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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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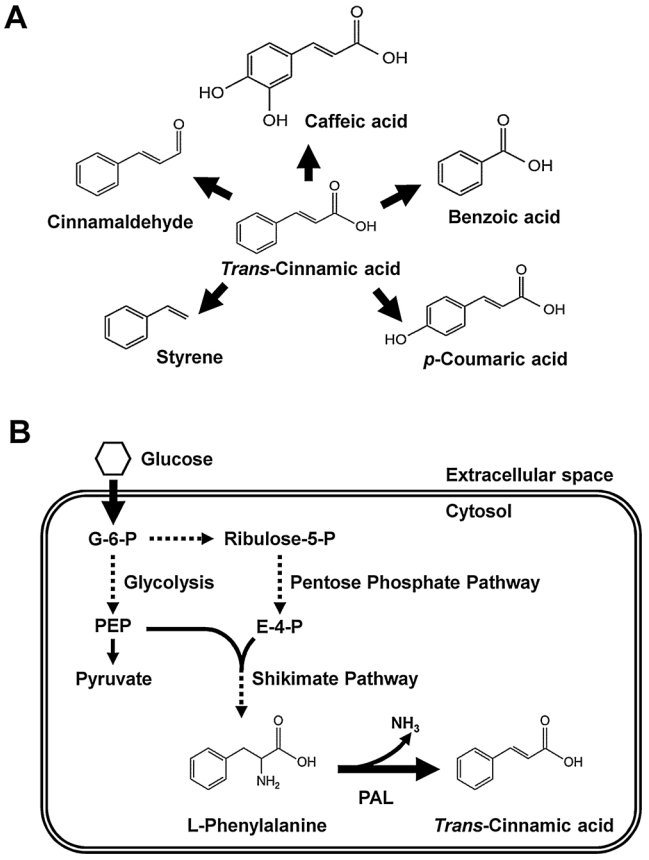

Fig. 1. Application and biosynthesis of *trans*-cinnamic acid. (A) Chemicals generated from the *trans*-cinnamic acid. (B) Schematic diagram of biosynthetic pathway of *trans*-cinnamic acid in *E. coli*.

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.

Table 2
List of oligonucleotides used in this study.

No.	Primer sequence[a] (5' → 3')
PAL-F1	**GCATCCATGGGGACCTTCGTTATTGAACTGGATATGAATGTTACCC**
PAL-R1	ATGC**GAATTC**TTATCACTTGTCATCGTCATCCTTGTAGTCGTGTGCTGCCACGGCTG
PAL-F2	GCAT**GAGCTC**TTGAACTTTAAGAAGGAGATATACATATGGGGACCTTCGTTATTGAACT
PAL-R2	ACT**GGTACCTCTAGA**TTATCACTTGTCATCGTCATCCTTGTAGTC

[a] Restriction enzyme sites are shown in bold.

2.3. Fed-batch cultivation

Fed-batch cultivations of *E. coli* YHP05 harboring pYHP and pHB-Trc-CA were performed in a 5 L bioreactor (BioCNS, Daejeon, Republic of Korea). The seed culture (200 mL) was prepared by cultivation in PHE medium containing 3 gL^{-1} yeast extract as a complex source. After cultivation for 12 h, cells were inoculated into 1.8 L of same fresh medium in a bioreactor with antibiotics (100 mgL^{-1} ampicillin and 40 mgL^{-1} kanamycin). The dissolved oxygen (DO) concentration was maintained at 40% by automatically increasing the agitation rate up to 1000 rpm and by mixing pure oxygen during the cultivation [21–23]. During cultivation, the temperature was maintained at 37 °C, and the culture pH was kept at 6.8 by the automatic addition of 25% (v/v) ammonia solution when the pH was lower than 6.77. Three different feeding solutions were supplied to the cultures: i) defined feeding solution (700 gL^{-1} glucose and 20 gL^{-1} $MgSO_4{\cdot}7H_2O$), ii) yeast extract feeding solution 1 (500 gL^{-1} glucose, 75 gL^{-1} yeast extract, and 20 gL^{-1} $MgSO_4{\cdot}7H_2O$), and iii) casamino acid feeding solution 2 (500 gL^{-1} glucose, 100 gL^{-1} casamino acid, and 20 gL^{-1} $MgSO_4{\cdot}7H_2O$) [24,25]. When the pH rose to a value greater than its set point (pH 6.8) by 0.06, an appropriate volume of feeding solution was automatically added to prevent glucose starvation.

2.4. Protein preparation and analysis

After culturing, the harvested cells were disrupted using BugBuster Master Mix (Novagen, San Diego, CA, USA). Upon cell lysis, 80 μL of each lysate was saved for the total fraction. The remaining lysate (520 μL) was centrifuged at 4 °C and 10,000 rpm for 10 min, and the supernatant was collected as a soluble fraction. Protein samples were run on 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting. Total and soluble fractions of lysates were mixed with 5X sample buffer containing SDS, bromophenol blue, and β-mercaptoethanol. After electrophoresis, all protein bands were transferred to a polyvinylidene fluoride (PVDF; Roche, Basel, Switzerland) membrane using Owl™ HEP-1 Semidry Electroblotter (Thermo Fisher Scientific, Marietta, OH, USA) at 100 mA per gel for 90 min. The membrane was blocked with 5% (w/v) skim milk in Tris-buffered saline containing Tween-20 (TBS-T; 10 mM Tris-Cl, 150 mM NaCl, and 0.05% Tween-20, pH 8.0) at 25 °C for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) dissolved in TBS-T with 5% (w/v) skim milk. After washing with TBS-T four times, ECL™ prime western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) was treated and immunoreactive bands were visualized on X-ray films.

2.5. Analytical methods

Optical density (OD) of cells was measured using a spectrophotometer (Optizen POP; Mecasys, Daejeon, Republic of Korea) at 600 nm. The concentration of *trans*-cinnamic acid in the culture broth was quantified by using HPLC (LC-20AD, CTO-20A, SPD-20A, SIL-30AC; Shimadzu, Kyoto, Japan) with Zorbax Eclipse AAA column (4.6 × 150 mm 3.5-μm; Agilent, Santa Clara, CA, USA). After cultivation, culture supernatants were filtered using 0.22 μm PVDF syringe filters (Futecs Co., Ltd., Daejeon, Republic of Korea). The detailed procedure of sample separation was described previously [19]. Briefly, binary nonlinear gradient method was used for the separation of supernatant samples with mobile phase A (0.1% (v/v) trifluoroacetic acid (TFA)) and mobile phase B (acetonitrile). Firstly, the column was equilibrated with 10% B for 1 min and the gradient was run from 10 to 70% B for next 19 min, followed by decrease of B from 70 to 10% for 5 min. Lastly, 10% B was run for 3 min. It was operated at 40 °C constantly and the flow rate was 1 mL/min. The samples were detected using UV detector (280 nm).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The harvested cells were resuspended in phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 at pH 7.2) and disrupted by sonication for 20 min (50% pulse, 20% amplitude). After cell lysis, total and soluble fractions were prepared by centrifugation as described earlier. Total and soluble fractions of samples were mixed with 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) as a final concentration of 5 μgL^{-1}, and 100 μL of each samples were loaded onto 96-well ELISA plate. After 2 h incubation at 37 °C, each well was washed with PBS-T (PBS containing 0.5% Tween-20) four times, followed by treatment of 100 μL of 5% BSA solution for 1 h at 25 °C. After washing, each well was treated with 100 μL of 1:5000-diluted horseradish peroxidase (HRP)-conjugated anti-FLAG antibody dissolved in PBS-T with 5% (w/v) skim milk for 1 h at 25 °C. Finally, the wells were washed, and 50 μL of tetramethylbenzidine (TMB) substrate (BD Biosciences, San Jose, CA, USA) was added for peroxidase reaction. The reaction was stopped by using 50 μL of 2 M H_2SO_4 and, the absorbance at 450 nm (Abs_{450}) was measured by a Tecan Infinite M200 Pro ELISA plate reader (Tecan Group Ltd., Männedorf, Switzerland).

3. Results and discussion

3.1. Gene expression with different promoters

Previously, we performed the metabolic engineering of *E. coli* for high-level production of L-phenylalanine, which is the main precursor of *trans*-cinnamic acid, and the engineered *E. coli* YHP05 harboring pYHP could produce L-phenylalanine as high as 4 gL^{-1} by flask cultivation [19]. From L-phenylalanine, *trans*-cinnamic acid can be synthesized by PAL-mediated deamination reaction (Fig. 1B) [26,27]. In previous works, several PAL enzymes were examined for the biosynthesis of *trans*-cinnamic acid, and PAL of *Streptomyces maritimus* (SmPAL) exhibited the highest activity [7,19]. Based on those results, we decided to use SmPAL in this work and, we sought to construct an efficient expression system of SmPAL in *E. coli* YHP05 harboring pYHP that can provide sufficiently high levels of L-phenylalanine for *trans*-cinnamic acid production. In general, gene expression in prokaryotic hosts is highly dependent on the promoter (strength, sequence, *etc*) [28,29]. For the efficient expression of SmPAL gene, we examined three different promoters including two IPTG-inducible promoters (P_{tac} and P_{trc}) and one arabinose-inducible BAD promoter (P_{BAD}), which also have different strengths and have been widely used for gene expression

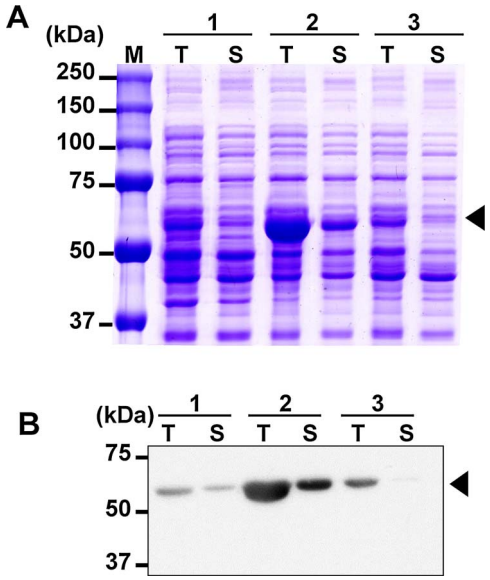

Fig. 2. Comparison of phenylalanine ammonia-lyase (SmPAL) production using three different promoters (P_{tac}, P_{trc}, or P_{BAD}). Expression level under each promoter was determined by (A) SDS-PAGE and (B) western blot analysis. Lane M, molecular size marker (kDa); lanes 1–3, pHB-Tac-CA, pHB-Trc-CA, and pHB-BAD-CA, respectively. Lanes T and S represent total and soluble protein fractions, respectively. Closed arrowheads (◀) indicate SmPAL (57 kDa).

in *E. coli* [30,31]. *E. coli* XL1-Blue harboring SmPAL expression systems using different promoters were cultivated in LB media and, SDS-PAGE and western blot analysis were performed for comparing the expression level of enzymes from each promoter system. Among the three promoter systems, P_{trc} promoter-based system (pHB-Trc-CA) showed the highest expression level although SmPAL was produced mostly as insoluble aggregates (Fig. 2). From both P_{BAD} and P_{tac} promoters, the expression levels were much lower than that under P_{trc} promoter and SmPAL was also mostly produced as insoluble aggregates. Therefore, we decided to use P_{trc}-based expression system (pHB-Trc-CA) for high-level production of *trans*-cinnamic acid.

3.2. Flask cultivation with different complex sources

Selection of the optimal medium composition is important for the high-level production of target products [25,32]. To determine the effect of complex source on the production of *trans*-cinnamic acid, four complex sources namely yeast extract, peptone, tryptone, and casamino acid were added into the PHE medium as final concentrations of 3 gL^{-1}. Tryptone, peptone, and casamino acid are formed from casein by enzymatic digestion or acid hydrolysis, so all those complex sources are useful for the supplementation of amino acids which are beneficial for protein synthesis in *E. coli* [25,33,34]. Yeast extract contains various components including nucleic acids, sugars, various ions, as well as amino acids, and it has been also widely used as a complex source in cultivation of *E. coli* [35]. Medium without any complex source was used as control. Among the four complex sources, higher cell growth and cell density was observed in yeast extract-containing medium (max. OD_{600} = 5.99) (Fig. 3A). Cultivations in tryptone (max. OD_{600} = 4.47) and casamino acid (max. OD_{600} = 3.97) showed similar cell growth but cell densities were relatively lower than that in yeast extract. The cultivations in peptone (max. OD_{600} = 2.60) and defined medium without any complex source (max OD_{600} = 2.34) showed much slower cell growth and final cell densities were also very lower than those in other complex sources. To confirm whether the expression levels of enzyme varied, the expression level of SmPAL in each cultivation was analyzed by ELISA. The cultivation in tryptone and casamino acids showed relatively higher expressions than those in cultivation with other complex

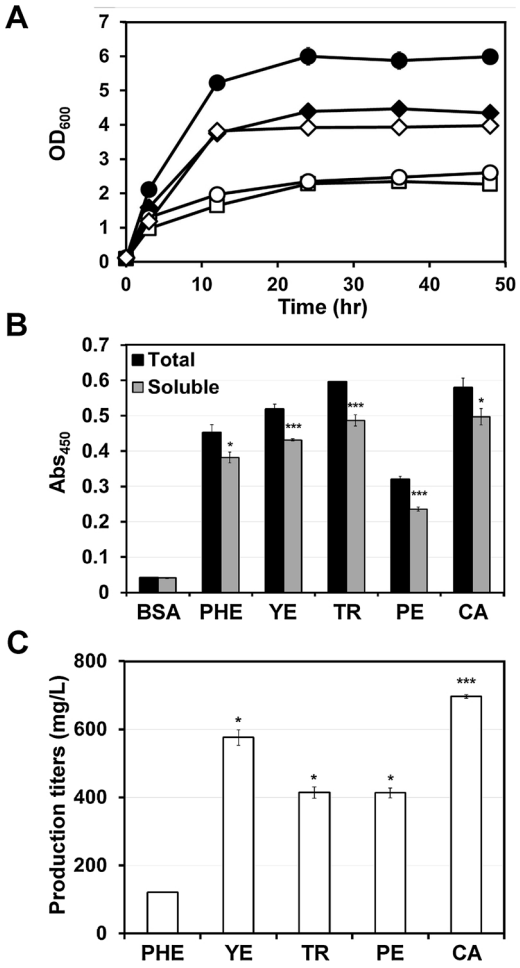

Fig. 3. Cell growth and *trans*-cinnamic acid production in flask cultivation using four different complex sources. (A) Time profiles of cell growth (OD_{600}). Symbols: Open square (□), No complex source; Closed circle (●), yeast extract; Open circle (○), peptone; Closed diamond (◆), tryptone; Open diamond (◊), casamino acid. (B) Analysis of SmPAL expression by ELISA. Black and gray bars represent the total and soluble proteins, respectively. BSA as a control. (C) Production titers of *trans*-cinnamic acid after 48 h cultivation. (B,C) PHE, no supplementation of complex source; YE, yeast extract; TR, Tryptone; PE, peptone; CA, casamino acid. Error bars represent standard deviation of the mean (n = 3) (*, $P < .05$; ***, $P < .001$).

sources, but under the supplementation of yeast extract, less expression of SmPAL gene was observed (Fig. 3B). Compared with earlier results shown in Fig. 2, it was observed that the protein solubilities in all cultivations were significantly improved (Fig. 3B). As one possible reason for higher solubility, we consider the effect of media. In Fig. 2, we used complex and rich LB medium but, in Fig. 3, we used a defined PHE medium with supplementation of complex sources. Although complex sources are supplied in the PHE medium, protein synthesis rates are generally slower in defined medium, which can prevent the protein aggregation. Compared with cultivation in LB medium, protein production levels in PHE medium were also much lower, which could not be detected by Coomassie brilliant blue staining but by western blotting. The slower and lower production of SmPAL in defined PHE medium might give a significant increase in the protein solubility. Finally, the production of *trans*-cinnamic acid in each cultivation was analyzed, and interestingly we found that the production titers of *trans*-cinnamic acid were not correlated with cell growth and SmPAL expression. When either casamino acid or yeast extract was supplied, the similarly high production (max. 697 mgL^{-1} or 576 mgL^{-1}, respectively) were achieved compared with those in cultivation with other nutrients: tryptone (max. 415 mgL^{-1}), peptone (max. 413 mgL^{-1}), and no complex source (max. 122 mgL^{-1}) (Fig. 3C). To explain the

production titer of *trans*-cinnamic acid in different complex sources, we need to consider both cell growth (Fig. 3A) and gene expression level (Fig. 3B). The supplementation of casamino acid exhibited higher gene expression and relatively good cell growth, so higher production titer could be obtained. When the yeast extract was supplied, the expression level was not high but it showed much higher cell growth, so volumetric production (mgL^{-1}) could be increased. In contrast, peptone exhibited relatively lower gene expression than casamino acid and cell growth rate was much lower than other complex sources. So, the titer of *trans*-cinnamic acid was not higher. In the case of tryptone, we don't know yet why the production titer was not high although both gene expression and cell growth were relatively good. Taken all these results, we decided to examine yeast extract and casamino acid in the following fed-batch cultivations.

3.3. Fed-batch cultivation with various feeding solutions

It is also well-known that production of desired products including recombinant proteins can be significantly affected by the cultivation conditions including temperature, pH, DO, nutrient feeding, etc. Regarding of temperature, it was previously reported that SmPAL showed higher activity at 37 °C than 30 °C [19] and it is also well known that *E. coli* can grow better at 37 °C [36]. SmPAL and *E. coli* cells also prefer the neutral pH condition, and 40% of DO is generally employed for the high cell density cultivation of *E. coli* [36,37]. Therefore, in the present study, those conditions were not further optimized but, we sought to optimize the nutrient feeding strategy which can also give a significant effect on the production [24,25,38,39]. In the aforementioned flask cultivations, supplementation with complex sources (yeast extract and casamino acid) increased the production titer of *trans*-cinnamic acid (Fig. 3C). Therefore, in order to find the optimal nutrient feeding solution in fed-batch cultivation, we also examined both nutrient solutions: yeast extract or casamino acid. First, as a control experiment, we performed the fed-batch cultivation with the defined feeding solution. In the pre-induction period, cells grew robustly with specific cell growth rate (μ) of $0.044\,h^{-1}$, but after IPTG induction at OD_{600} = 60, cell growth rate decreased gradually (Fig. 4A). Maximum cell density as high as OD_{600} of 69.4 was obtained at 62 h cultivation. Immediately after IPTG induction, the expression of SmPAL enzyme started and similar level of enzyme content was maintained in the entire post-induction period (Fig. 4B). Following enzyme production, it was also clearly observed that *trans*-cinnamic acid was produced, and its production titer continued to increase up to $4.4 \pm 1.7\,gL^{-1}$ at the end of fermentation (86 hrs) with productivity of $0.088 \pm 0.034\,gL^{-1}h^{-1}$ and yield of 0.013 ± 0.004 g/g in glucose (Table 3).

Next, a yeast extract-containing nutrient solution was supplied as feeding solution in the fed-batch cultivation. Compared with the aforementioned fermentation with defined feeding solution, higher cell growth rate ($\mu = 0.054\,h^{-1}$) was achieved upon using yeast extract (Fig. 5A). However, after IPTG induction at OD_{600} = 60, cells continued to grow with similar growth rate, and maximum cell density (OD_{600} = 89.4) was obtained at 50 hrs cultivation. The induction resulted in the expression of SmPAL, followed by production of *trans*-cinnamic acid (Fig. 5B). The production titer of *trans*-cinnamic acid increased gradually in the post-induction period, and at the end of fermentation (86 hrs), the maximal titer of *trans*-cinnamic acid ($5.0 \pm 1.2\,gL^{-1}$) could be obtained at a productivity of $0.100 \pm 0.024\,gL^{-1}h^{-1}$ and yield of 0.020 ± 0.004 g/g in glucose (Table 3).

Lastly, casamino acid was used as a nutrient feeding solution in the fed-batch cultivation. Cells showed slightly higher specific growth rate compared to the former yeast extract feeding condition ($\mu = 0.077\,h^{-1}$), but after induction, cell density did not increase further. The maximum cell density (OD_{600} = 74) could be achieved at 55 h (Fig. 6A). After IPTG induction at OD_{600} = 60, SmPAL was robustly

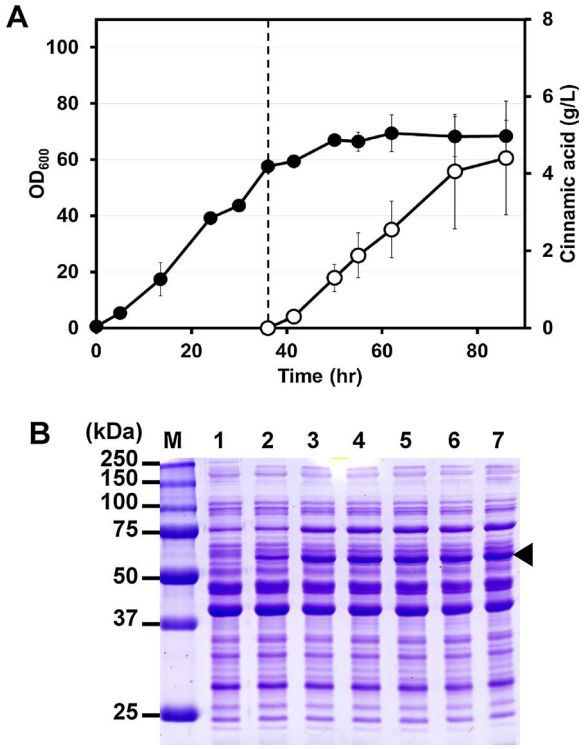

Fig. 4. Fed-batch cultivation with defined feeding solution. (A) Time profiles of cell growth (OD_{600}) and production titer of *trans*-cinnamic acid. Closed (●) and open circles (○) indicate cell growth (OD_{600}) and the titer of *trans*-cinnamic acid, respectively. The dashed line indicates the induction point. Error bars represent standard deviation of the mean (n = 2). (B) SDS-PAGE analysis of harvested samples after induction point. Lane M represents the molecular size markers (kDa). Lanes 1–7 represent the samples at 0 h, 5.5 h, 14 h, 19 h, 26 h, 39.5 h, 50 h after induction. Closed arrowhead (◀) indicates SmPAL.

Table 3
Fermentative *trans*-cinnamic acid production by different feeding solutions.

Complex source in feeding solution[1]	Specific growth rate (h^{-1})	*Trans*-cinnamic acid concentration (gL^{-1})	Productivity ($gL^{-1}h^{-1}$)	Yield (g/g in glucose)
No (defined solution)	0.044	4.4 ± 1.7	0.088 ± 0.034	0.013 ± 0.004
Yeast extract	0.054	5.0 ± 1.2	0.100 ± 0.024	0.020 ± 0.004
Casamino acid	0.077	6.9 ± 0.1	0.138 ± 0.002	0.028 ± 0.001

[1] Basically, all feeding solution contains glucose ($500\,gL^{-1}$)and $MgSO_4{\cdot}7H_2O$ ($20\,gL^{-1}$).

produced (Fig. 6B) and *trans*-cinnamic acid titer began to increase. At the end of fermentation (86 hrs), the maximum titer of *trans*-cinnamic acid was $6.9 \pm 0.1\,gL^{-1}$, the productivity was $0.138 \pm 0.002\,gL^{-1}h^{-1}$, and yield of 0.028 ± 0.001 g/g in glucose which were 1.38-fold, 1.38-fold, and 1.4-fold higher than that by fed-batch fermentation with a yeast-extract feeding solution, respectively (Table 3).

In all fed-batch fermentations, cell growth were slowed as *trans*-cinnamic acid concentrations reached about $2–3\,gL^{-1}$ (Figs. 4–6). These retardation of cell growth may be caused by the toxicity of *trans*-cinnamic acid. It is known that *E. coli* has tolerance to low concentration of *trans*-cinnamic acid (below $400\,mgL^{-1}$), and cells could not grow well at the concentrations higher than $600\,mgL^{-1}$ [40]. But, in those experiments, *trans*-cinnamic acid was added in the media before inoculation, and at this condition, cells could get damaged more

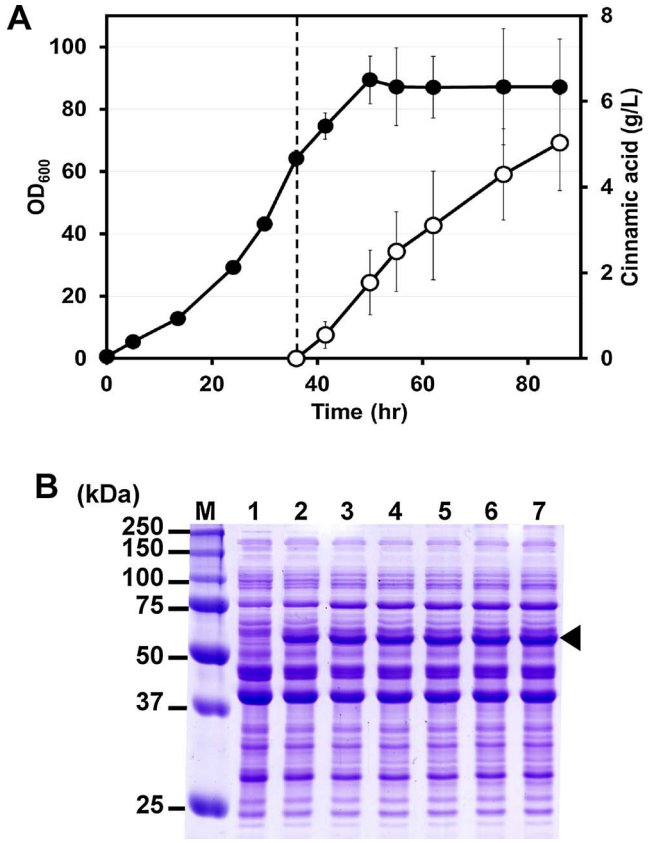

Fig. 5. Fed-batch cultivation with yeast extract feeding solution 1. (A) Time profiles of cell growth (OD_{600}) and production titer of *trans*-cinnamic acid. Closed (●) and open circles (○) indicate cell growth (OD_{600}) and the titer of *trans*-cinnamic acid, respectively. The dashed line indicates the induction point. Error bars represent standard deviation of the mean (n = 2). (B) SDS-PAGE analysis of harvested samples after induction point. Lane M represents the molecular size markers (kDa). Lanes 1–7 represent the samples at 0 h, 5.5 h, 14 h, 19 h, 26 h, 39.5 h, 50 h after induction. Closed arrowhead (◀) indicates SmPAL.

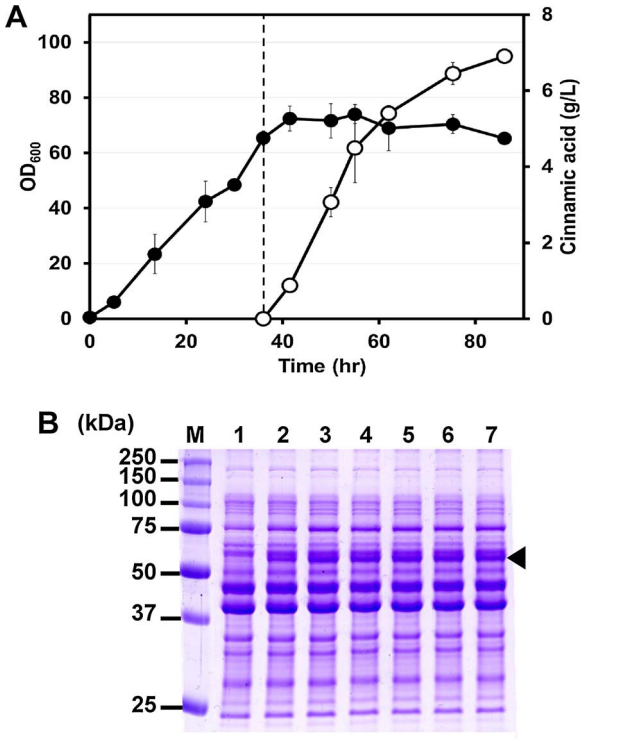

Fig. 6. Fed-batch cultivation with casamino acid feeding solution 2. (A) Time profiles of cell growth (OD_{600}) and production titer of *trans*-cinnamic acid. Closed (●) and open circles (○) indicate cell growth (OD_{600}) and the titer of *trans*-cinnamic acid, respectively. The dashed line indicates the induction point. Error bars represent standard deviation of the mean (n = 2). (B) SDS-PAGE analysis of harvested samples after induction point. Lane M represents the molecular size markers (kDa). Lanes 1–7 represent the samples at 0 h, 5.5 h, 14 h, 19 h, 26 h, 39.5 h, 50 h after induction. Closed arrowhead (◀) indicates SmPAL.

because cell density was not high. We performed the toxicity experiment where *trans*-cinnamic acids (0.5–10 gL^{-1}) were added at mid-log growth phase (OD_{600} = 2.5). We observed that cells could grow well under 2 gL^{-1} of *trans*-cinnamic acid as cell growth under no addition of *trans*-cinnamic acid (Fig. 7). However, at 4 gL^{-1}, cells growth was significantly decreased and at higher concentrations (6–10 gL^{-1}), cells did not show any growth (Fig. 7), which results were well coincided with our fermentation results (Figs. 4–6). By cell engineering strategies such as adaptive evolution, the tolerance of *E. coli* against *trans*-cinnamic acid can be further improved, and with the engineered strain, more increased production of *trans*-cinnamic acid may be achieved.

4. Conclusions

Herein, we developed an *E. coli* platform for the enhanced production of *trans*-cinnamic acid. Using the optimized expression system and casamino acid feeding solution, we achieved the production of *trans*-cinnamic acid as high as 6.9 gL^{-1} in fed-batch cultivation. To the best of our knowledge, this is the highest production titer obtained in microbial production systems [7,18], and we believe that the engineered *E. coli* strain and the fermentation process can be major contributors in the commercial production of *trans*-cinnamic acid in the bio-industry. To further increase the productivity of *trans*-cinnamic acid, we also consider the engineering of key enzyme (SmPAL). Although the crystal structure of SmPAL is not reported yet, the similar model can be used for mutagenesis, and by combination of enzyme engineering and metabolic engineering, production titer would be improved [41].

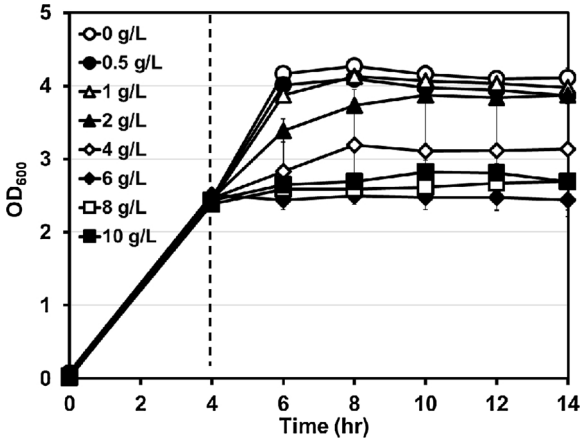

Fig. 7. Toxicity of *trans*-cinnamic acid to host strain. During the mid-log phase (OD_{600} = 2.5), *trans*-cinnamic acid was added (dashed line) as final concentrations of 0 gL^{-1}, 0.5 gL^{-1}, 1 gL^{-1}, 2 gL^{-1}, 4 gL^{-1}, and 6 gL^{-1}, 8 gL^{-1}, 10 gL^{-1}. Error bars represent standard deviation of the mean (n = 3).

Moreover, many useful building-block compounds such as styrene and caffeic acid can be derived from *trans*-cinnamic acid (Fig. 1A); therefore, the current platform can be potentially used for the production of such high-value compounds.

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