, 40 mM catechol, 60 mM pyruvic acid and 60 mM ammonium chloride. The reaction condition was 35 ℃, pH 7.5, and the reaction was allowed to proceed for 12 h at 150 rpm. After the reaction, the mixture was boiled (2 min) to inactivate the enzyme, and then centrifuged (8000 g, 2 min) to remove the bacterial cells. The concentration of various compounds in the reaction solution were determined by HPLC.

Optimization of pH and temperature

Strain 4 was inoculated into flask with TB medium. The flask was filled with 20 mL reaction system and the reaction time was 1 h. Reaction system comprised 50 g/L wet cells, 40 mM catechol, 60 mM pyruvic acid, 60 mM glucose and 60 mM ammonium chloride. To optimize pH, the temperature and shaker speed were 37 °C and 150 rpm. The pH levels were adjusted to 5.5, 6, 6.5, 7, 7.5, 8, 8.5 using phosphate buffer and Tris-HCL buffer. To optimize temperature, the pH and shaker speed were 7.5 and 150 rpm. The temperature was varied to 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, respectively.

Large-scale culture of recombinant *Escherichia coli* in 5 L bioreactor

Strain 4 was inoculated into flask with TB medium. The culture condition was 37 °C, 220 rpm for 12 h. Following this, 50 mL seed solution was inoculated into 3 L TB medium. The temperature was adjusted to 37 °C, the initial ventilation was adjusted to 5 L/min, and the dissolved oxygen level was maintained at 20% by controlling the stirring speed of 100–500 rpm. The pH of culture medium was adjusted by sodium hydroxide and hydrogen chloride. When the OD_{600} reached 15, 0.4 mM IPTG (final concentration) was added into 3 L TB medium, and the culture temperature was adjusted to 25 °C. The wet cells were obtained by centrifugation (4 °C, 8000 g, 5 min).

Scaled-up production of hydroxytyrosol in 5 L bioreactor

The biotransformation system was 1.5 L and consisted of 50 g/L wet cells, 60 mM glucose, 60 mM ammonium chloride, 60 mM pyruvic acid and 40 mM catechol. The temperature and pH were maintained at 25 °C and 7.5, respectively. The pH was adjusted by adding sodium hydroxide and hydrogen chloride. The speed of air supplied was 1 L/min, and the dissolved oxygen level was regulated at 3% by modulating the stirring speed of 100–500 rpm. In the process of transformation, feeding methods were divided into feeding every 8 h, feeding every 4 h, feeding every 2 h and no feeding. After each feeding, the content of catechol reached 40 mM. Samples were taken every 2 h and centrifuged at 4 °C, 8000 g for 5 min. The supernatant was diluted and filtered by microporous membrane, and then the content of various substances was determined by HPLC.

Analytical methods

The concentrations of products and substrates were detected by HPLC (Shimadzu). GALAK column was used to separate the substances in the sample, and the injection volume of each sample was 10 μL. The temperature of column was maintained at 35 °C. Gradient elution was used after injection. The eluting mobile phase was methanol and water containing 0.1% formic acid. The elution procedure was set as follows: 0–15 min, 10% methanol was increased to 100% methanol; 15–20 min, the column was washed with 100% methanol; 20–30 min, the column was balanced with 10% methanol. The detection wavelength of the system is 280 nm.

Results

Construction of hydroxytyrosol synthesis pathway

To construct strain 1, we utilized pCDFDuet-1, pETDuet-1 and pRSFDuet-1 to express *TPL*, *aadL*, *KAD*, *yahK* and *gdh* (Fig. 2). The pRSFDuet-1 (high copy number plasmid) was used to express *KAD* and *aadL*, pETDuet-1 (medium copy number plasmid) was used to express *TPL* and *yahK*, and pCDFDuet-1 (low copy number plasmid) was used to express *gdh*. After the conversion, 9.32 mM hydroxytyrosol was produced. To verify the production of hydroxytyrosol, the products were detected by HPLC (Fig. 3) and UPLC-MS (Fig.S1). The peak time, mass-to-charge ratio and UV absorption spectra is the same as that of hydroxytyrosol standard.

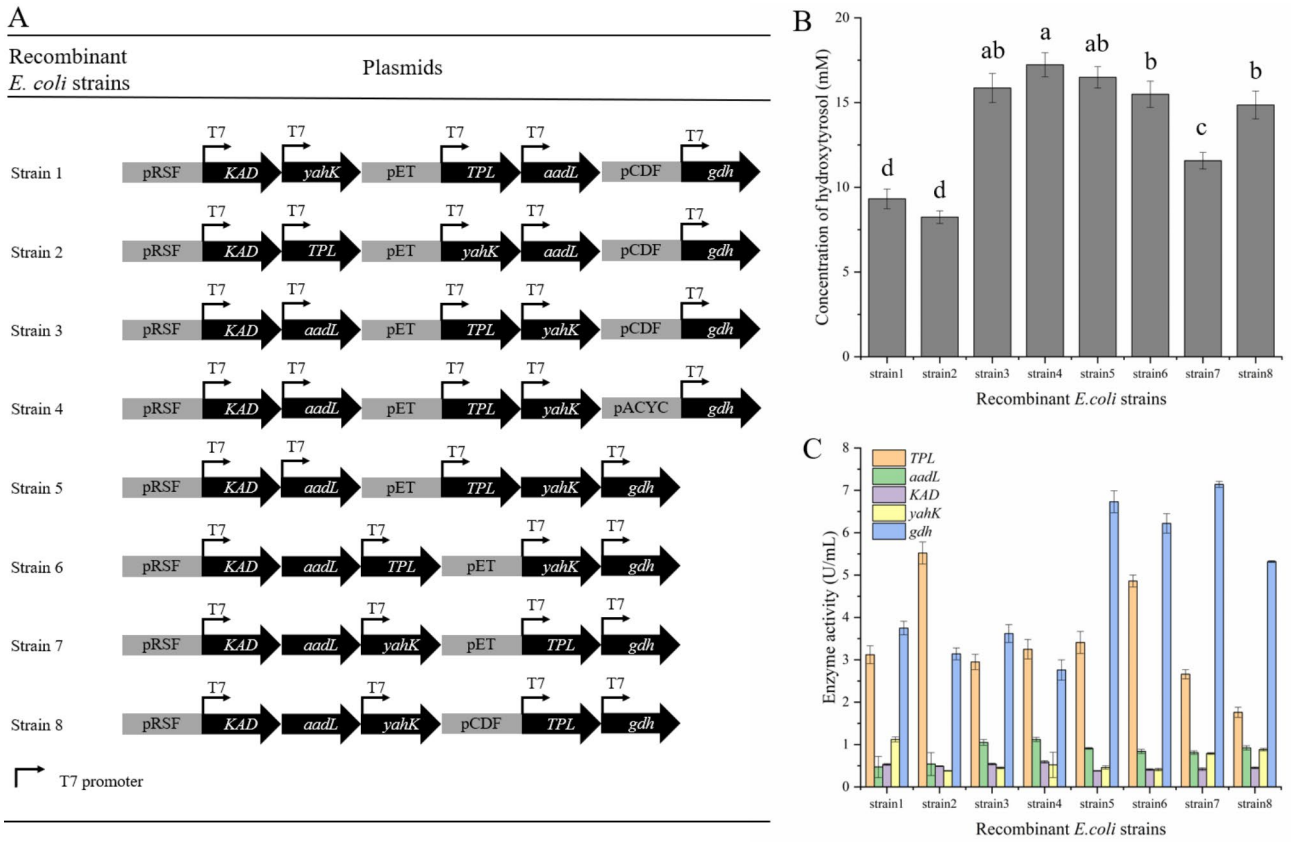

Fig. 2. whole cell biotransformation from engineering *Escherichia coli*. (**A**) Construct composite plasmids for co-expressing *KAD*, *yahK*, *TPL*, *aadL* and *gdh*; (**B**) Hydroxytyrosol production from engineered strains; (**C**) Enzyme activity of *KAD*, *yahK*, *TPL*, *aadL* and *gdh* in each strain.

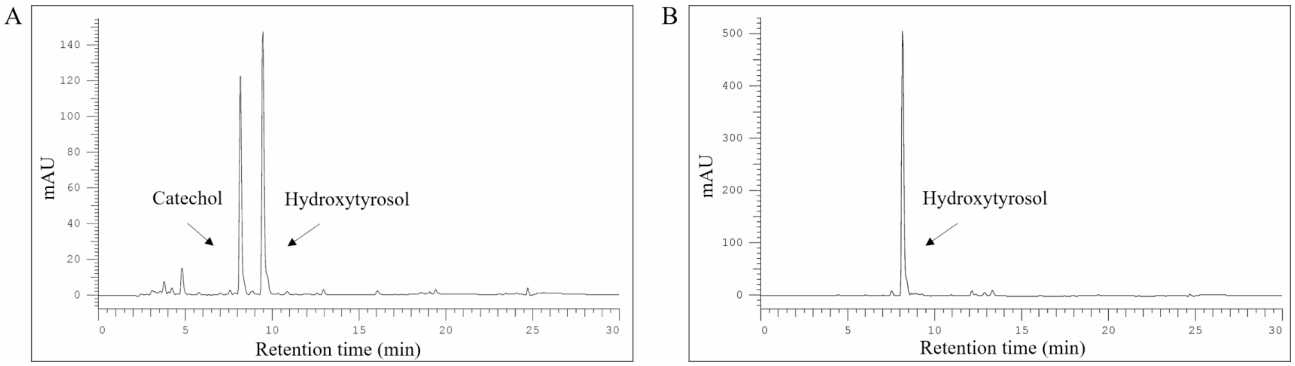

Fig. 3. HPLC analyses, (**A**) Hydroxytyrosol produced in strain 1, (**B**) Hydroxytyrosol standard.

Combination of different copy number plasmids to improve production of hydroxytyrosol

Previous studies have shown that changing combinations between plasmids with different copy numbers and target genes can improve the production capacity of engineered strains[22,23]. We used plasmids with different copy numbers to express pathway genes (Fig. 2A). These plasmids encompassed PACYCDuet-1, pCDFDuet-1, pETDuet-1 and pRSFDuet-1. The expression levels of transcription for the five genes within the eight engineered strains of *E. coli* were individually measured through reverse transcriptase-quantitative PCR (RT-qPCR), and the illustration of their relative transcript abundances is provided in Figure S2. The recombinant *E. coli* strains was used to produce hydroxytyrosol and the reaction time was 5 h (Fig. 2B).

When strain 2 (pRSF-*KAD*-*TPL*, pET-*yahK*-*aadL*, pCDF-*gdh*) and strain 1 (pRSF-*KAD*-*yahK*, pET-*TPL*-*aadL*, pCDF-*gdh*) were compared, *TPL* in strain 2 was expressed by high copy plasmid of pRSFDuet-1. *TPL* in strain 1 was expressed using a medium intensity plasmid of pETDuet-1, and the activity of TPL was higher in strain 2 (5.52 U/mL) than in strain 1 (3.12 U/mL) (Fig. 2C). The *TPL* relative transcription level of strain 2 is also

higher than that of strain 1 (Fig. S2). HPLC analysis showed that the hydroxytyrosol production of strain 2 (8.24 mM) was slightly lower than that of strain 1 (9.32 mM) (Fig. 2B). To study the effect of aadL on the production of hydroxytyrosol, strain 3 (pRSF-*KAD-aadL*, pET-*TPL-yahK*, pCDF-*gdh*) was constructed. *KAD* and *aadL* in strain 3 were expressed by high copy plasmid of pRSFDuet-1. *TPL* and *yahK* were expressed by medium copy plasmid of pETDuet-1. The *aadL* relative transcription level of strain 3 is higher than that of strain 1 (Fig. S2). HPLC analysis showed that the yield of hydroxytyrosol in strain 3 (15.86 mM) increased sharply compared with strain 1 (23.3%) and strain 2 (20.6%). It can be concluded that strong overexpression of *aadL* is essential for the synthesis of hydroxytyrosol. When strain 4 (pRSF-*KAD-aadL*, pET-*TPL-yahK*, pACYC-*gdh*) was compared with strain 3, the *gdh* in strain 4 was expressed by the ultra-low copy plasmid of pACYCDuet-1. HPLC analysis showed that the yield of hydroxytyrosol in strain 4 (43.08%) was higher than that in strain 3 (39.65%).

Then we constructed strain 5 (pRSF-*KAD-aadL*, pET-*TPL-yahK-gdh*). *KAD* and *aadL* in strain 5 were expressed by high copy plasmid of pRSFDuet-1. *TPL*, *yahK* and *gdh* were expressed by medium copy plasmid of pETDuet-1. The strain 6 (pRSF-*KAD-aadL-TPL*, pET-*yahK-gdh*) was also constructed. *KAD*, *aadL* and *TPL* in strain 6 were expressed by high copy plasmid of pRSFDuet-1, *yahK* and *gdh* were expressed by medium copy plasmid of pETDuet-1. When strain 4 and strain 5 were compared, *gdh* in strain 5 was expressed by medium intensity plasmid, and the activity of gdh was significantly enhanced in strain 5 (6.73 U/mL) than in strain 1 (3.75 U/mL) (Fig. 2C). HPLC analysis showed that the yield of hydroxytyrosol of strain 5 (41.23%) was slightly decreased compared with strain 4 (43.08%). In addition, HPLC analysis showed that the yield of hydroxytyrosol of strain 6 (38.73%) was lower than that of strain 5 (41.23%). Then strain 7 (pRSF-*KAD-aadL-yahK*, pET-*TPL-gdh*) was constructed. *KAD*, *aadL* and *yahK* in strain 7 were expressed by high copy plasmid of pRSFDuet-1, *TPL* and *gdh* were expressed by low copy plasmid of pCDFDuet-1. HPLC analysis showed that the yield of hydroxytyrosol of strain 7 (28.93%) was significantly lower than that of strain 6 (38.73%). Finally, strain 8 (pRSF-*KAD-aadL-yahK*, pCDF-*TPL-gdh*) was constructed. *KAD*, *aadL* and *yahK* in strain 8 were expressed by high copy plasmid of pRSFDuet-1, *TPL* and *gdh* were expressed by low copy plasmid of pCDFDuet-1. The results of HPLC analysis showed that the yield of hydroxytyrosol of strain 8 (37.15%) was higher than that of strain 7 (28.93%). Overall, strain 4 (17.23 mM) was able to produce the highest amount of hydroxytyrosol relative to other strains. The hydroxytyrosol yield of strain 4 (43.08%) increased by 84% compared with that of strain 1 (23.3%).

Optimization of pH and temperature

Culture conditions are crucial for whole cell biotransformation. For pH optimization, hydroxytyrosol gradually increased when the pH was from 5.5 to 7.5. Conversely, the production of hydroxytyrosol decreased gradually at pH ranging from 7.5 to 8.5. The optimum pH was 7.5 (Fig. 4A). For temperature optimization, hydroxytyrosol gradually increased when the temperature was from 15 °C to 25 °C. The production of hydroxytyrosol decreased gradually at temperatures ranging from 25 °C to 40 °C. The optimum reaction temperature was 25 °C, yielding 17.38 mM hydroxytyrosol (Fig. 4B).

Scaled-up production of hydroxytyrosol using fed-batch biotransformation

Previous studies have shown that high concentrations of catechol can inhibit enzyme activity and reduce the yield of production[19]. To increase the yield of hydroxytyrosol, the fed-batch biotransformation was performed by feeding catechol, pyruvate and ammonium chloride in 5 L bioreactor.

The initial concentration of catechol was added by 40 mM. Without feeding, the total amount of catechol was added by 40 mM. The yield of hydroxytyrosol was 30.8 mM within 14 h (Fig. 5A). When feeding every 2 h, the total amount of catechol was added by 88.6 mM. The yield of hydroxytyrosol was 55.3 mM within 14 h (Fig. 5B). When feeding every 4 h, the total amount of catechol was added by 80.5 mM. The yield of hydroxytyrosol was

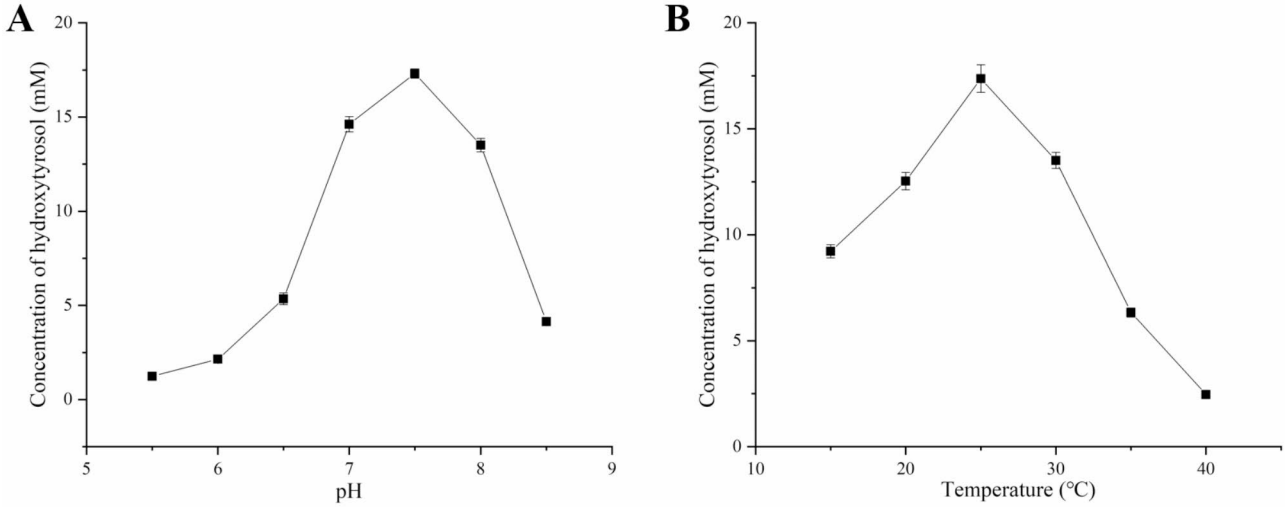

Fig. 4. Effect of pH and temperature on hydroxytyrosol production of strain 4, (**A**) pH, (**B**) temperature.

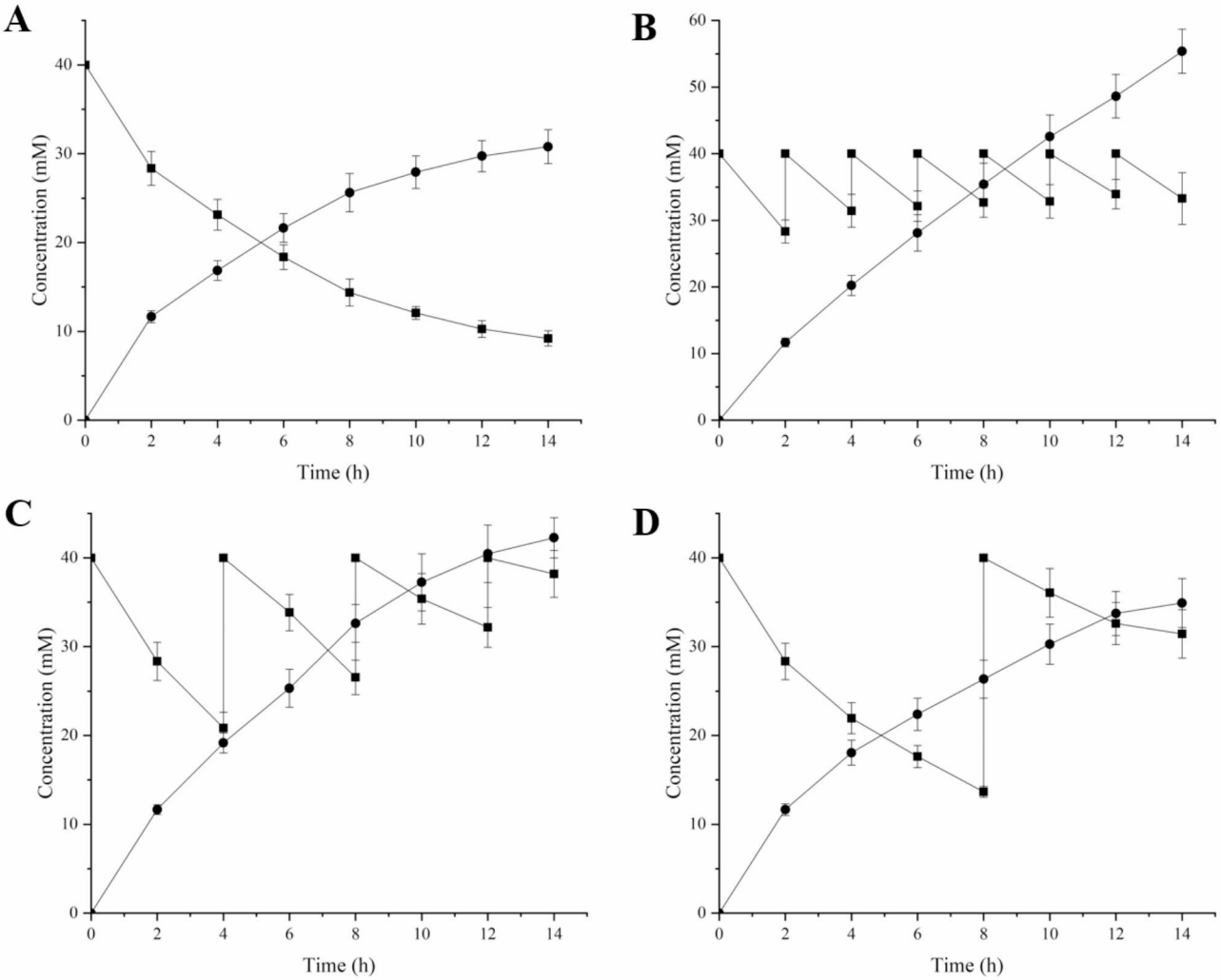

Fig. 5. Scaled-up production of hydroxytyrosol using fed-batch biotransformation. (**A**) No feeding. (**B**) Feeding every 2 h. (**C**) Feeding every 4 h. (**D**) Feeding every 8 h. ▪, catechol; ●, hydroxytyrosol.

42.2 mM within 14 h (Fig. 5C). When feeding every 8 h, the total amount of catechol was added by 66.3 mM. The yield of hydroxytyrosol was 34.9 mM within 14 h (Fig. 5D). The maximum yield of hydroxytyrosol was obtained by feeding every 2 h. The hydroxytyrosol yield of feeding every 2 h is 1.8 times that of no feeding.

Discussion

Cascade reactions play an important role in synthetic chemical products and can avoid waste formation, time consumption and expensive purification operations. With the rapid development of bioinformatics and protein engineering, whole cell biotransformation has been widely used in the production of various foods, drugs and daily necessities and has achieved huge economic and social benefits[9].

Hydroxytyrosol is a valuable bioactive compound. Many biosynthetic methods of hydroxytyrosol have been reported in the past. Hydroxytyrosol could be produced at 1.3 mM, 1.7 mM, 8.1 mM and 12.3 mM with tyrosine as substrate[10,15,24,25]. 10.4 mM, 16 mM and 24.9 mM hydroxytyrosol could be produced using tyrosol as substrate[26–28]. The productivity of hydroxytyrosol is low using tyrosine and tyrosol as substrates due to the low activity of hydroxylase, which is difficult to meet the requirements of industrial production. L-DOPA can also be used as a substrate to synthesize hydroxytyrosol by recombinant *Escherichia coli*[17]. However, L-DOPA is more expensive and high pH will cause the decomposition of L-DOPA, resulting in the loss of L-DOPA[29]. Thus, a cascade for transformation of catechol and pyruvate to hydroxytyrosol is desired. Recently, zhao et al. envisions a cascade biocatalytic approach to convert lignin-derived catecholins and phenols into hydroxytyrosol by integrating C-C coupling, hydroxylation, decarboxylation, deamination, and reduction. The maximum titers of 20.6 mM (3.18 g/L) and 18.5 mM (2.85 g/L) were obtained from 30 mM catechol and phenol by batch feeding of recombinant *Escherichia coli* resting cells[30]. In this study, the yield of hydroxytyrosol was significantly higher than in previous studies, 55.3 mM hydroxytyrosol was efficiently produced by fed-batch biotransformation using catechol and pyruvate as substrates.

The balance of various combination enzymes is important in whole cell biotransformation. Previous studies have shown that different copy numbers plasmids can balance the expression of enzymes[31,32]. In this study, we used different copy numbers plasmids to express target genes. The results showed that strain 4, which was constructed with high copy plasmids that strongly overexpressed *KAD* and *aadL*, medium copy plasmids that moderately overexpressed *TPL* and *yahK*, and low copy plasmids that weakly expressed *gdh*, could produce the highest yield of hydroxytyrosol. The weak expression of *gdh* can meet the requirement of NADH regeneration due to the high activity[33]. During the fed-batch biotransformation, higher conversion efficiencies were observed when catechol was maintained at approximately 30 mM. This may be attributed to the existence of a narrow optimal concentration window for catechol, where higher conversion efficiencies can be achieved when the catechol concentration is maintained within a certain range.

Conclusions

In this study, catechol, sodium pyruvate and ammonium chloride were utilized as substrates to produce hydroxytyrosol. The expression of pathway genes was regulated through different copy numbers plasmid which enhanced the production capacity of hydroxytyrosol. Furthermore, the yield of hydroxytyrosol was increased by implementing a batch feeding method. This study has a strong industrial application prospect.

Data availability

All data included in this study are available upon request by contact with the corresponding author.

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Author contributions

X.T.Z. designed and initiated the experiments. Y.H.D.; L.J.L.; B.D.Y. and L.X.M. conducted experiments. L.X.M., L.W. and F.G.Y.; Formal analysis; X.T.Z. and L.W. wrote manuscripts. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

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