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High-level production of *trans*-cinnamic acid by fed-batch cultivation of *Escherichia coli*

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ABSTRACT

Trans-cinnamic acid is a phenylpropanoid that is widely used in cosmetics, anti-bacterial compounds, anti-cancer, and flavoring agents. Previously, we succeeded in the generation of L-phenylalanine-high producing *Escherichia coli* strain by metabolic engineering of the L-phenylalanine biosynthesis pathway. Using this engineered strain, in this study, we developed an *E. coli* platform for the enhanced production of *trans*-cinnamic acid that can be generated from L-phenylalanine by phenylalanine ammonia-lyase (PAL)-mediated deamination reaction. To increase the production titer of *trans*-cinnamic acid, three different promoters and four different nutrient solutions were examined and, using the optimized system (gene expression under Trc promoter and supplementation of casamino acid), we achieved the production of *trans*-cinnamic acid as high as 697 mgL^{-1} in shake flask cultivation. Finally, pH-stat fed-batch fermentations were performed in a lab-scale (2 L) bioreactor with three different feeding solutions (glucose, yeast extract, and casamino acid). When casamino acid was supplied as feeding solution, the production of *trans*-cinnamic acid as high as 6.9 gL^{-1} was achieved.

1. Introduction

Trans-cinnamic acid (also called 3-phenylprop-2-enoic acid) is the major naturally occurring phenolic compound in plants that serves as a precursor of various phenylpropanoids such as lignin and flavonoids [1]. Further, it has broad biological activities including use in flavoring agents, anti-bacterial compounds, anti-cancer, cosmetics, and pharmaceuticals especially for the treatment of malaria [2–5]. In addition, *trans*-cinnamic acid is regarded as 'Generally Recognized as Safe' compound by the Food and Drug Administration (FDA), enabling its use as a food additive [6]. Furthermore, *trans*-cinnamic acid can be converted to various high-value compounds such as styrene, p-hydroxycinnamic acids, cinnamaldehyde, and caffeic acid, which have been widely used as materials for preparing fragrances for cosmetics, anti-nematode agents, thermoplastics, and pharmaceuticals (Fig. 1A) [7]. With the increasing demand for *trans*-cinnamic acid, it is also necessary to develop a cost-effective and economical approach to produce *trans*-cinnamic acid.

Currently, two methods have been used to produce *trans*-cinnamic acid; (i) direct extraction from plant sources such as cinnamon bark and (ii) chemical synthesis [8,9]. However, both manufacturing methods have shortcomings that are not suitable for large-scale production in industry. In case of direct extraction from plants, the natural amount of *trans*-cinnamic acid in plants varies depending on environmental and geographic conditions and the extraction yield is not high enough. In case of chemical synthesis, *trans*-cinnamic acid can be synthesized via condensation reaction between petrochemical benzaldehyde and organic acids (acetic acid and malonic acid) [10]. However, this process is energy intensive (requirement of high temperature and fossil resources) and the contamination by toxic byproduct and racemate production make the downstream process more difficult [11,12]. Furthermore, there is a huge public pressure to replace petroleum-derived *trans*-cinnamic acid synthesis with natural production. Therefore, a more facile method is desired for the commercial production of *trans*-cinnamic acid, and as an alternative strategy, the microbial production of *trans*-cinnamic acid has been attracting attention. In the natural biosynthesis, *trans*-cinnamic acid can be synthesized from L-phenylalanine. The L-phenylalanine is synthesized *de novo* through the shikimate pathway, which is a major source of cellular aromatic compounds, and it is deaminated to *trans*-cinnamic acid by the phenylalanine ammonia-lyase (PAL), which catalyzes non-oxidative deamination of ammonia from L-phenylalanine (Fig. 1B). With the evolved and various metabolic engineering strategies, various microorganisms such as *Escherichia coli*, *Streptomyces lividans*, *Saccharomyces cerevisiae*, and *Pseudomonas putida*

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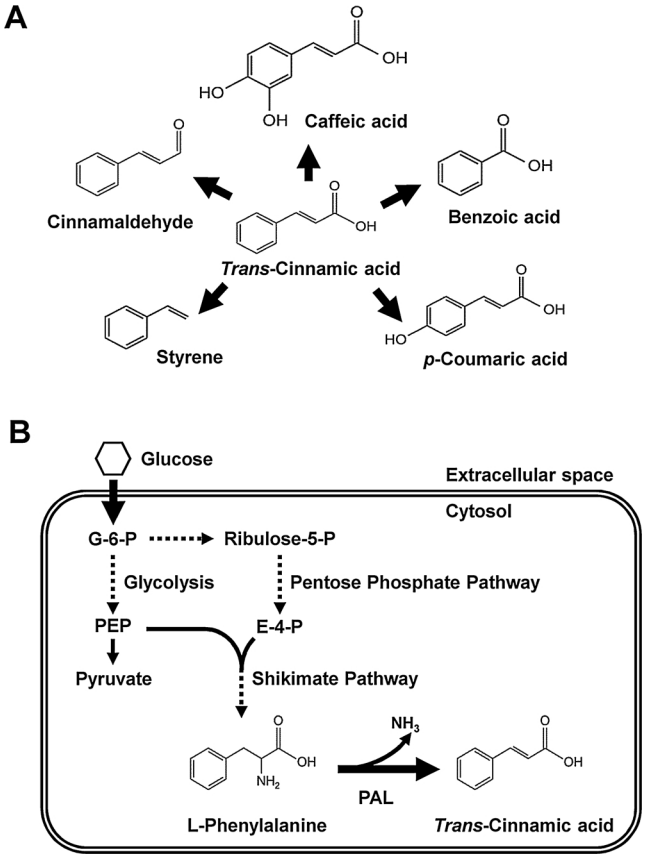

Fig. 1. Application and biosynthesis of *trans*-cinnamic acid. (A) Chemicals generated from the *trans*-cinnamic acid. (B) Schematic diagram of biosynthetic pathway of *trans*-cinnamic acid in *E. coli*.

have been used to produce *trans*-cinnamic acid as high as 0.8 gL^{-1} [7,12–17]. Recently, Masuo et al. reported an alternative pathway using D-phenyllactate instead of L-phenylalanine to produce *trans*-cinnamic acid without PAL enzyme, and much improved production titer as high as 1.7 gL^{-1} was achieved, which is the highest record in a microbial production system [18]. Although there has been considerable progress in the microbial production of *trans*-cinnamic acid, the productivity still requires further improvement.

In our previous study, we successfully developed an L-phenylalanine-overproducing *E. coli* by metabolic engineering, and the engineered *E. coli* could produce L-phenylalanine as high as 4 gL^{-1} in flask cultivation [19]. By introducing a PAL expression system into this engineered strain, we sought to develop a new *E. coli* platform for the efficient production of *trans*-cinnamic acid. To increase the expression level of PAL, we examined three different promoters and to subsequently increase the production titer, we assessed different nutrient solutions in flask cultivation. Finally, we performed fed-batch cultivation in lab-scale bioreactor (2 L working volume) and two different nutrient feeding solutions were examined for high-level production of *trans*-cinnamic acid.

2. Materials and methods

2.1. Bacterial strains and plasmids

All strains and plasmids used in this study were listed in Table 1. *E. coli* XL1-Blue was used as the host for gene cloning and plasmid maintenance. *E. coli* YHP05 that was previously engineered from *E. coli* W3110 [19] was used as a host for the production of *trans*-cinnamic acid. All DNA manipulations including restriction enzyme digestions, ligations, and transformations were performed following standard

Table 1
Bacterial strains and plasmids used in this study.

Strain	Description	Reference or Source
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacI^{q}Z ΔM15 Tn10 (Tet^r)]*	Stratagene[1]
W3110	*F$^-$ l$^-$ rph-1 INV(rrnD, rrnE)*	CGSC No.4474[2]
YHP05	W3110 *Δcrr ΔtyrR ΔtrpE ΔtyrA ΔpykA*	[19]

Plasmid	Description	Reference or Source
pTac15k	Km^R, p15A origin, *tac* promoter, 4.0 kb	[29]
pTrc99A	Amp^R, pBR322 origin, *trc* promoter, 4.2 kb	Pharmacia[3]
pBAD33	Cm^R, p15A origin, *araBAD* promoter, 5.3 kb	[28]
pHB-Tac-CA	pTac15k derivatives, P_{tac}-*Sm*PAL	This study
pHB-Trc-CA	pTrc99A derivatives, P_{trc}-*Sm*PAL	[19]
pHB-BAD-CA	pBAD33 derivatives, P_{BAD}-*Sm*PAL	This study
pYHP	pTac15k derivatives, P_{tac}-aroG8/15-ydiB-aroK- pheA^{fbr,dm} P_{pc113} − glk − T_{lpp}, P_{pc113} − galP − T_{lpp}	[19]

[1] Stratagene Cloning Systems, La Jolla, CA, USA.
[2] The Coli Genetic Stock Center, Yale University, USA.
[3] Pharmacia Biotech, Uppsala, Sweden.

protocols [20]. Polymerase chain reaction (PCR) was performed using C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with PrimeStar HS Polymerase (Takara Bio Inc., Shiga, Japan). The nucleotide sequences of all primers used in this study were listed in Table 2. All restriction enzymes were purchased from Enzynomics™ (Daejeon, Republic of Korea). For the expression of SmPAL gene under the Tac promoter or BAD promoter, SmPAL gene was amplified from pHB-Trc-CA by PCR using primers PAL-F2 and PAL-R2. The PCR product was digested with restriction enzymes *Sac*I and *Kpn*I, followed by ligation into pTac15k and pBAD33, yielding pHB-Tac-CA and pHB-BAD-CA, respectively.

2.2. Flask cultivation

For the promoter examination, *E. coli* cells were cultivated in Luria-Bertani (LB) liquid medium (10 gL^{-1} NaCl, 10 gL^{-1} tryptone, and 5 gL^{-1} yeast extract) containing 2% (w/v) glucose and appropriate antibiotics (100 mgL^{-1} ampicillin or 40 mgL^{-1} kanamycin or 35 mgL^{-1} chloramphenicol). After overnight cultivation at 37 °C with agitation at 200 rpm, the inoculum was transferred into fresh LB medium. When the optical density (OD_{600}) reached 0.6 (exponential growth phase), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for the induction of gene expression. In pHB-BAD-CA that contains arabinose-inducible BAD promoter, L-arabinose was added to a final concentration of 0.2% (w/v) for the induction of gene expression. After incubation for 6 h, cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The supernatants were filtered by 0.22 μm PVDF syringe filters, and the cell pellets were stored at −20 °C until further analysis. For the examination of complex sources, cells were cultivated in PHE medium [19] containing 20 gL^{-1} glucose, 3 gL^{-1} KH_2PO_4, 5 gL^{-1} $(NH_4)_2SO_4$, 1 gL^{-1} NaCl, 1.5 gL^{-1} sodium citrate, 3 gL^{-1} $MgSO_4{\cdot}7H_2O$, 0.015 gL^{-1} $CaCl_2{\cdot}2H_2O$, 0.075 gL^{-1} thiamine-HCl, 0.01125 gL^{-1} $FeSO_4{\cdot}7H_2O$, 0.3 gL^{-1} L-tyrosine, 0.03 gL^{-1} tryptophan, and 1.5 mL/L Trace Element Solution (TES): TES was composed of 2.0 gL^{-1} $AL_2(SO_4)_3{\cdot}18H_2O$, 0.75 gL^{-1} $CoSO_4{\cdot}7H_2O$, 2.5 gL^{-1} $CuSO_4{\cdot}5H_2O$, 0.5 gL^{-1} H_3BO_3, 14.64 gL^{-1} $MnSO_4{\cdot}H_2O$, 12 gL^{-1} $CaCO_3$, 3 gL^{-1} $Na_2MoO_4{\cdot}2H_2O$, 2.5 gL^{-1} $NiSO_4{\cdot}6H_2O$, 15 gL^{-1} $ZnSO_4{\cdot}7H_2O$, and 10 mL/L HCl. All other conditions including temperature, induction with IPTG or arabinose and cultivation time were same as cultivation in LB medium.