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Engineering of a Tyrosol-Producing Pathway, Utilizing Simple Sugar and the Central Metabolic Tyrosine, in *Escherichia coli*

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Supporting Information

ABSTRACT: Metabolic engineering was applied to the development of *Escherichia coli* capable of synthesizing tyrosol (2-(4-hydroxyphenyl)ethanol), an attractive phenolic compound with great industrial value, from glucose, a renewable carbon source. In this strain, tyrosine, which was supplied not only from the culture medium but also from the central metabolism, was converted into tyrosol via three steps: decarboxylation, amine oxidation, and reduction. The engineered strain synthesized both tyrosol and 4-hydroxyphenylacetate (4HPA), but disruption of the endogenous phenylacetaldehyde dehydrogenase gene shut off 4HPA production and improved the production of tyrosol as a sole product. The engineered mutant strain was capable of producing 0.5 mM tyrosol from 1% (w/v) glucose during a 48 h shake flask cultivation.

KEYWORDS: *tyrosol (2-(4-hydroxyphenyl)ethanol), Ehrlich pathway, metabolic engineering, Escherichia coli*

INTRODUCTION

Tyrosol (2-(4-hydroxyphenyl)ethanol) is an attractive phenolic compound of great industrial value and is marketed as a fine chemical. This compound and its derivatives are important synthons for the production of various organic compounds. For example, tyrosol can be used for the preparation of commercially available pharmaceutical agents such as betaxolol[1] and metoprolol,[2] which are selective β_1 receptor blockers and used in the treatment of hypertension, angina, heart failure, and glaucoma.[3] Moreover, tyrosol is a possible precursor for hydroxytyrosol, (2-(3,4-dihydroxyphenyl)ethanol),[4−9] which is an antioxidant presenting several interesting aspects for human health[10] and polymer production.[11,12] Tyrosol itself has also been reported to have biologically relevant properties, including a role in the prevention of cardiovascular diseases,[13,14] osteopenia,[15] and melanin pigmentation,[16] as well as anti-inflammatory activity.[17] In addition, tyrosol can be used as an additive of a bitter ingredient to improve the taste of Japanese rice wine (sake) because this is much easier than development of a brewery yeast producing tyrosol in large quantity.[18]

For industrial purposes, tyrosol is often produced chemically,[19−21] although it is a naturally occurring compound that has been found in olive oil,[13,22] wine,[14] sake,[18] and so on. It is known that virgin olive oil contains tyrosol as a minor component, 2.6 mg/kg of tyrosol and 22.4 mg/kg of dialdehydic form of elenolic acid linked to tyrosol;[22] hence, totally 25 mg/kg of tyrosol can be obtained if the latter is degraded. It would, however, be hard to purify it at an industrial scale because of its low concentration, the existence of other types of phenols, and the volatility of the composition according to cultivar, harvesting time, health of the olives, and technology used in the extraction process. Olive oil mill wastewater, a polluting byproduct of the olive oil production process, is also a potential source of tyrosol; however, the development of effective separation methods from the wastewater would be required.[23]

The tyrosol biosynthesis pathway in *Saccharomyces cerevisiae*, starting from tyrosine, was studied by Ehrlich; hence, it is termed the Ehrlich pathway (Figure 1).[24,25] In this pathway, tyrosine is converted into tyrosol as follows: (i) transamination of tyrosine by aminotransferase, (ii) decarboxylation of 4-hydroxyphenylpyruvate by pyruvate decarboxylase, and (iii) reduction of 4-hydroxyphenylacetaldehyde (4HPAA) by alcohol dehydrogenase (ADH).

In this paper, we present the metabolic engineering of *Escherichia coli* to produce tyrosol via a novel biosynthetic pathway. The production of tyrosol is attempted not only by the supplementation of a direct precursor, tyrosine, but also by using central metabolic tyrosine as a substrate without any tyrosine supplementation. Without any further engineering for tyrosine overproduction, we have achieved 0.5 mM tyrosol production from simple sugar as a sole feedstock.

MATERIALS AND METHODS

Bacterial Strains and Cultures. *E. coli* DH10B (Invitrogen, Carlsbad, CA) was routinely used for plasmid construction. For tyrosol production, *E. coli* BW25113 and its *feaB*-knockout mutant, JW1380, in the Keio collection, a single-gene knockout mutants library,[26] were employed. The strain JW1380 was used after elimination of the

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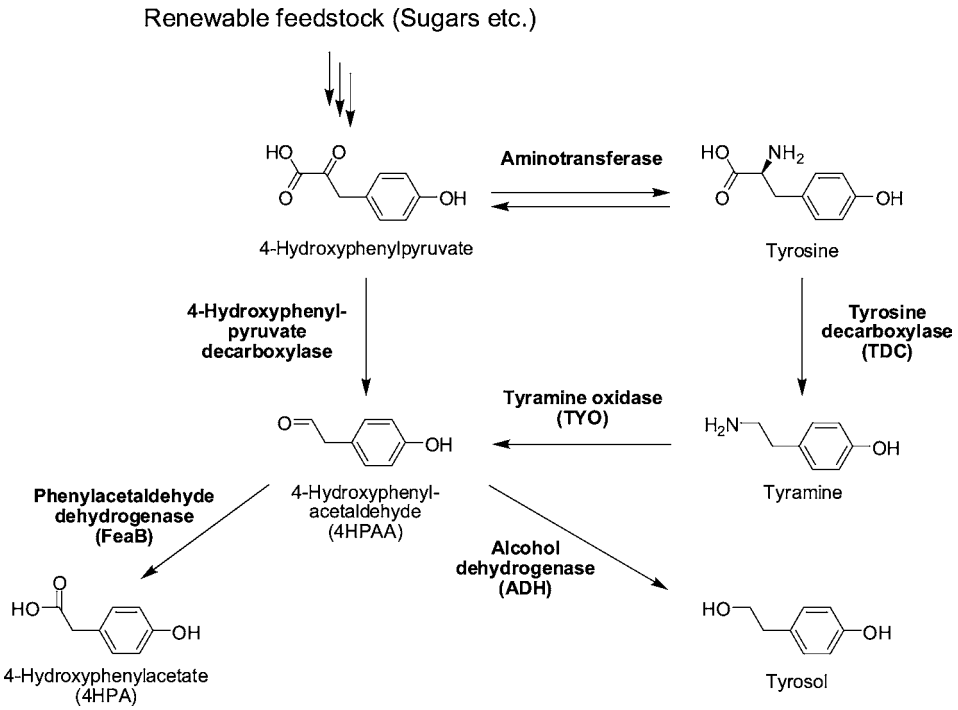

Figure 1. Pathways for the production of tyrosol.

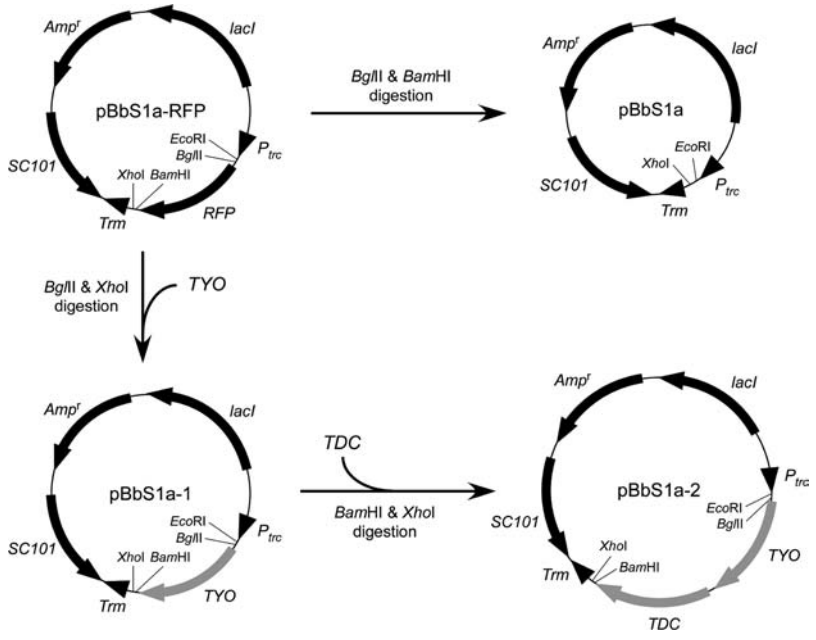

Figure 2. Schematic representation of plasmids used for tyrosol production. P_{trc}, *trc* promoter; Trm, terminator; *SC101*, SC101 origin; *lacI*, lac repressor gene; *Amp*^{r}, ampicillin/carbenicillin resistant gene; *RFP*, red fluorescent protein gene; *TYO*, tyramine oxidase gene; *TDC*, tyrosine decarboxylase gene.

kanamycin-resistance gene on the chromosome, as described by Datsenko and Wanner.[27]

The media used were LB broth medium (Lennox; Becton, Dickinson and Co., Franklin Lakes, NJ) and M9 minimal medium (M9 minimal salts (Becton, Dickinson and Co.), 1% (w/v) glucose, 5 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.025% (w/v) of yeast extract (M9Y media). Carbenicillin was added to the media at 100 μg mL^{-1} to maintain plasmids.

Plasmid Construction. To enable rapid cloning and assembly of genes, we employed the BglBrick cloning strategy.[28,29] The tyramine oxidase (TYO) gene from *Micrococcus luteus* (accession no. AB010716)[30] and tyrosine decarboxylase (TDC) gene from *Papaver somniferum* (accession no. U08598),[31] which were optimized for codon usage in *E. coli* and to which was attached a ribosome-binding site, by using Gene Designer 2.0 software (DNA2.0 Inc., Menlo Park, CA), were purchased from GeneScript USA Inc. (Piscataway, NJ). The expression of these genes in *E. coli* and the in vivo activity of TDC were confirmed by SDS-PAGE and HPLC analyses, respectively (see Figure S1 in the Supporting Information). The TYO gene was subcloned into the BglBrick compatible vector pBbS1a-RFP (SC101 origin, *trc* promoter, *lacI*, Amp^r, red fluorescent protein gene)[29] to construct pBbS1a-1 as shown in Figure 2. To construct an artificial operon of TYO and TDC based on BglBrick strategy, the TDC gene was inserted downstream of the TYO gene in pBbS1a-1; this construct

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was designated pBbS1a-2 (Figure 2). For the construction of a control plasmid pBbS1a, the gene encoding red fluorescent protein was deleted in the pBbS1a-RFP vector (Figure 2).

Tyrosol Production. *E. coli* harboring pBbS1a-derived plasmids was cultured in LB medium for 16 h at 37 °C. Thereafter, 100 μL aliquots were inoculated into 5 mL of fresh LB medium and cultured at 30 °C for 3 h; subsequently, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After an addition 5 h of culturing, the cells were harvested and washed once with 5 mL of M9Y medium. The cells were then cultured for 20 h, at 30 °C, in 5 mL of M9Y medium supplemented with 1 mM tyramine or tyrosine, and the supernatants were then analyzed by HPLC, as described below.

Shake flask experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of M9Y medium. The aliquots (50 μL) of overnight culture of *E. coli* harboring pBbS1a derivatives were inoculated into 2 mL of fresh LB medium and cultured at 30 °C, for 4 h. After the cells were harvested and washed once, with the same amount of M9Y medium, the complete cell pellets were inoculated into 50 mL of M9Y medium (0 days in Figure 4). IPTG was added to a final concentration of 0.5 mM after 3 h of cultivation at 30 °C, shaking at 160 rpm. Samples (1 mL) were collected at appropriate time points and analyzed by HPLC. Optical density (OD) measurements at 600 nm were also taken using a Beckman Coulter spectrophotometer (Beckman Coulter Inc., Brea, CA).

HPLC Analysis. Samples of culture supernatants (2 μL) were analyzed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc., Santa Clara, CA), equipped with a Discovery HS F5 column (15 cm × 2.1 mm i.d., 3 μm; Sigma-Aldrich Co. LLC, St. Louis, MO). Buffer A (0.1% (v/v) formic acid solution) and buffer B (acetonitrile with 0.1% (v/v) formic acid) were used as a mobile phase, and compounds were eluted at 35 °C and a flow rate of 0.3 mL min^{-1}, with increasing concentrations of buffer B as follows: 5%, 0−2 min; 5−30%, 2−22 min.

Eluted compounds were detected by a diode array spectrophotometer (Agilent Technologies) measuring absorbance at 280 nm or a time-of-flight mass spectrometer (Agilent Technologies). Tyrosine, tyramine, tyrosol, and 4-hydroxyphenylacetate (4HPA) (Sigma-Aldrich) were used as standards.

RESULTS

We first assessed whether *E. coli* is able to produce tyrosol from tyrosine or not. When cultured in M9Y media supplemented with 1 mM tyrosine, tyrosol production was not observed. This finding indicated that *E. coli* BW25113 did not have the ability to convert tyrosine into tyrosol.

We next attempted to construct a metabolic pathway for the production of tyrosol. We considered 4-hydroxyphenylacetaldehyde (4HPAA) as a possible precursor for tyrosol production, because several types of ADHs exist in *E. coli*;[32] however, 4HPAA is not available commercially. On the other hand, tyramine oxidase (TYO) from *M. luteus* can convert tyramine into 4HPAA and is specific for tyramine and dopamine.[33] Hence, we assessed whether tyrosol was produced from tyramine in *E. coli* expressing the *M. luteus* TYO gene from the pBbS1a-1 plasmid. As shown in Figure 3 and Table 1, we found that the strain harboring pBbS1a-1 was able to produce 0.7 mM tyrosol from 1 mM tyramine, under the cultivation conditions, demonstrating that the host cell can potentially convert 4HPAA to tyrosol.

Moreover, a peak with a retention time of 11.8 min was detected on HPLC analysis of the cultures of the *E. coli* harboring pBbS1a-1 (Figure 3). We speculated that the compound was 4HPA because the *E. coli* genome encodes an enzyme, phenylacetaldehyde dehydrogenase (FeaB or PadA), which catalyzes the oxidation of 4HPAA to 4HPA with H_2O as a substrate and NAD^+ as a coenzyme.[34,35] As expected, we then confirmed that 4HPA eluted at the same retention time.

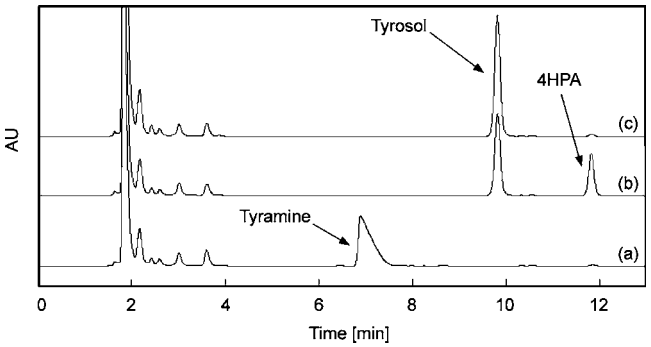

Figure 3. HPLC analysis of compounds produced by engineered *E. coli* strains in M9Y medium supplemented with tyramine: (a) BW25113 harboring pBbS1a; (b) BW25113 harboring pBbS1a-1; (c) JW1380 harboring pBbS1a-1.

Table 1. Tyrosol Production from Tyramine by Engineered *E. coli*[a]

strain and plasmid	tyramine (mM)	tyrosol (mM)	4HPA (mM)
BW25113			
pBbS1a	0.94 ± 0.005	0	0
pBbS1a-1	0	0.70 ± 0.008	0.35 ± 0.008
JW1380			
pBbS1a	0.93 ± 0.011	0	0
pBbS1a-1	0	1.02 ± 0.008	0

[a]Data are expressed as mean values ± SD derived from three independent experiments.

As shown in Table 1, the total yield of tyrosol and 4HPA synthesized by the *E. coli* harboring pBbS1a-1 was approximately 1 mM, which indicated that tyramine added to the media was almost completely converted. In contrast, *E. coli* BW25113 harboring the control vector did not catabolize any tyramine to tyrosol. Thus, we showed that the TYO construct achieved its purpose and that the 4PHAA formed was metabolized to both the alcohol and the acid by internal enzyme(s) in the cell.

For the effective production of tyrosol, the 4HPA synthesis pathway should be shut off. To confirm that the *feaB* gene product was related to 4HPA formation, we analyzed a culture of *E. coli* JW1380, a *feaB*-knockout mutant derived from BW25113 in the Keio collection,[26] harboring pBbS1a-1, by HPLC. As shown in Figure 3, the mutant expressing TYO successfully produced tyrosol from tyramine and the peak representing 4HPA disappeared, indicating that FeaB plays a major role in the formation of 4HPA. Moreover, tyramine was completely converted to tyrosol (Table 1). As a result, we used the *feaB*-knockout mutant for further analysis.

Then, we attempted to synthesize tyrosol from tyrosine. To facilitate tyramine formation from tyrosine, we constructed a plasmid, pBbS1a-2, which contained an artificial operon encoding TDC from *P. somniferum*[31] downstream of the TYO gene. As shown in Table 2, the *E. coli feaB*-knockout mutant harboring pBbS1a-2 produced 1.22 mM tyrosol from 1 mM tyrosine. This indicated that the artificial pathway successfully and effectively converted tyrosine into tyrosol. It was interesting that the yield of tyrosol from this pathway exceeded 100%. To elucidate the question, the *E. coli feaB* mutant harboring pBbS1a-2 was tested in M9Y medium

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Table 2. Tyrosol Production from Tyrosine by Engineered *E. coli*[a]

strain and plasmid	tyrosine (mM)	tyramine (mM)	tyrosol (mM)	4HPA (mM)
JW1380				
pBbS1a	0.54 ± 0.010	0	0	0
pBbS1a-2	0	0	1.22 ± 0.004	0
pBbS1a-2[b]	0	0	0.31 ± 0.133	0

[a]Data are expressed as mean values ± SD derived from three independent experiments. [b]Without tyrosine.

without tyrosine. Under these cultivation conditions, the strain could produce 0.31 mM tyrosol (Table 2), indicating that it was able to utilize the central metabolic tyrosine in the cell for the production of tyrosol.

Finally, we attempted tyrosol biosynthesis from glucose on flask scale. As shown in Figure 4, the *feaB*-knockout mutant harboring pBbS1a-2 successfully produced 0.50 mM tyrosol

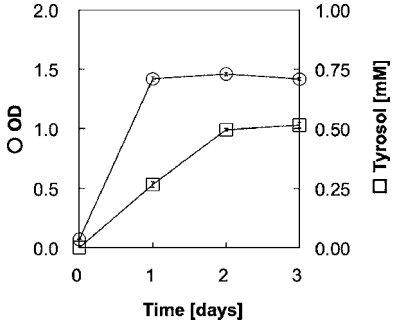

Figure 4. Time course changes of tyrosol production by *E. coli* JW1380 harboring pBbS1a-2 from glucose: (○) optical density (OD); (□) tyrosol. Data are expressed as mean values derived from three independent experiments (SD < ±0.02).

during 2 days of cultivation, when cultured on M9Y media containing 1% (w/v) glucose. The product was also confirmed using LC/MS as the $[M + Na]^+$ fragment at m/z 161.

DISCUSSION

In this study, we successfully engineered a novel metabolic pathway in *E. coli* capable of producing an industrially valuable phenolic compound, tyrosol, efficiently from tyrosine provided in the culture media and also from simple sugar that is converted to tyrosine via the central metabolic pathway.

Although *S. cerevisiae* can potentially convert tyrosine to tyrosol from an intermediate in the tyrosine biosynthesis pathway, 4-hydroxyphenylpyruvate, through a decarboxylation and a reduction reaction in the Ehrlich pathway,[24,25] a native *E. coli* cannot produce tyrosol even when supplemented with tyrosine (Table 2). This indicates that the Ehrlich pathway does not operate in *E. coli*; however, our metabolically engineered *E. coli* was able to produce tyrosol efficiently.

Tyrosine and glucose were used for tyrosol production in this study. As shown in Table 2, tyrosine is a more preferred starting material for tyrosol production; however, the price is about 10 times higher than that of glucose. Because *E. coli* requires nutrients for its growth, glucose is a more appropriate carbon source and has been used for the fermentation production.

To translate our innovation into a practical industrial application, however, the capacity of the strain for tyrosol production from glucose needs to be improved. One potential way of doing this is to reinforce the precursor pathway in the cell bearing tyrosol pathway. Tyrosine is an attractive compound as a dietary supplement and a valuable precursor compound for various industrial and pharmaceutical applications, and tyrosine-overproducing strains have already been developed.[36−38] If the engineered *E. coli* strains capable of producing about 10 g/L of tyrosine were used as host cells, they could produce 7.6 g/L of tyrosol. This yield is about 3 times higher than the tyrosol yield extracted from olive oil mill wastewater as described below.[23] By employing these strains for tyrosol production, higher titer production of tyrosol from simple sugar would be achieved for industrial application.

As a natural source of tyrosol, virgin olive oil may be considered; however, its low concentration (25 mg/kg tyrosol and its derivatives)[22] would not be appropriate as a feedstock for industrial-scale production. On the other hand, olive oil mill wastewater, which is a byproduct generated from the olive oil production process and represents a severe environmental problem, has recently drawn much attention as a cheap source of bioactive phenolic compounds such as tyrosol and hydroxytyrosol, and various methods to recover the compounds from the wastewater have been developed.[23,39−42] Although De Macro et al. could extract 2.5 g of tyrosol from 1 L of the olive oil mill wastewater using organic solvent, its purification has not been achieved,[23] indicating that an effective separation process of tyrosol from the mixture of the phenolic compounds is challenging. In contrast, the use of an engineered strain specifically producing tyrosol at the order of several grams per liter enables us to develop a simpler and more effective tyrosol-producing route with a higher quality.

E. coli laboratory strains demonstrate different assimilation activities for aromatic compounds.[43] The *E. coli* B, C, and W strains are able to grow using 3-hydroxyphenylacetate (3HPA) or 4HPA as the sole carbon and energy source, whereas *E. coli* K-12 cannot.[43] In *E. coli* C and W strains, the HPAs are hydroxylated at the beginning of the assimilation process by the action of an aromatic hydroxylase, to form homoprotocatechuate (3,4-dihydroxyphenylacetate), and are then degraded to pyruvate and succinate via a meta-cleavage pathway. Indeed, we confirmed that *E. coli* BLR(DE3), a strain B derivative, did not accumulate 4HPA in the culture (data not shown), which suggested that the 4HPA was catabolized via the meta-cleavage pathway. The accumulation of 4HPA in the culture of *E. coli* BW25113, a strain K-12 derivative, is likely to be due to the absence of the genes responsible for the HPA degradation pathway. Therefore, disruption of the *feaB* gene involved in 4HPA formation would be requisite for the effective production of tyrosol in any host strain.

In addition, the initial step of HPA degradation via the meta-cleavage pathway in *E. coli* B, C, and W strains is the hydroxylation of HPAs by the aromatic hydroxylase encoded by the *hpaBC* genes.[43] Broad substrate specificity has been reported for this enzyme,[44] and especially HpaBC from *Pseudomonas aeruginosa* has been reported for tyrosol hydroxylation in hydroxytyrosol synthesis.[45] Because these results suggest that unexpected hydroxylation of tyrosol may occur, deletion of the *hpaBC* genes from the chromosome may need to be considered when using *E. coli* B, C, or W strains as host cells for the specific production of tyrosol. In this study, we employed *E. coli* K-12 derivatives, which have neither *hpaB* gene nor its homologous genes in the chromosome, as the host cell. Considering that hydroxytyrosol was eluted at the retention time of 6.5 min under our HPLC condition, we

could conclude that unexpected hydroxylation of tyrosol did not occur in the host cell.

On the other hand, hydroxytyrosol is a powerful antioxidant found in olive extracts and presents several interesting aspects for human health.[10] Because there has been no cost-effective way to obtain hydroxytyrosol, many efforts have been devoted to the development of relevant chemical, biological, and extraction processes.[4−9,23,39−42] In certain biological processes, hydroxytyrosol was synthesized from tyrosol as a substrate, using mushroom tyrosinase[9] and whole bacterial cells of *Halomonas* sp.[45,46] as well as *P. aeruginosa*[7,8] as catalysts, but tyrosol itself is not an inexpensive compound. As an alternative, Brouk and Fishman reported a method using engineered toluene monooxygenases for the double hydroxylation of phenylethanol, which is less expensive than tyrosol.[47] We, therefore, expect that a tyrosol-overproducing *E. coli* strain which possesses the capability to hydroxylate tyrosol would be a cost-effective platform for hydroxytyrosol production, especially when renewable feedstocks can be used as carbon source.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

4HPAA, 4-hydroxyphenylacetaldehyde; 3HPA, 3-hydroxyphenylacetate; 4HPA, 4-hydroxyphenylacetate; IPTG, isopropyl-β-D-thiogalactopyranoside; NAD^+, nicotinamide adenine dinucleotide; ADH, alcohol dehydrogenase; FeaB, PadA, phenylacetaldehyde dehydrogenase; HpaBC, aromatic hydroxylase; TDC, tyrosine decarboxylase; TYO, tyramine oxidase; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; OD, optical density; SD, standard deviation.

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