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# Production of Cinnamaldehyde through Whole-Cell Bioconversion from *trans*-Cinnamic Acid Using Engineered *Corynebacterium glutamicum*

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**ABSTRACT:** Cinnamaldehyde (CAD) has various applications in foods and pharmaceuticals and has gained prominence as a potent nematicide in agricultural research owing to its nematicidal activity. However, conventional methods of CAD production, including extraction from plants or organic chemical synthesis, are environmentally hazardous and limit its utilization for downstream applications. Here, we engineered *Corynebacterium glutamicum* as a whole-cell biocatalyst for the efficient bioconversion of *trans*-cinnamic acid (*t*-CA) into CAD. An expression module of *Mycobacterium phlei* carboxylic acid reductase was constructed for the conversion of *t*-CA to CAD. Additionally, the putative dehydrogenase-related genes (*dkgA*, *adhC*, and *cg*1176) responsible for the conversion of CAD to cinnamyl alcohol were deleted from the engineered *C. glutamicum* strain to prevent the loss of CAD. Furthermore, as the conversion is NADPH-dependent, we investigated the conversion efficiency by exchanging the putative promoter region for the *zwf* gene, which encodes glucose-6-phosphate dehydrogenase, with a strong promoter to increase the NADPH pool. Finally, a bioconversion platform using *C. glutamicum* as a whole-cell biocatalyst was developed by deleting the *vdh* gene, which is involved in the reverse conversion of CAD to *t*-CA. Taken together, a 100% conversion yield of 1.1 g/L CAD from 1.2 g/L *t*-CA was obtained within 30 min.

**KEYWORDS:** *Corynebacterium glutamicum, cinnamaldehyde, carboxylic acid reductase, whole-cell biocatalyst*



## INTRODUCTION

Cinnamaldehyde (CAD) is a major component of cinnamon essential oils produced by cinnamon plants and has been traditionally used as a food additive owing to its distinct aroma. In recent years, the functional characteristics of CAD have been studied. CAD has been found to inhibit food pathogens/food spoilage bacteria and prevent infection of plants by parasitic nematodes, one of the causes of crop productivity loss.[1,2] Furthermore, in terms of medical applications, CAD has been reported to be effective in inhibiting cancer cells that have metastasized to the lungs, ovaries, and central nervous system, as well as to promote exothermic and metabolic reactions that are important in downregulating obesity in humans and mice.[3,4] Owing to the broad applicability of CAD, there has been a significant increase in research on CAD production recently. CAD can be extracted from cinnamon plants; however, CAD production in plants is dependent on the plant's life cycle, climatic conditions, and susceptibility to plant diseases. Meanwhile, organic chemical synthesis of CAD is cost-effective and accounts for the majority of CAD production so far. Organic chemical synthesis is the primary method for CAD production, where a benzaldehyde derivative reacts with acetaldehyde under alkaline conditions; however, byproducts produced during the synthesis can act as factors causing environmental pollution and have the potential to cause safety issues in downstream applications.[5] In this regard, alternative strategies for high-efficiency CAD production using biological methods that rely on microorganisms are drawing attention.

Previously, we used metabolic engineering in *Escherichia coli* for the accumulation of L-phenylalanine (L-Phe) from glucose and assembled a *de novo* synthesis pathway capable of converting L-Phe to CAD, achieving a CAD production titer of approximately 75 mg/L.[6] However, compared to the L-Phe or *trans*-cinnamic acid (*t*-CA) production titers, the CAD production titer still needs to be improved. The low CAD production titer may be attributed to the unknown properties of endogenous genes that may be involved in the synthesis process from L-Phe to CAD, as well as optimal biosynthetic conditions that compete with each other in the metabolic pathway can inhibit high CAD production. Furthermore, the potential toxicity of CAD due to the reactivity of the aldehyde group hinders the accumulation of CAD during the cultivation.[7] In this regard, a new fermentation process based on a whole-cell biocatalyst system that directly produces CAD from *t*-CA in a single step can be a new alternative to achieve high CAD production using microorganisms.

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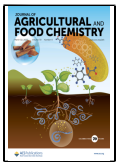

*Corynebacterium glutamicum* is an industrial microorganism that was used to produce L-amino acids, proteins, and compounds in cosmetics.[8−10] *C. glutamicum* is regarded as a promising host for industrially relevant biotechnological production owing to the following characteristics: (i) it has a capability for high-cell-density growth resulting in a high yield of target products; (ii) being recognized as a safe host for production; (iii) recent advances in optimized genetic tools for *C. glutamicum* enable a wide range of industrial applications.[11] As a result, many studies have recently reported a whole-cell biocatalyst design using *C. glutamicum* to produce various chemicals, including phenylpropanoid-derived compounds.[12−17]

We previously reported on the engineering of *C. glutamicum* for the biosynthesis of *t*-CA from L-Phe.[18] An efficient bioconversion process with a conversion yield of 75% was successfully developed by combining *C. glutamicum* as a biocatalyst with a micromembrane-based cell recycling system. Here in the presented study, we developed a whole-cell biocatalyst system in engineered *C. glutamicum* to efficiently produce CAD from *t*-CA (Figure 1). We first constructed an

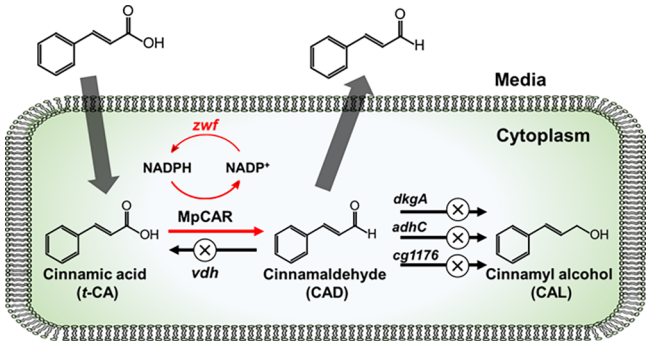


**Figure 1.** Schematic of *C. glutamicum* engineering for the efficient conversion of *t*-CA to CAD with a whole-cell biocatalyst system.

expression system for the *Mycobacterium phlei* carboxylic acid reductase (MpCAR) gene for the direct conversion of *t*-CA to CAD (Figure 2A). The putative genes involved in the conversion of CAD to cinnamyl alcohol (CAL) were then deleted in *C. glutamicum* in order to increase the CAD bioconversion yield. Furthermore, we examined the conversion efficiency by increasing the pool of NADPH, a cofactor required for *t*-CA conversion. Finally, the *vdh* gene was deleted to prevent the reverse conversion of the produced CAD to *t*-CA, and the bioconversion process with *C. glutamicum* as a whole-cell biocatalyst was developed with high CAD conversion efficiency.

## MATERIALS AND METHODS

**Bacterial Strains and Cultivation.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) competent cells were used for recombinant DNA work, while *C. glutamicum* ATCC13032 was used as the host for CAD production. *E. coli* XL1-Blue was cultivated in a Luria-Bertani medium (Becton-Dickinson, Franklin Lakes, NJ, USA) at 37 °C and 200 rpm. *C. glutamicum* was cultivated in a brain heart infusion (BHI; Becton-Dickinson) medium or modified CGXII medium (3 g/L $K_2HPO_4$, 1 g/L $KH_2PO_4$, 2 g/L urea, 10 g/L $(NH_4)_2SO_4$, 2 g/L $MgSO_4$, 200 μg/L biotin, 5 mg/L thiamine, 10 mg/L calcium pantothenate, 10 mg/L $FeSO_4$, 1 mg/L $MnSO_4$, 1 mg/L $ZnSO_4$, 200 μg/L $CuSO_4$, 10 mg/L $CaCl_2$, and 20 g/L glucose)[19] supplemented with a 15 g/L BHI (Becton-Dickinson) medium and 7

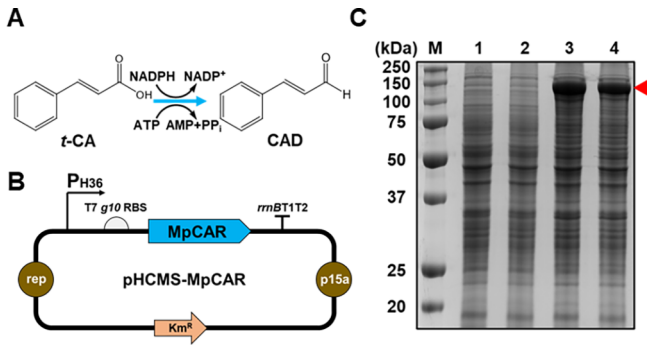


**Figure 2.** Expression of MpCAR in *C. glutamicum* for CAD production. (A) Schematic of conversion of *t*-CA to CAD by CAR. (B) Schematic diagram of the plasmid pHCMS-MpCAR constructed for the expression of the gene encoding MpCAR in *C. glutamicum*. $P_{H36}$, strong synthetic promoter; *rrnB*T1T2, transcriptional terminator; rep, replication origin of *C. glutamicum*; p15a, replication origin of *E. coli*. (C) SDS-PAGE analysis for the expression of MpCAR in *C. glutamicum* wild-type and strain MpV1. Lane 1, total protein fractions of wild-type; Lane 2, soluble protein fractions of wild-type; Lane 3, total protein fractions of MpV1; Lane 4, soluble protein fractions of MpV1; Lane M, molecular weight markers (kDa). The arrowhead (red triangle) indicates the band of MpCAR (~127 kDa).

g/L casamino acids (Becton-Dickinson). In all cultivations, kanamycin (25 μg/mL) was added to the culture medium as the sole antibiotic.

**Strain and Plasmid Construction.** For the gene expression in *C. glutamicum*, pHCMS, the high-copy-number plasmid derivative of pCES208 was used as a backbone plasmid.[20] Polymerase chain reaction (PCR) was performed using a C1000™ thermal cycler (Bio-Rad, Richmond, CA, USA) and PrimeSTAR HS Polymerase (Takara Bio Inc., Shiga, Japan). All the oligonucleotides used for PCR are listed in Table 2.

For the MpCAR expression, the *MpCAR* gene was amplified from the genomic DNA of *M. phlei* ATCC11758 via PCR with two primers (F-MpCAR and R-MpCAR). The PCR product was digested with two restriction enzymes (*Nde*I and *Not*I) and cloned into pHCMS, yielding pHCMS-MpCAR.

All genome engineering (replacement or deletion) in *C. glutamicum* was performed with the double crossover method using pK19mobsacB.[21] For deletion of the *dkgA* gene in the chromosome, pK19mobsacB-*dkgA* was constructed. The *dkgA* gene and its upstream region were amplified from the chromosome of *C. glutamicum* through PCR with the primers F-dkgAUp and R-dkgAUp. The primers F-dkgADown and R-dkgADown were then used to amplify the downstream region and a portion of the *dkgA* gene of *C. glutamicum*. A third PCR using the primers F-dkgAUp and R-dkgADown was used to join the two PCR products. The final PCR product was digested with *Sph*I and *Eco*RI and cloned into pK19mobsacB digested with the same restriction enzymes, yielding pK19mobsacB-*dkgA*. After the transformation of pK19mobsacB-*dkgA* into wild-type *C. glutamicum*, the deletion of *dkgA* was confirmed through colony PCR using the two primers F-dkgAUp and R-dkgADown. The *dkgA*-deleted strain was named *C. glutamicum* VS-1.

For deletion of the *adhC* gene, pK19mobsacB-*adhC* was constructed. The primers F-adhCUp and R-adhCUp were used to amplify the upstream region and a portion of the *adhC* gene from *C. glutamicum*. The primers F-adhCDown and R-adhCDown were then used to amplify the downstream region and a portion of the *adhC* gene of *C. glutamicum*. The two PCR products were then subjected to a third PCR using the primers F-adhCUp and R-adhCDown. The final PCR product was digested with *Sph*I and *Eco*RI and cloned into pK19mobsacB, yielding pK19mobsacB-*adhC*. After the transformation of pK19mobsacB-*adhC* into *C. glutamicum* VS-1, the deletion of *adhC* was confirmed through colony PCR using the two primers F-


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**Table 1. Bacterial Strains and Plasmids Used in This Study**

| strains or plasmids | description | reference |
|---|---|---|
| strains | | |
| *E. coli* XL1-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacIqZDM15 Tn10* ($Tet^R$)] | Stratagene[a] |
| *M. phlei* ATCC 11758 | the wild-type strain | ATCC |
| *C. glutamicum* ATCC13032 | the wild-type strain | ATCC |
| V5-1 | *C. glutamicum* Δ*dkgA* | this study |
| V5-2 | *C. glutamicum* Δ*dkgA* Δ*adhC* | this study |
| V5-3 | *C. glutamicum* Δ*dkgA* Δ*adhC* Δ*cg*1176 | this study |
| V7-3 | V5-3 + replacement of *zwf* own promoter with $P_{H36}$ and T7 *g*10 RBS | this study |
| V9-3 | V7-3 + deletion of *ncgl*2578 | this study |
| MpV1 | *C. glutamicum* harboring pHCMS-MpCAR | this study |
| MpV5-3 | *C. glutamicum* V5-3 harboring pHCMS-MpCAR | this study |
| MpV7-3 | *C. glutamicum* V7-3 harboring pHCMS-MpCAR | this study |
| MpV9-3 | *C. glutamicum* V9-3 harboring pHCMS-MpCAR | this study |
| plasmids | | |
| pCES208 | *E. coli-C. glutamicum* shuttle vector, $Km^R$ | 40 |
| pCES-PLPV | pCES208 derivative; MCS and *rrn* terminator, $Km^R$ | 41 |
| pHCMS | pCES-PLPV derivative; *parB* nonsense mutation, $Km^R$ | 20 |
| pHCMS-MpCAR | pHCMS derivative; $P_{H36}$, T7 *g*10 RBS, MpCAR, $Km^R$ | this study |
| pCES-H36-GFP | pCES208 derivative; $P_{H36}$, eGFP, $Km^R$ | 22 |
| pK19mobsacB | mobilizable vector, $Km^R$ | 21 |
| pK19mobsacB-*dkgA* | pK19mobsacB derivative for *dkgA* deletion | this study |
| pK19mobsacB-*adhC* | pK19mobsacB derivative for *adhC* deletion | this study |
| pK19mobsacB-*cg*1176 | pK19mobsacB derivative for *cg*1176 deletion | this study |
| pK19mobsacB-$P_{H36}$-*zwf* | pK19mobsacB derivative, exchange vector with H36 promoter and T7 *g*10 RBS flanked by upstream and downstream regions of *zwf* | this study |
| pK19mobsacB-*vdh* | pK19mobsacB derivative for *ncgl*2578 deletion | this study |

[a]Stratagene Cloning Systems, La Jolla, CA, USA

adhCUp and R-adhCDown. The *adhC*-deleted strain was named *C. glutamicum* V5-2.

For deletion of the *cg*1176 gene, the primers F-cg1176Up and R-cg1176Up were used to amplify the upstream region and a portion of the *cg*1176 gene from *C. glutamicum* via PCR. The primers F-cg1176Down and R-cg1176Down were then used to amplify the downstream region and a portion of the *cg*1176 gene of *C. glutamicum*. The two PCR products were subjected to a third PCR using the primers F-cg1176Up and R-cg1176Down. The final PCR product was digested with *Hind*III and *Eco*RI and cloned into pK19mobsacB, yielding pK19mobsacB-*cg*1176. After the transformation of pK19mobsacB-*cg*1176 into *C. glutamicum* V5-2, the deletion of *cg*1176 was confirmed through colony PCR using the primers F-cg1176Up and R-cg1176Down. The *cg*1176-deleted strain was named *C. glutamicum* V5-3.

For integration of the *zwf* gene expression cassette under the synthetic promoter ($P_{H36}$),[22] pK19mobsacB-$P_{H36}$-*zwf* was constructed. The primers F-zwfUp and R-zwfUp were used to amplify the upstream region of the *zwf* gene from *C. glutamicum*. The primers F-zwfDown and R-zwfDown were then used to amplify the downstream region and a portion of the *zwf* gene of *C. glutamicum*. The two PCR products were then overlapped with the region of the $P_{H36}$ promoter in the pCES208-H36-GFP[22] vector using a third PCR with the primers F-zwfUp and R-zwfDown. The final PCR product was digested with *Sph*I and *Xma*I and cloned into pK19mobsacB, yielding pK19mobsacB-$P_{H36}$-*zwf*. After the transformation of pK19mobsacB-$P_{H36}$-*zwf* into *C. glutamicum* V5-3, the 104 bp putative promoter region of *zwf* was exchanged into the H36 promoter,[22] and the T7 gene 10 leader sequence as the ribosome-binding site (RBS) was confirmed via colony PCR using the two primers F-zwfUp and R-zwfDown. The resulting strain was named *C. glutamicum* V7-3.

For deletion of the *vdh* gene, the primers F-vdhUp and R-vdhUp were used to amplify the upstream region and a portion of the *ncgl*2578 gene from *C. glutamicum*. The primers F-vdhDown and R-vdhDown were then used to amplify the downstream region and a portion of the *ncgl*2578 gene of *C. glutamicum*. The two PCR products underwent a third PCR using the primers F-vdhUp and R-vdhDown. The final PCR product was digested with *Hind*III and *Eco*RI and cloned into pK19mobsacB, yielding pK19mobsacB-*vdh*. After the transformation of pK19mobsacB-*vdh* into *C. glutamicum* V7-3, the deletion of *ncgl*2578 was confirmed through colony PCR using the two primers F-vdhKO and R-vdhKo. The *ncgl*2578-deleted strain was named *C. glutamicum* V9-3.

**Protein Preparation and Analysis.** SDS-PAGE gels were run using the total and soluble protein fractions of *C. glutamicum* wild-type and strain MpV1. Cells were grown in a BHI (Becton-Dickinson) medium for 12 h at 30 °C. After that, cells were centrifuged (3300*g*, 5 min, 4 °C) and disrupted by sonication (7 min, 5 s on, and 3 s off). Soluble protein fractions in the supernatant were prepared by centrifugation of crude cell lysates with 15,000*g* for 5 min at 4 °C. Protein samples were loaded onto 12% polyacrylamide gel, and electrophoresis was performed. Then, the gel was stained with Coomassie brilliant blue (Bio-Rad, Richmond, CA, USA) for 30 min and then destained with a destaining solution (10% [v/v] methanol, 10% [v/v] acetic acid).

**Whole-Cell Bioconversion in a Shake Flask.** To analyze CAD production, *C. glutamicum* strains were first inoculated into a BHI (Becton-Dickinson) medium and grown at 30 °C for 12 h with shaking at 200 rpm. The fully grown cultures were then transferred at a concentration of 1/100 into 50 mL of modified CGXII medium in 250 mL baffled flasks, and cells were cultivated at 30 °C with shaking (200 rpm). The $OD_{600}$ of the cells was measured with a spectrophotometer (Optizen POP; Mecasys, Daejeon, Republic of Korea) for determining cell growth. After the cells reached the stationary phase with 16 h cultivation, 12.5 mL of fermentation broth containing 6 g/L of *t*-CA[23] was added to the cells for the conversion of *t*-CA into CAD. To prepare the fermentation broth containing *t*-CA as a cell-free substrate, we prepared a fermentation broth containing *t*-CA by performing fed-batch fermentation of engineered *E. coli* YHP05 harboring pYHP and pHB-Trc-CA that was previously developed for the enhanced production of *t*-CA.[23] Then, the fermentation broth was centrifuged (25,000*g*, 20 min, 4 °C) and filtered through a 0.22 μm membrane filter (Whatman, Maidstone, UK). All conversion reactions were executed in a shaking incubator (200 rpm) at 30 °C.

To analyze the reverse conversion profiles of CAD to *t*-CA, after the cells were cultured as described above, 5 mL of 13.2 g/L CAD in dimethyl sulfoxide was added to the cells for the conversion of CAD to *t*-CA.

**$NADP^+$/NADPH Quantification Assay.** To analyze the $NADP^+$/NADPH concentration, *C. glutamicum* strains were inoculated and grown in the same manner as during the shake. After the cells reached the stationary phase with 16 h cultivation, the cells were harvested by centrifugation (3300*g* for 10 min at 4 °C). Cells were then washed with ice-cold PBS (phosphate-buffered saline), lysed with $NADP^+$/NADPH extraction buffer (Biovision, Milpitas, CA, USA) in a microcentrifuge tube, and kept on ice for 10 min. The crude extracts were centrifuged at 15,000*g* for 10 min to obtain the supernatant. The amount of $NADP^+$/NADPH in the cells was measured using the $NADP^+$/NADPH Quantitation Colorimetric Kit (Biovision).



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**Table 2. Oligonucleotides Used in This Study**

| primer | sequence (5′ to 3′) |
|---|---|
| F-MpCAR | TACATATGATGGCATCAGAATCCCGTGAC |
| R-MpCAR | GCGGCCATGTCTTCCTATTAATGGTGGTGATGATGGTGGAGCAGCCCGAGCAGCCG |
| F-dkgAUp | CGATTAGCATGCCTGCTTCTTCGGCGGAGTT |
| R-dkgAUp | AAACCGTTCTGCAGGTGCCCTTGAACACACCAAAACCAAGCT |
| F-dkgADown | GGCACCTGCAGAACGGTTT |
| R-dkgADown | CGTAATGAATTCCGTGGATCAAGAAAAGCTCATCA |
| F-adhCUp | CATTAGCATGCTCACAGCTGAAGAACTCACAGTGC |
| R-adhCUp | GTACTTGTCGACTGGGATGGATGTCCGACGTTTCCGCGAAG |
| F-adhCDown | CATCCATCCCAGTCGACAAGTAC |
| R-adhCDown | CTAATGAATTCATCCAAAGGCTATTTTACATCACTTAGA |
| F-cg1176Up | CATTAAAGCTTAACGCAACGAAGGCAAAAGA |
| R-cg1176Up | GTTTGGCACCGAAGCCGGCGCCGAATACATTGACCTC |
| F-cg1176Down | CGGCTTCGGTGCCAAACC |
| R-cg1176Down | CTAATGAATTCACGTGGTAGCCGACCTCGA |
| F-zwfUp | GATTAGCATGCTCCACTCTGTGGCTTCCTTCTT |
| R-zwfUp | TGAACGCCGGAGGATCAGCTACTTCAGGCGAGCTTCCATG |
| F-zwfDown | AACTTTAAGAAGGAGATATACATGTGAGCACAAACACGACCCC |
| R-zwfDown | GTAATCCCGGGTCTTCGGTGGATTCAGCCAT |
| F-vdhUp | CGCGGATCCCGCCACATCACCGACGGAC |
| R-vdhUp | GGATCGGCATCAAGCGCAGC |
| F-vdhDown | GCTGCGCTTGATGCCGATCCCTCCACCCACTGACCTCCG |
| R-vdhDown | CCCAAGCTTTGCACTTCCCGGAGGCTACC |
| F-vdhKO | TCAACAGAATTCCTCGAGTGCAAAAATTCCT |
| R-vdhKO | GCATGGTTCACATCAATGACCTCACC |

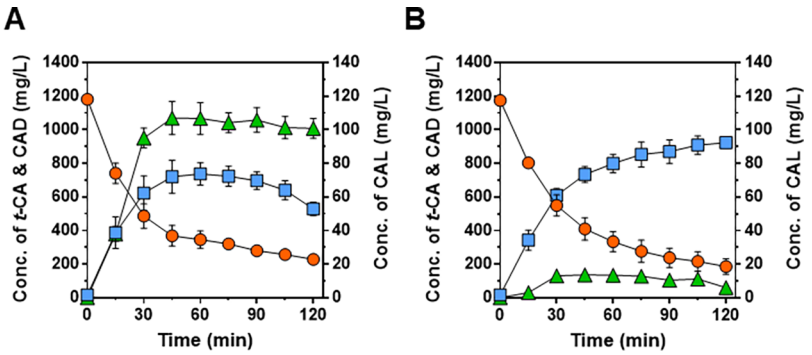


**Figure 3.** CAD production profiles of *C. glutamicum* MpV1 and MpV5-3 in shake-flask cultures. (A) Conversion profiles of *t*-CA to CAD in MpV1. (B) Conversion profiles of *t*-CA to CAD in MpV5-3. (A) and (B) symbols: *t*-CA, circle; CAD, square; CAL, triangle. Results represent mean values and standard deviations obtained from two independent experiments.

**Whole-Cell Bioconversion in a Bioreactor.** *C. glutamicum* strains were seeded in a BHI medium. After cultivation for 12 h, the cells were transferred into 200 mL of modified CGXII medium in four 250 mL baffled flasks. The seed culture was poured into 1.8 L of fresh modified CGXII medium in a 5 L jar bioreactor (BioCNS, Daejeon, Republic of Korea). The cultivation temperature was set at 30 °C until the cells reached the stationary phase. While the cells were growing up to the stationary phase, the pH was controlled at pH 7 with 20% (v/v) sulfuric acid and 5 M ammonia solution. After the cells reached the stationary phase, i.e., after 8 h of cultivation, 500 mL of fermentation broth containing 6 g/L of *t*-CA[23] was added to the cells for the conversion of *t*-CA to CAD. The fermentation broth containing *t*-CA was prepared as a cell-free substrate in the same manner as described above. All conversion reactions were performed at 200 rpm and 30 °C.

**High-Performance Liquid Chromatography (HPLC) Analysis.** The amounts of *t*-CA, CAD, and CAL in the conversion reactions were quantified by HPLC. The cells were harvested by centrifugation at 15,000*g* for 5 min at 4 °C. Then, the supernatant was prepared by filtration with a 0.22 μm syringe filter (Futecs, Daejeon, Republic of Korea) and diluted 1:10 with 10% (v/v) acetonitrile. The samples were measured by HPLC (UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Zorbax Eclipse AAA column (150 × 4.6 mm 3.5 μm; Agilent Technologies, PA, CA, USA). The samples were separated with eluent A (0.1% (v/v) trifluoroacetic acid) and eluent B (acetonitrile). The flow rate was 1 mL/min, and the temperature was set at 40 °C. The gradient was as follows: 1 min 90% A, 20 min 30% A, 25 min 90% A, and 28 min 90% A. The samples were detected at 210 nm wavelength.

## RESULTS AND DISCUSSION

**Expression of the *MpCAR* Gene in *C. glutamicum* for CAD Bioconversion.** To establish the single-step bioconversion process for CAD production, carboxylic acid reductase (CAR, EC 1.2.1.30) was considered as a suitable catalyst to be introduced in *C. glutamicum*. In microorganisms, CAR catalyzes the reduction of aliphatic fatty acids and aromatic carboxylic acids to the corresponding aldehyde forms at the expense of ATP (adenosine triphosphate) and NADPH to produce AMP (adenosine monophosphate), $PP_i$ (pyrophos-


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phate), and $NADP^+$ as byproducts[24] (Figure 2A). In addition, owing to the broad-substrate specificity for phenylpropanoids including benzoic acid, ferulic acid, and even *t*-CA, CAR has the potential of being a part of a toolbox in the production of aromatic aldehydes such as vanillin, benzaldehyde, and CAD via microbial cell factories.[25,26] *M. phlei* carboxylic acid reductase (MpCAR) has been reported to have higher enzymatic activity than CARs from other bacteria or fungi.[24] Hence, we employed MpCAR for the construction of a bioconversion system for CAD. The *MpCAR* gene was cloned into a high-copy-number plasmid, pHCMS,[20] in which the *MpCAR* gene was expressed under the control of a synthetic constitutive promoter ($P_{H36}$)[22] (Figure 2B). Additionally, for the stable expression of the *MpCAR* gene in *C. glutamicum*, the phage T7 gene 10 leader sequence as RBS was introduced since 5′ UTR (5′ untranslated region) can affect gene expression irrespective of the transcription start site[27] (Figure 2B). Based on the constructed plasmid (pHCMS-MpCAR), we checked for the synthesis of the soluble heterologous protein (~127 kDa) in *C. glutamicum* (Figure 2C).

Next, to ensure economic feasibility, we evaluated CAD production using the crude *t*-CA substrate present in the fermentation broth by performing fed-batch fermentation of engineered *E. coli* YHP05 harboring pYHP and pHB-Trc-CA.[23] After the transformation of *C. glutamicum* using pHCMS-MpCAR, the recombinant strain MpV1 was evaluated in a shake flask. Cells were grown for 16 h to reach the stationary phase. After that, the fermentation broth containing *t*-CA[23] was immediately added to the cells to analyze the CAD production efficiency. As shown in Figure 3A, 1.2 g/L of *t*-CA was rapidly converted to CAD, and 0.7 g/L of CAD was produced with a conversion rate of 0.9 g/L/h and a yield of 65% (0.65 mol/mol) at 45 min. However, the yield of CAD gradually decreased over time (Figure 3A), and CAL was gradually produced up to 100 mg/L during the bioconversion reaction (Figure 3A). In this respect, we hypothesized that intracellular metabolic pathways in *C. glutamicum* converted the synthesized CAD to CAL.

**Enhancing the CAD Yield by Deleting the CAL Pathway.** Endogenous reductases and/or dehydrogenases in bacterial hosts can spontaneously convert aldehyde molecules to alcohols. As a result, many studies have proposed different approaches to remove putative genes in order to increase the efficiency of aldehyde biosynthesis.[28−30] For example, in *E. coli* MG1655, the deletion of seven genes (encoding three aldo-keto reductases, three aldehyde dehydrogenases, and one transcription activator) that are involved in the conversion of benzaldehyde to benzyl alcohol resulted in a 55-fold higher production yield for benzaldehyde compared to that in the wild-type host.[31] Another example is the deletion of five aldehyde reductase genes involved in the reduction of a wide range of aldehyde types (C2−C12) to alcohols; this deletion substantially increased aldehyde production titers.[30] Based on BLAST search and genome sequence analysis, we predicted that the following three genes are related to convert CAD to CAL in *C. glutamicum*: *adhC* encoding alcohol dehydrogenase, *dkgA* encoding 2,5-diketo-D-gluconate reductase, and *cg*1176 encoding a short-chain dehydrogenase. All these genes were deleted from the chromosome of MpV1, and the resulting engineered strain was named MpV5-3. As shown in Figure 3B, in contrast to the loss of CAD after 45 min in MpV1, MpV5-3 exhibited continuous production of CAD, and the production of CAL was reduced to approximately 10 mg/L (Figure 3B). From 1.2 g/L of *t*-CA, 0.9 g/L of CAD was produced with a conversion rate of 0.45 g/L/h and a yield of 84% (0.84 mol/mol) at 120 min (Figure 3B). Based on these findings, it was evident that blocking the CAL conversion pathway could contribute to an increased CAD production yield. However, it was observed that CAD bioconversion did not consume the entire 1.2 g/L of *t*-CA, and production of CAD was stopped with approximately 0.2 g/L of *t*-CA remaining in both MpV1 and MpV5-3 (Figure 3A,B).

**Examination of CAD Bioconversion by Increasing the NADPH Pool.** In biological systems, many enzymes require a continuous supply of cofactors such as ATP and NADPH, and various strategies for cofactor supplementation have been successfully employed to improve production.[32,33] CAR, the enzyme that converts *t*-CA to CAD, also requires ATP and NADPH,[34] and we assumed the incomplete consumption of *t*-CA, as observed in MpV5-3, could be attributed to the insufficient supplementation of cofactors. Therefore, we subsequently focused on increasing the NADPH pool. In *C. glutamicum*, glucose-6-phosphate dehydrogenase is an enzyme involved in NADPH production via the pentose phosphate pathway.[35] To increase the NADPH pool in the cytoplasm of the MpV5-3 strain, we replaced the putative promoter region of the *zwf* gene encoding glucose-6-phosphate dehydrogenase with the $P_{H36}$ promoter,[22] resulting in strain MpV7-3 (Figure 4A). As shown in Figure 4B, the concentration of NADPH in the cytoplasm of MpV7-3 increased approximately 1.5-fold compared to MpV5-3. After that, the bioconversion of *t*-CA to CAD with MpV7-3 was evaluated in a 2 L bioreactor cultivation. MpV7-3 was cultivated for 7 h until it reached an $OD_{600}$ of 50 (Figure S1). After reaching the stationary

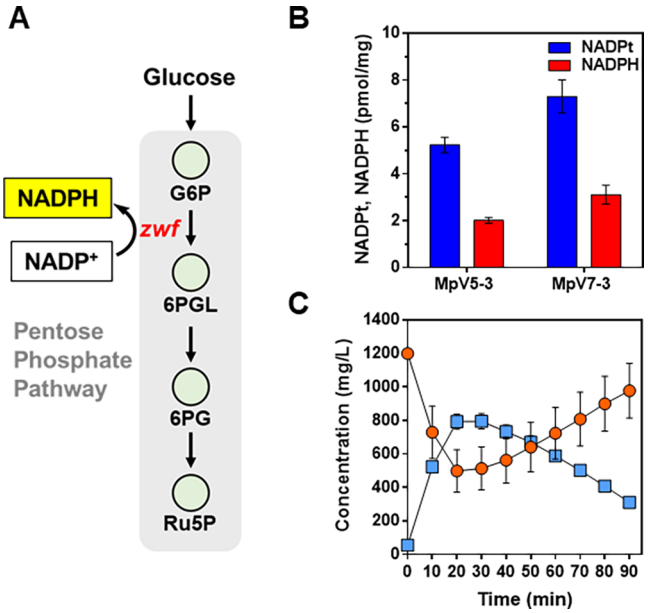


**Figure 4.** Performance analysis for CAD production in *C. glutamicum* MpV7-3. (A) Scheme of the engineering strategy. Abbreviations: G6P, glucose-6-phosphate; 6PGL, 6-phosphoglucono-1,5-lactone; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate. (B) NADPt ($NADP^+$ + NADPH) and NADPH concentration in *C. glutamicum* MpV5-3 and MpV7-3. (C) CAD production profile of *C. glutamicum* MpV7-3 in reactor-scale cultivation. Symbols: *t*-CA, circle; CAD, square. Results represent mean values and standard deviations obtained from two independent experiments.


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phase and incubating further for 1 h, 1.2 g/L of *t*-CA was then added to the cells for its bioconversion to CAD. From 1.2 g/L of *t*-CA, MpV7-3 produced 0.8 g/L of CAD in 20 min, 25 min faster than MpV5-3. However, after the bioconversion by MpV7-3 for 20 min, the remaining 0.5 g/L of *t*-CA was not consumed, and the amount of converted CAD rapidly decreased, while the *t*-CA concentration substantially increased (Figure 4C). As shown in Figure 4C, cofactor supplementation in MpV7-3 could accelerate the conversion of *t*-CA into CAD, and we assumed that regeneration of *t*-CA was caused by an unknown reverse reaction from CAD to *t*-CA at high CAD concentrations. When we checked the titer of CAL, the generation of CAL was also observed, but its titer (below 30 mg/L) was much lower than that of CAD (Figure S3A). This result means that the blocking of the CAL conversion pathway by deletion of *adhC*, *dkgA*, and *cg*1176 was also effective on bioconversion in a large-scale bioreactor.

**Prevention of Reverse Conversion to *t*-CA through Deletion of *vdh*.** Vanillin dehydrogenase (VDH) (EC 1.2.1.67) is an enzyme that uses vanillin (4-hydroxy-4-methoxybenzaldehyde) to produce vanillic acid (4-hydroxy-3-methoxybenzoic acid).[36] The putative VDH gene (*ncgl*2578) in *C. glutamicum* has been reported to exhibit catalytic activity with a broad range of substrates, including *p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, *o*-phthaldialdehyde, syringaldehyde, benzaldehyde, and even CAD.[37] Given that the catabolism of various aromatic compounds is dependent on the *vdh* gene, VDH is regarded as a crucial enzyme involved in the utilization of lignin-derived aromatic compounds as an alternative carbon source to sugars in *C. glutamicum*.[37] Moreover, with respect to its significant contribution to the formation of protocatechuate, which can be assimilated into the TCA cycle by catalyzing aldehyde forms of various aromatic compounds into carboxylic acid forms,[38] we considered the *vdh* gene as a key factor involved in reverse conversion to *t*-CA from CAD. Therefore, we examined the deletion of the *vdh* gene in *C. glutamicum* to prevent the reverse conversion of CAD into *t*-CA. We constructed the MpV9-3 strain by deleting the *vdh* gene from the MpV7-3 strain and then analyzed the performance of the bioconversion between *t*-CA and CAD (Figure 5A). Shake-flask cultures with MpV7-3 and MpV9-3 were used to analyze the reverse conversion profiles. When the cells reached the stationary phase, 1.2 g/L CAD was added. As shown in Figure 5B, CAD added to MpV7-3 continued to decrease, whereas *t*-CA gradually increased. After 4 h of reaction, 0.28 g/L of CAD remained in the culture, and approximately 0.4 g/L of *t*-CA was produced (Figure 5B). In contrast, in MpV9-3, the reduction width of the same amount of CAD was significantly reduced compared to that of MpV7-3 (Figure 5C). In addition, unlike MpV7-3, *t*-CA production was not observed in MpV9-3; this result indicates that the inactivation of *vdh* contributes to suppressing the reverse conversion of CAD to *t*-CA (Figure 5C). Meanwhile, we found that added CAD was gradually decreased although the reverse conversion to *t*-CA was not observed (Figure 5C). One possible reason might be due to the leakage to CAL that was not completely blocked by the unknown endogenous reductases and/or dehydrogenases as we showed in the earlier conversion reaction with MpV7-3 (Figure S3A).

Next, we performed a conversion reaction for the production of CAD in a bioreactor. After MpV9-3 was fully grown to an $OD_{600}$ of 50 at 30 °C for 7 h, the cells were further incubated

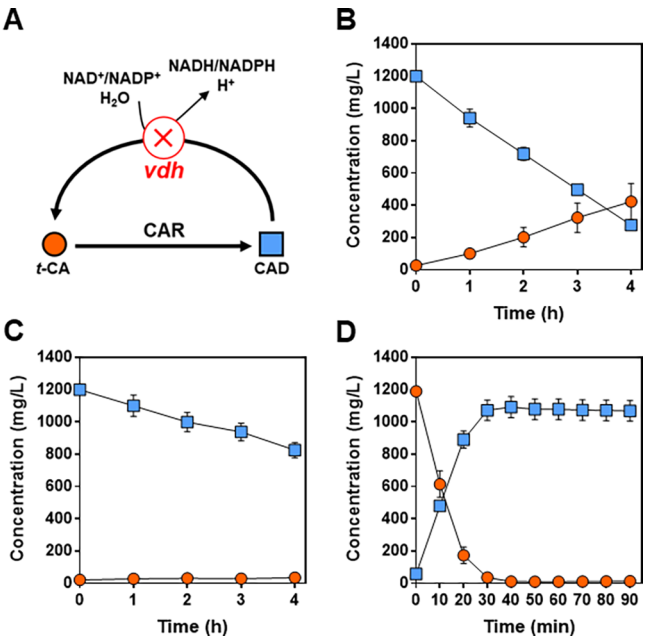


**Figure 5.** Performance analysis for bioconversion in *C. glutamicum* MpV9-3. (A) Scheme of the engineering strategy. Reverse conversion profiles in (B) *C. glutamicum* MpV7-3 and (C) *C. glutamicum* MpV9-3 in shake-flask cultures. (D) CAD production profile of *C. glutamicum* MpV9-3 in reactor-scale cultivation. (B–D) Symbols: *t*-CA, circle; CAD, square. Results represent mean values and standard deviations obtained from two independent experiments.

for 1 h, and 1.2 g/L of *t*-CA was added to the cells (Figure S2). In the conversion reaction, MpV9-3 showed enhanced CAD bioconversion efficiency compared to MpV7-3. For 1.2 g/L of *t*-CA, complete consumption of *t*-CA occurred in 30 min, with a conversion rate of 2.2 g/L/h, and up to 1.1 g/L CAD was produced with a conversion yield of 100% (1.02 mol/mol) (Figure 5D). In this conversion, the generation of CAL was also observed, but its titer was low (below 30 mg/L), similar to that of MpV7-3 (Figure S3B). In the whole-cell biocatalyst platforms, degradation of substrates can be a factor that hinders the development of efficient conversion processes. In *C. glutamicum*, lignin-derived aromatic compounds, including phenylpropanoids can be utilized as carbon and energy sources by the catabolic pathway inherent in the host. For example, the *phd* gene cluster was known to be involved in the catabolism of phenylpropanoids via the CoA-dependent, *β*-oxidative deacetylation route.[39] Among the gene cluster, transcriptional regulator PhdR activated by the phenylpropanoid such as *p*-coumaric acid, caffeic acid, and ferulic acid leads to a derepression of the Phd pathway.[39] Even though PhdR is not reactive with *t*-CA, it was identified that *t*-CA and its intermediates can be used as a substrate in the Phd pathway,[39] which may cause the loss of the *t*-CA substrate and consequently reduce the conversion efficiency. However, we could achieve 100% conversion without loss of the *t*-CA substrate, and we think that the interference from the substrate's degradation pathway may be overcome by the rapid completion of conversion within 30 min (Figure 5D).

In conclusion, we describe the development of a highly efficient bioconversion process for CAD production from *t*-CA using *C. glutamicum*. CAD could be synthesized by constructing a strong expression system for the *MpCAR* gene and supplementing it with NADPH, and the conversion yield


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and rate of CAD could be increased further by deleting the pathways for conversion into CAL as well as reverse conversion into *t*-CA. To the best of our knowledge, this is the first report of CAD production in *C. glutamicum*, and our bioconversion system with the robust *C. glutamicum* can provide a highly competitive and stable platform compared with microbial cultivation, which requires relatively long cultivation (2 or more days) and has the limitation of cell growth due to CAD toxicity.[6] As demonstrated here, the crude *t*-CA produced by microbial cultivation can be directly supplied without further separation, so the economic bioprocess platform can be established using a very cheap substrate. Previously, we also developed a bioconversion platform with *C. glutamicum* for the production of *t*-CA from L-Phe.[18] By connecting two bioconversion systems, the bioprocess from L-Phe to CAD can be established, and we believe it would be a substantial contributor to the commercial CAD production.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c07398.

Time profile of the reactor-scale cultivation of *C. glutamicum* MpV7-3; time profiles of the reactor-scale cultivation of *C. glutamicum* MpV9-3; CAL profiles of the reactor scale *t*-CA conversion of *C. glutamicum* MpV7-3 and *C. glutamicum* MpV9-3 (PDF)

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### Author Contributions

J.S. and K.J.J. designed the overall experiment, interpreted the data, and wrote the manuscript. J.S., J.W.C., M.G.S., H.J.Y., and E.J.J. conducted plasmid construction. J.S., I.H.C., and C.G.L. conducted cultivation and analysis products. J.S., J.H.J., and H.B.B. analyzed the data. J.S., S.C.K., and K.J.J. conceived of the study, revised the manuscript, and approved the final version for publication. All authors read and approved the final manuscript.


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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

L-Phe, L-phenylalanine; *t*-CA, *trans*-cinnamic acid; CAD, cinnamaldehyde; CAL, cinnamyl alcohol; CAR, carboxylic acid reductase; MpCAR, carboxylic acid reductase from *Mycobacterium phlei*; VDH, vanillin dehydrogenase

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