A Novel Process for Obtaining Phenylpropanoic Acid Precursor Using *Escherichia coli* with a Constitutive Expression System

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Abstract Phenylpropanoids are widely used in food supplements, pharmaceuticals, and cosmetics with diverse benefits to human health. *Trans*-cinnamic acid or *p*-coumaric acid is usually used as the starting precursor to produce phenylpropanoids. Synthetic bioengineering of microbial cell factories offers a sustainable and flexible alternative method for obtaining these compounds. In this study, a constitutive expression system consisting of *Rhodotorula glutinis* phenylalanine/tyrosine ammonia lyase was developed to produce a phenylpropanoic acid precursor in *Escherichia coli*. To improve *trans*-cinnamic acid and *p*-coumaric acid production, BioBrick optimization was investigated, causing a 7.2- and 14.2-fold increase in the yield of these compounds, respectively. The optimum strain was capable of *de novo* producing 78.81 mg/L of *trans*-cinnamic acid and 34.67 mg/L of *p*-coumaric acid in a shake flask culture. The work presented here paves the way for the development of a sustainable and economical process for microbial production of a phenylpropanoic acid precursor.

Keywords: phenylpropanoic acid precursor, phenylpropanoids, *escherichia coli*, bioprocess design, constitutive expression

Introduction

Phenylpropanoids, which include stilbenoids, flavonoids, curcuminoids, and cinnamyl anthranilate, are a diverse group of secondary metabolites synthesized from L-phenylalanine or L-tyrosine. These compounds exhibit different bioactivities including antioxidant, antiviral, antibacterial, and anticancer (1–3). Thus, phenylpropanoids are widely used in pharmaceuticals, cosmetics, and food supplements. With the rapid development of market demands, phenylpropanoid supply is facing new opportunities and challenges (4). Phenylpropanoids are traditionally acquired through phytoextraction from mosses or angiosperms (5–7). However, the extraction yield of these compounds can hardly exceed 1% of the dry weight (8). Recently, synthetic biologists have attempted to explore biological phenomena and natural product synthesis by artificial-biological systems (9). Phenylpropanoids biosynthesis in recombinant *Escherichia coli* and *Saccharomyces cerevisiae* has achieved great progress, especially the biosynthesis of several bioactive compounds such as caffeic acid (10), naringenin (11,12), anthocyanin (13), resveratrol (14), pinocembrin

(15,16), pinosylvin (17), and styrene (18) by engineered *E. coli* fermentation. *E. coli*, which is the most genetically tractable microbial host, has been used as a microbial factory for the artificial biosynthesis of different types of molecules (19). The engineering strategy for the biosynthetic pathway of phenylpropanoids in microorganisms is shown in Fig. 1. This strategy involves embedding phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL) to convert endogenous/extrinsic L-phenylalanine into *trans*-cinnamic acid or L-tyrosine into *p*-coumaric acid. Different pivotal enzymes then convert *trans*-cinnamic acid or *p*-coumaric acid into different phenylpropanoids.

So far, the development of phenylpropanoid biosynthesis in *E. coli* is limited by some problems. Firstly, *trans*-cinnamic acid or *p*-coumaric acid is usually used as the starting precursor to produce phenylpropanoids because of the low turnover numbers of the reaction catalyzed by PAL or TAL, but the cost is more than that incurred using amino acids. Therefore, producing phenylpropanoids from L-tyrosine/L-phenylalanine or a carbon source has attracted the attention of academic and industrial researchers. Secondly, expression

Fig. 1. Engineering strategy for the biosynthetic pathway of phenylpropanoids in microorganisms.

system inducers such as isopropyl- β -D-1-thiogalactopyranoside (IPTG) are toxic and unsuitable for the industrial production of phenylpropanoids. By contrast, constitutive expression systems are appropriate for industrial needs. Such systems can be actively induced by the carbon source during fermentation and allow the continuous transcription of heterogeneous genes. For instance, the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (pGAP) system has been successfully used to produce *trans*-resveratrol in engineered *E. coli* (14). Thirdly, different strains with different expression vectors have different effects (20,21). BioBrick assembly parts such as different ribosomal binding sites (RBSs), replication regions, promoters, terminators, and polycistronic transcriptional assemblies are also limiting factors that need to be resolved (22).

In this study, the *Rhodotorula glutinis* RgPAL/TAL enzyme, which has dual substrate specificity and high enzymatic activity in *E. coli* (23), was selected for heterogeneous expression. The kinetic analyses were conducted using L-phenylalanine and L-tyrosine as substrates to explore the substrate preference of RgPAL/TAL. The constitutive expression, BioBrick assembly, and strain were optimized to biosynthesize *trans*-cinnamic acid and *p*-coumaric acid in *E. coli*. To the best of our knowledge, this is the first to explore the possibility of using a constitutive expression system for phenylpropanoid precursor bioproduction in *E. coli*. Moreover, this synthetic process may be further used for industrial food bioproduction of phenylpropanoids in *E. coli*.

Materials and Methods

Reagents, chemicals, and strains *E. coli* DH5 α was used for plasmid cloning. *E. coli* ATCC31884 and other strains (Table 1) were used for

recombinant molecule production. *R. glutinis* GIM2.156 was used for total RNA isolation. PrimeScript reverse transcription polymerase chain reaction (RT-PCR) kit, ExTaq polymerase, vector plasmid pMD18-T, restriction enzymes, and T4 DNA ligase were purchased from Takara Biochemicals Inc, Dalian, China. Vector plasmids pQE-30 and pREP4 were purchased from Qiagen Inc, Shanghai, China. DNA plasmids were prepared from stock strains using a Tiangen plasmid miniprep kit, Beijing, China. DNA fragments were isolated through gel extraction using a Tiangen gel DNA recovery kit (Tiangen Biotech Co., Ltd., Beijing, China). L-tyrosine, L-phenylalanine, *trans*-cinnamic acid, and *p*-coumaric acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

RgPAL/TAL complementary DNA (cDNA) cloning Total RNA was extracted in accordance with the hot phenol method. The first-strand cDNA of RgPAL/TAL was synthesized by using the PrimeScript RT-PCR kit in accordance with the manufacturer's protocol. The pF_PAL/TAL_clone and pR_PAL/TAL_clone specific primers listed in Table 1, based on existing PAL sequences (Genebank entry KF770992), were used to amplify the complementary strand. The RT-PCR products were purified and ligated into the pMD18-T vector to generate pMD18-RgPAL/TAL for sequencing.

Expression plasmid construction Plasmid pMD18-pGAP was provided by Bioengineering Laboratory stocks. The gap promoter (pGAP) was cloned from pMD18-pGAP. The *rrnB* T1 terminator was cloned from pQE-30. RgPAL/TAL was cloned from pMD18-RgPAL/TAL. The total RgPAL/TAL operon (fusion of three DNA fragments) was amplified through touchdown overlap extension PCR. Different sequences of RBS were added using specific primers. Different RgPAL/TAL operon fragments were digested by *Hind*III and *Xba*I and independently inserted to the *Hind*III/*Xba*I site of pQE-30 or pREP4.

Table 1. Plasmids, bacterial strains, primers, and RBS sequence used in this study

Plasmids/Strains	Characteristics	Source
<i>Plasmids</i>		
pMD18	mpMB1 replication region; Amp ^R High copy number	Takara Biochemicals Inc.
pQE-30	pBR322 replication region; Amp ^R Moderate copy number	Qiagen Inc.
pREP4	p15A replication region; Kan ^R Low copy number	Qiagen Inc.
pMD18-pGAP	pMD18 with pGAP	This study
pMD18-RgPAL/TAL	pMD18 with RgPAL/TAL	This study
pTAL	pMD18 with RgPAL/TAL and weak RBS in a pGAP operon	This study
pTBS	pMD18 with RgPAL/TAL and strong RBS in a pGAP operon	This study
pTAL1	pQE-30 with RgPAL/TAL and weak RBS in a pGAP operon	This study
pTBS1	pQE-30 with RgPAL/TAL and strong RBS in a pGAP operon	This study
pTK15	pREP4 with RgPAL/TAL and strong RBS in a pGAP operon	This study
<i>Strains</i>		
<i>Rhodotorula glutinis</i> GIM2.156	Produces phenylalanine	Guangdong Microbiology Culture Center, China
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80lacZ Δ M15, Δ (lacZYA-argF) U169, deoR, recA1, endA1, hsdR17 (r _k ⁻ m _k ⁺), phoA, supE44, λ ⁻ , thi-1, gyrA96, relA1	Laboratory preservation
<i>E. coli</i> BW27784	F ⁻ , Δ (araD-araB)567, Δ lacZ4787(::rrnB-3), λ ⁻ , Δ (araH-araF)570(::FRT), Δ araEp-532::FRT, ϕ Pcp18araE533, Δ (rhaD-rhaB)568, hsdR514	<i>E. coli</i> Genetic Stock Center, New Haven, CT
<i>E. coli</i> MG1665	F ⁻ , λ ⁻ , ilvG ⁻ , rfb-50, rph-1, “wild type” K-12 strain	<i>E. coli</i> Genetic Stock Center, New Haven, CT
<i>E. coli</i> BL21 (DE3)	F ⁻ , ompT, gal, dcm, lon, hsdSB(rB ⁻ mB ⁻), λ (DE3 [lacI, lacUV5-T7, gene1, ind1, sam7, nin5])	Novagen
<i>E. coli</i> MC1061	hsdR2, hsdM ⁺ , hsdS ⁺ , araD139 Δ (ara-leu)7697, Δ (lac)X74, galE15, galK16, rpsL (StrR), mcrA, mcrB1	Laboratory preservation
<i>E. coli</i> M15	Host strain for QIA expression system	Qiagen Inc.
<i>E. coli</i> TOP10	F ⁻ , mcrA Δ (mrr-hsdRMS-mcrBC), ϕ 80lacZ Δ M15, Δ lacX74 nupG, recA1, araD139, Δ (ara-leu)7697, galE15galK16, rpsL(StrR), endA1, λ ⁻	Laboratory preservation
<i>E. coli</i> ATCC31884	aroH367, tyrR366, tna-2, lacY5, aroF394 ^{fb} , malT384, pheA101 ^{fb} , pheO352, aroG397 ^{fb}	American Type Culture Collection
MGTAL	<i>E. coli</i> MG1665 with pTAL	This study
MGTBS	<i>E. coli</i> MG1665 with pTBS	This study
MGTAL1	<i>E. coli</i> MG1665 with pTAL1	This study
MGTBS1	<i>E. coli</i> MG1665 with pTBS1	This study
MGTK15	<i>E. coli</i> MG1665 with pTK15	This study
BLTBS1	<i>E. coli</i> BL21(DE3) with pTBS1	This study
DHTBS1	<i>E. coli</i> DH5 α with pTBS1	This study
BWTBS1	<i>E. coli</i> BW27784 with pTBS1	This study
MCTBS1	<i>E. coli</i> MC1061 with pTBS1	This study
M1TBS1	<i>E. coli</i> M15 with pTBS1	This study
TOTBS1	<i>E. coli</i> TOP10 with pTBS1	This study
ATTBS1	<i>E. coli</i> ATCC31884 with pTBS1	This study
<i>Primers/RBS</i>		
<i>Primers</i>		
pF_PAL/TAL_clone	5'ATGGCCCCCTCCGTCGACT3'	
pR_PAL/TAL_clone	5'CTATGCCATCATCTTGACGAGGACG3'	
pF_pGAP_weak RBS (<i>Hind</i> III)	5'AAGCTTGTGCGTAATGCTTAGGCACAGGA3'	
pR_pGAP_weak RBS	5'CGGAGGGGGCCATATATCCACCAGC3'	
pR_pGAP_strong RBS	5'ATGTATATCTCCTTCTAGTGAATAAAAGGTTGC3'	
pF_TEM	5'CAAGATGATGGCATAGAGGCATCAAATAAACGAA3'	
pR_TEM (<i>Xba</i> I)	5'CGGGATCCTCTAGAGATTTTACAGACAAGCTGT3'	
pF_RgPAL/TAL_Overlap_weak RBS	5'GCTGGTGGAATATATGCCCCCTCCGT3'	
<i>RBS</i>		
weak RBS from <i>gpd</i> in <i>E. coli</i>	5'TAGCTGGTGGAATAT3'	
strong RBS from pET32a	5'GAAGGAGATATACAT3'	

N-109aa Trx-Tag™ thioredoxin protein fusion was prepared for RgPAL/TAL in pET-32a to isolate purified recombinant protein. This process was conducted through PCR amplification using restriction tail primers and cloning between *Bam*HI and *Hind*III restriction sites downstream of the T7 promoter.

All constructed plasmids were verified via colony PCR and sequencing. All plasmids, primers, and RBS sequences are listed in Table 1.

Protein purification and *in vitro* assays *E. coli* BL21 (DE3) harboring the expression vector pET-32a-RgPAL/TAL was grown at 37°C in lysogeny broth medium containing 100 µg/mL ampicillin antibiotics to isolate recombinant RgPAL/TAL protein. Cells were cultured until Optical Density (OD)₆₀₀ reached to 0.6–0.8. Cultures were then induced with 0.5 mM IPTG and grown overnight at 20°C. The purified protein was prepared through a column of Ni-NTA His-Bind™ Resin (Qiagen) containing Ni²⁺ as an affinity ligand. Protein concentration was determined by using the Bradford method with bovine serum albumin as the standard.

Recombinant RgPAL/TAL activity was assayed with different

concentrations of L-phenylalanine (1–9 mM) and L-tyrosine (0.25–1.25 mM). The concentration of the second substrate was the saturation point. The experiment was conducted three times with 4.71 µg of purified enzyme in a final volume of 500 µL of reaction buffer containing 100 mM Tris-HCl (pH 7.0). The reaction mixture was incubated at 35°C for 30 min, and the reaction was terminated by adding 500 µL of methanol. The samples were quantified via HPLC. The Lineweaver-Burk plots of the data were used to derive the apparent K_m and k_{cat} values.

Shake flask conditions The culture medium consisted of yeast extract M9 medium (YM9) adjusted to pH 7.0 (1×M9 salts, 10 g/L yeast extract, 5% glycerol) along with 100 µg/mL ampicillin antibiotic. Recombinant *E. coli* cells containing different expression vectors were precultured overnight at 37°C in 10 mL YM9 medium. Preinoculums were diluted into 100 mL fresh YM9 medium according to the 1% volume fraction of bacteria liquid. Shaking flask fermentation was performed at 150 RPM/min and 35°C, and then different concentrations of L-tyrosine or L-phenylalanine were supplemented. The samples were collected at different times for HPLC detection.

Fig. 2. HPLC analyses of a phenylpropanoid precursor produced by engineered *E. coli*. Authentic standard of *trans*-cinnamic acid (A); extract from the culture broth of the negative control (B); extract from the culture broth of the recombinant (C); authentic standard of *p*-coumaric acid (D); extract from the culture broth of the negative control (E); extract from the culture broth of the recombinant (F).

Phenylpropanoic acid precursor bioproduction analysis All samples extracted from the cultures were centrifuged at 13,000×*g* (micro liter rotor size: 24×2 mL) for 5 min. The supernatants were analyzed on an HPLC system with a phenomenex ODS reversed-phase C-18 column (5 μm, 250×4.6 mm) and an LC2030 UV Detector. The solvent profile was acetonitrile (buffer A) and 0.1% (v/v) acetic acid in water (buffer B) as mobile phases with a flow rate of 1.0 mL/min. The injection volume was 20 μL. For *p*-coumaric acid, *trans*-cinnamic acid, and *trans*-resveratrol detection, the samples were separated with the mobile-phase composition profile fixed at 35% buffer A/65% buffer B and 50% buffer A/50% buffer B, respectively. The characteristic UV absorption spectra of the samples were 308 nm and 272 nm, respectively. The metabolites were confirmed by comparing their retention times with those of authentic standards (i.e., 4.847 and 5.560 min).

Results and Discussion

Identifying the best constitutive BioBrick combination for phenylpropanoic acid precursor bioproduction in *E. coli* BioBricks are originally defined as DNA sequences that conform to a restriction-enzyme assembly standard; these sequences contain a combination of a promoter, RBS, operon, terminator, and plasmid backbone. In bacteria, RBS is an effective control element for translation (24), and thereby, protein expression. The replication region determines the copy number of vectors and the expression level of heterogeneous genes. So we hoped for the increasing accumulation of phenylpropanoic acid precursors via BioBrick combinatorial optimization.

In this study, five constitutive expression vectors with different RBSs and replication regions were constructed and transferred into *E. coli* MG1665 for shake flask studies. The supernatant of the cultured broth was analyzed via HPLC. A peak was detected at the same retention time of *trans*-cinnamic acid and *p*-coumaric acid standards, whereas no peak appeared in the corresponding HPLC chromatogram in the negative control (Fig. 2).

As shown in Table 2, when 1 mM L-phenylalanine or L-tyrosine was used as a substrate, the maximum titer of *trans*-cinnamic acid and *p*-

coumaric acid was detected in the strain MGTBS1. With the same RBS, the production of the strain with the pBR322 replication region (moderate copy number) plasmid was higher than the strain with the p15A replication region (low copy number) plasmid and the mpMB1 replication region (high copy number) plasmid. This result may be related to the fact that the low-copy plasmid was more stable than the high-copy vector and the plasmid-specific expression of the low-copy plasmid was higher than the high-copy plasmid (25). The production of the strain with the strong RBS (RBS of pET32a) plasmid was higher than the strain with the weak RBS (RBS of *gpd* in *E. coli*) plasmid (comparison between MGTAL1 and MGTBS1 with the same replication region) because the strong RBS plasmid utilized ribosomal binding and transcriptional initiation. Therefore, different BioBrick compositions directly influenced the titers of metabolic production. We decided to use the strong RBS plasmid with moderate copy number constructs (pTBS1) for further experiments.

Determining the best strain for phenylpropanoic acid precursor biosynthesis For different strain selection, we investigated pTBS1 in eight general lab strains and observed strain-to-strain variations in *p*-coumaric acid and *trans*-cinnamic acid titers (Fig. 3A). When 1 mM L-phenylalanine or L-tyrosine was used as the substrate, the strain ATTBS1 produced higher yields of *p*-coumaric acid and *trans*-cinnamic acid in comparison with other strains, and the maximum yield was 3.6- and 7.4-fold the minimum yield (when compared with strains ATTBS1 and MCTBS1), respectively. These results demonstrate that the different strains had varied capacities for phenylpropanoic acid precursor biosynthesis. The preferable metabolic capability of *E. coli* ATCC31884 cells may be associated with its developed shikimic acid metabolism.

Next, we explored the capacities of phenylpropanoic acid precursor biosynthesis in the engineered strain ATTBS1 by shake flask culturing with no substrate added. As shown in Fig. 3B, the accumulation of *p*-coumaric acid/*trans*-cinnamic acid and the value of OD₆₀₀ were detected at different times. The production of *p*-coumaric acid continuously increased before 36 h. The highest yield of *p*-coumaric acid was 34.67±3.71 mg/L, but this amount decreased after 36 h. During cultivation, *trans*-cinnamic acid was also detected and the

Table 2. Effect of different BioBrick assembly parts on phenylpropanoic acid precursor production

Strains	Ribosome bind site ¹⁾	Replication origin	<i>trans</i> -Cinnamic acid		<i>p</i> -Coumaric acid		PAL/TAL
			Concentration (mg/L)	Fold change ²⁾	Concentration (mg/L)	Fold change ²⁾	
MGTAL	RBS2	mpMB1	11.02±0.18 ^{d3)}	1.0	1.29±0.08 ^c	1.0	8.54
MGTBS	RBS1	mpMB1	27.79±0.23 ^c	2.5	5.71±0.13 ^b	4.4	4.86
MGTAL1	RBS1	pBR322	41.77±0.17 ^b	3.7	8.53±1.04 ^b	6.5	4.89
MGTBS1	RBS2	pBR322	79.71±0.51 ^a	7.2	18.43±0.33 ^a	14.2	4.32
MGTK15	RBS2	p15A	36.39±0.75 ^b	3.3	6.29±0.56 ^b	4.8	5.78

¹⁾RBS1 is RBS of *gpd* in *E. coli*, RBS2 is RBS of pET-32a system.

²⁾Fold change relative to MGTAL.

³⁾All values are the average from three independent experiments. Values within column followed by the same letter are not significantly different at *p*<0.05.

Fig. 3. (A) Effects of different *E. coli* strains on phenylpropanoic acid precursor production. (B) Dynamics of the production of *p*-coumaric acid (black square), *trans*-cinnamic acid (black circle), and OD₆₀₀ (black triangle) with strain ATTBS1.

titer of production continuously increased before 60 h. The highest yield of *trans*-cinnamic acid was 78.81 ± 4.96 mg/L, but this amount decreased after 60 h. The OD₆₀₀ obviously decreased after 24 h possibly because of the growth inhibition by *p*-coumaric acid and *trans*-cinnamic acid.

These results highlight that we have successfully evolved an *E. coli* strain that is able to produce a phenylpropanoic acid precursor from its shikimic acid metabolism without any additive toxic inducers or amino acids. Increasing the production of L-tyrosine/L-phenylalanine is an effective and feasible strategy for the improvement of *p*-coumaric acid/*trans*-cinnamic acid biosynthesis. Recent studies have used genetically engineered *E. coli* for L-tyrosine/L-phenylalanine overproduction (26,27). This expression system can be used for these engineered *E. coli* and can facilitate the low-cost direct production of phenylpropanoids.

Moreover, to change the proportion of L-tyrosine/L-phenylalanine according to the need of synthesis by nitrogen source regulation in the culture medium was another process for product enhancement (28).

Characterization analysis of RgPAL/TAL We explored the substrate preference of RgPAL/TAL by a kinetic study to explain why the titer amount of *trans*-cinnamic acid is higher than that of *p*-coumaric acid. A kinetic study of RgPAL/TAL was conducted with L-tyrosine and L-phenylalanine as substrates. As shown in Table 3, K_m of RgPAL/TAL for

L-tyrosine (0.55 ± 0.04 mM) was lower than that of L-phenylalanine. This result indicates that RgPAL/TAL exhibits a higher affinity toward L-tyrosine than L-phenylalanine. However, k_{cat}/K_m of RgPAL/TAL was significantly greater for L-phenylalanine than for L-tyrosine. This result is consistent with previous research (23,29). However, the main strategy of synthetic bioengineering for phenylpropanoid biosynthesis is to use *p*-coumaric acid as the initial substrate rather than *trans*-cinnamic acid. *Trans*-cinnamic acid can be transformed into *p*-coumaric acid via cinnamate 4-hydroxylase (C4H), a P450 enzyme, and cannot be effectively expressed in *E. coli* (30–32). L-tyrosine can be directly converted into *p*-coumaric acid under the catalysis of TAL, thereby avoiding the C4H pathway. Consequently, TAL is suitable for phenylpropanoid biosynthesis in *E. coli* to establish a high conversion platform. Codon optimization of the *tal* gene had a good effect on *p*-coumaric acid production (10,33). Engineering an *E. coli* strain that overproduces L-tyrosine can increase the yield of phenylpropanoids through vast substrate accumulation (10). Rational enzyme reconstruction or highly active and specific TAL screening can also improve the catalytic efficiency (34); thus, this process needs to be performed in the future.

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Table 3. The kinetic properties of the RgPAL/TAL at pH 7.0 and 35°C

Substrate	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m
L-Phenylalanine	$1.72 \pm 0.12^{a1)}$	$4.45 \pm 0.57 \times 10^{3a}$	$2.57 \pm 0.14 \times 10^{3a}$
L-Tyrosine	0.55 ± 0.04^b	$0.14 \pm 0.02 \times 10^{3b}$	$0.25 \pm 0.02 \times 10^{3b}$

¹⁾All values are the average from three independent experiments. Values within column followed by the same letter are not significantly different at $p < 0.05$.

Disclosure The authors declare no conflict of interest.

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