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High-Level Production of Catechol from Glucose by Engineered *Escherichia coli*

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Abstract: Catechol (CA) is an aromatic compound with important applications in the fine chemical and pharmaceutical fields. As an alternative strategy to petroleum-based chemical synthesis, the production of catechol by using microbial cell factories has attracted great interest. However, the toxicity of catechol to microbial cells significantly limits the efficient production of bio-based catechol via one-step fermentation. Therefore, in this study, a two-step strategy for the efficient synthesis of CA was designed. Protocatechuic acid (PCA) was first efficiently produced by the engineered *Escherichia coli* strain AAA01 via fermentation, and then PCA in the fermentative broth was converted into CA by the whole-cell biocatalyst AAA12 with PCA decarboxylase. By optimizing the expression of flavin isoprenyl transferases and protocatechuic acid decarboxylases, the titer of CA increased from 3.4 g/L to 15.8 g/L in 12 h through whole-cell biocatalysis, with a 365% improvement; after further optimizing the reaction conditions for whole-cell biocatalysis, the titer of CA achieved 17.7 g/L within 3 h, which is the highest titer reported so far. This work provides an effective strategy for the green biomanufacturing of toxic compounds by *Escherichia coli* cell factories.

Keywords: *Escherichia coli*; catechol; toxicity; two-step strategy; flavin isoprenyl transferase; protocatechuic acid decarboxylase

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1. Introduction

Catechol (1,2-dihydroxybenzene, CA) is an important aromatic compound with many applications. It can be used as a reagent in photography, dyes, electroplating, rubber, and plastic production, and also used as a precursor in the synthesis of fine chemicals such as cis and cis-muconic acid (ccMA) [1–3]. ccMA can be used to produce a variety of valuable polymers and drugs, including adipic acid and terephthalic acid [4,5]. In the past few decades, CA has continued to be obtained mainly via chemical conversion of petroleum derivatives, although some catechol is distilled from coal tar [6]. The manufacturing of CA or many other chemicals based on petroleum, a non-renewable resource, is increasingly constrained in many ways due to the growing scarcity of resources and the degradation of the natural environment. Therefore, it is urgently necessary to create a cost-effective and environmentally friendly method for producing CA.

Microbial cell factories have been created as a result of the ongoing development of biotechnology for the environmentally friendly production of organic alcohols, amino acids, organic acids, vitamins, and natural products made from renewable resources, including L-phenylalanine [7–9], p-coumaric acid [10,11] and p-hydroxybenzoic acid [12]. For instance, Draths et al. initially described the biomanufacturing of 2 g/L catechol from glucose using an engineered *Escherichia coli* strain in 1995 [6]. The precursor of catechol, 3-dehydroshikimic acid (DHS), is synthesized from glucose after three steps of catalytic phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) (which are precursors

of DHS), and then protocatechuic acid (PCA) and CA are successively synthesized via exogenous DHS dehydratase and PCA decarboxylase. However, low titer, preventing biomanufacturing catechol from replacing chemical synthesis, has to be addressed as the main challenge, even though the research progress on this compound supports the feasibility of CA manufacture by *E. coli* cell factories.

Two main factors contribute to the low titer of CA production in microbial cell factories: One is the low activity of PCA decarboxylase; the other is the toxicity of CA to microbial cells. Despite several works successfully improving the production of the precursor, DHS, and PCA [13–16], the titer of CA remained less than 10 g/L, indicating a major limitation in the synthesis of CA from PCA catalyzed via nonoxidative PCA decarboxylases. These enzymes belong to the hydroxy aromatic decarboxylase/phenol carboxylase family and consist of three distinct subunits encoded by the *kpdB*, *aroY,* and *kpdD* genes [17]. Weber et al. expressed the three subunits of which only *aroY* optimized, achieving CA production by *Saccharomyces cerevisiae* under aerobic conditions [17]. Further research confirmed that the combined expression of *kpdB* and *aroY* resulted in a 14-fold increase in the activity of PCA decarboxylase compared to *aroY* alone [18]. However, the mechanism by which *kpdB* promotes *aroY* catalysis has not been elucidated. Recently, the resolution of *KpdB* protein function revealed that it is a highly homologous protein with *ubix* in *E. coli*, involved in the synthesis of a new cofactor-isoprenyl riboflavin cofactor, which plays an important role in the decarboxylation reaction of *aroY* [19,20]. Then, through a protein comparison, Johnson et al. combined the expression of *aroY* and *ecdB* (encodes flavin isoprenyl transferase) from *Enterobacter cloacae* and increased ccMA titer by more than threefold [21]. Taken together, these works demonstrated that optimization of flavin isoprenyl transferase and PCA decarboxylase is the key to further improving CA titer in microbial cell factories.

Moreover, the toxicity of CA on cells also affects the CA production in microbial cell factories. For *E. coli* cell factories, cell growth was significantly inhibited when the titer of CA reached 1 g/L [1]; when the CA titer was 0.275 g/L, no colony grew on LB plates; and when CA titer reached 2.75 g/L, the titer of DHS decreased almost fivefold compared with no CA added [6]. Therefore, addressing the toxicity of catechol in microbial cell factories is essential for efficient CA production. Recently, extensive research was reported to address the impact of targets toxicity on production, including enhancing the tolerance of microbial cell factories through adaptive laboratory evolution [22], selecting more tolerant microbial cell factories as chassis [14], isolating cell growth and targets production by using dynamic regulation [23], removing toxic targets from culture media [3], whole-cell biocatalysis [24], or cell-free bioconversion [25]. For example, to reduce the titer of CA in a culture medium by using in situ resin-based extraction, Li et al. increased the titer of CA from 4.2 g/L to 8.5 g/L [3]. However, this strategy increases the cost of large-scale production of CA. The approach of whole-cell biocatalysis has been widely used for efficient biosynthesis of fine and bulk chemicals due to its unique advantages [26]. For instance, Wang et al. created a cofactor self-sufficient whole-cell biocatalysis system, achieving the efficient production of 2-phenyl ethanol (9.14 g/L), which is toxic to cells [27]. Therefore, whole-cell biocatalysis will play an important role as an effective strategy for CA production in microbial cell factories.

To achieve high-level production of CA from glucose by engineered *E. coli*, an engineered *E. coli* strain AAA03 directly producing CA was constructed based on a PCA cell factory AAA01. However, under one-step fermentation conditions, the cell growth and the titer of CA were significantly affected due to the toxicity of CA. To address this issue, we designed a two-step strategy for improving CA production using different engineered *E. coli* strains at each step. Optimizing the expression of flavin isoprenyl transferase and PCA decarboxylase increased the activity of PCA decarboxylase. Finally, the high-level production of CA by optimizing whole-cell biocatalytic reaction conditions was achieved.

2. Materials and Methods

2.1. Strains and Plasmids

The strains and plasmids used in this study are listed in Figure 1 and Table 1.

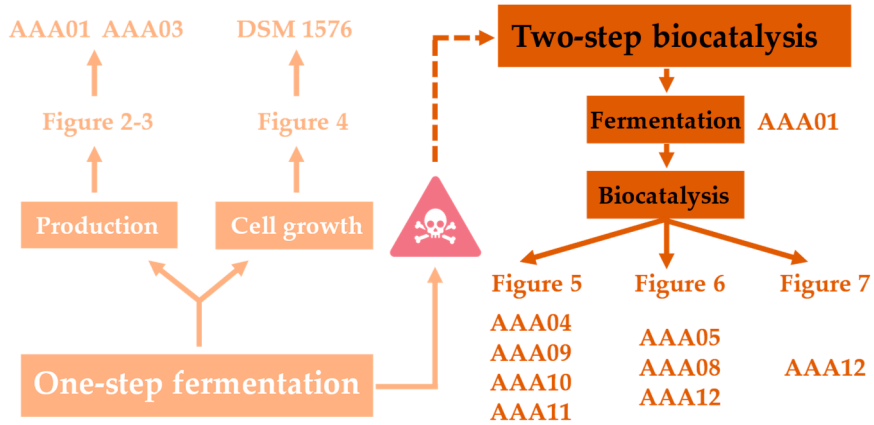

Figure 1. The flowchart of engineered *E. coli* strains using in each step of this study.

Table 1. Strains and plasmids used in this study.

Name.	Description	Source
	Strains	
E. coli DSM 1576	Wild type	Lab collection
E. coli AAA01	DSM 1576 M1-12-*aroE*^{TTG} M1-30-*aroF*^{fbr} Δ*tyrR* M1-93-*tktA* Δ*ptsI* M1-12-*galP* M1-93-*glk* M1-12-*pykF*^{TTG} M1-12-*pykA*^{TTG} M1-12-*pgi*^{TTG} M1-93-*aroB*^{opt}	Lab collection for PCA production
E. coli AAA03	AAA01 Δ *ptsI* :: M1-93 -*aroY*	This study
E. coli AAA04	BL21 (DE3) with pET30a-T7-aroY	This study
E. coli AAA05	BL21 (DE3) with pET30a-T7-*kpdB*	This study
E. coli AAA06	BL21 (DE3) with pET30a-T7-*ecdB*	This study
E. coli AAA07	BL21 (DE3) with pET30a-T7-*ubiX*	This study
E. coli AAA08	BL21 (DE3) with pET30a-T7-*EcaroY*	This study
E. coli AAA09	BL21 (DE3) with pET30a-T7-*kpdB*-RBS-*aroY*	This study
E. coli AAA10	BL21 (DE3) with pET30a-T7-*ecdB*-RBS-*aroY*	This study
E. coli AAA11	BL21 (DE3) with pET30a-T7-*ubiX*-RBS-*aroY*	This study
E. coli AAA12	BL21 (DE3) with pET30a-T7-*kpdB*-RBS-*EcaroY*	This study
	Plasmids	
pET30a-T7-*kpdB*	pET30a expressing *kpdB* with T7 promoter	This study
pET30a-T7-*ecdB*	pET30a expressing *ecdB* with T7 promoter	This study
pET30a-T7-*ubiX*	pET30a expressing *ubiX* with T7 promoter	This study
pET30a-T7-*aroY*	pET30a expressing *aroY* with T7 promoter	This study
pET30a-T7-*EcaroY*	pET30a expressing *EcaroY* with T7 promoter	This study
pET30a-T7-*kpdB*-RBS-*aroY*	pET30a expressing *kpdB* and *aroY* with T7 promoter	This study
pET30a-T7-*ecdB*-RBS-*aroY*	pET30a expressing *ecdB* and *aroY* with T7 promoter	This study
pET30a-T7-*ubiX*-RBS-*aroY*	pET30a expressing *ubiX* and *aroY* with T7 promoter	This study
pET30a-T7-*kpdB*-RBS-*EcaroY*	pET30a expressing *kpdB* and *EcaroY* with T7 promoter	This study
pET30a-T7-*ecdB*-RBS-*EcaroY*	pET30a expressing *ecdB* and *EcaroY* with T7 promoter	This study

2.2. Plasmid Construction

First, according to the codon bias of *E. coli*, *ecdB* and *EcaroY* from *Enterobacter cloacae* [21] and *KpdB* from *Klebsiella pneumoniae* [17] were codon-optimized and sent to Beijing Tsingke Biotechnology Co. for gene synthesis, generating three plasmids (pET30a-T7-*kpdB*/*ecdB*/*EcaroY*). PCR amplified the *ubiX* DNA fragment from *E. coli* and the *aroY* DNA fragment from *Klebsiella pneumoniae* and ligated them to the pET30a fragment containing the T7 promoter, generating pET30a-T7-*ubiX* and pET30a-T7-*aroY*. Subsequently, to evaluate the effect of flavin isoprenyl transferases on CA production, codon-optimized *aroY* (laboratory collection) with RBS was inserted into three plasmids (pET30a-T7-*kpdB*/*ecdB*/*ubiX*) and formed pET30a-T7-*kpdB*/*ecdB*/*ubiX*-RBS-*aroY*; similarly, EcaroY with RBS was inserted

into pET30a-T7-*kpdB*, generating plasmid pET30a-T7-*kpdB*-RBS-*EcaroY* for optimizing CA production via two-step whole-cell biocatalysis.

2.3. Strain Construction

In order to construct the engineered strain for CA production via one-step fermentation, we selected the PCA-producing engineered *E. coli* AAA01 (laboratory collection) as a chassis. The targeting fragment (ptsI40-*cat-sacB*-ptsI40) with 40 bp upstream and downstream homologous arms of *ptsI* was amplified using *cat-sacB* (laboratory collection) as a template, and the other targeting fragment (ptsI40-M1-93 -*aroY*-ptsI40) was obtained by overlapping PCR M1-93 (laboratory collection) [28] fragment and *aroY* fragment, which amplified using pET30a-T7-*kpdB-aroY* as a template. Based on the λ-Red two-step homologous recombination method [29], the engineered *E. coli* strain AAA03 was successfully for CA production built by sequentially integrating two targeting fragments.

2.4. Media and Growth Conditions

E. coli BL21 (DE3) was used for genetic cloning and recombinant protein expression as well as whole-cell biocatalysis. Strains were cultured at 30 °C in LB liquid medium (10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone) or on agar plates. Corresponding antibiotics (34 μg/mL chloramphenicol, 50 μg/mL kanamycin, and 100 μg/mL ampicillin) were added to the culture medium to avoid plasmid loss.

For protein expression in a shake flask, the single colony of biocatalysts cells was picked from the plate in tubes containing 2 mL of LB medium (with kanamycin antibiotic) and incubated overnight at 30 °C, 250 rpm. The overnight seeds were then transferred to 1 L shake flasks containing 100 mL LB with kanamycin antibiotics and incubated at 30 °C, 250 rpm until OD_{600} was about 0.6, and then adding 0.2 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) to induce protein expression at 30 °C for 10 h at 250 rpm. Lastly, cells were harvested via centrifugation at 5000 g for 10 min at 4 °C.

For CA or PCA production using shake-flask fermentation, the engineered *E. coli* AAA01/AAA03 was cultured in tubes containing 2 mL of LB medium from their plate at 37 °C, 250 rpm. Then, the overnight seeds were transferred to 100 mL shake flasks containing 10 mL of NBS media (20 g/L glucose, 6.5 g/L $K_2HPO_4 \cdot 3H_2O$, 3.5 g/L KH_2PO_4, 3.5 g/L $(NH_4)_2HPO_4$, 0.12 g/L $MgSO_4$, 0.16 mg/L $FeCl_3 \cdot 6H_2O$, 11 mg/L $CaCl_2$, 0.02 mg/L $CoCl_2 \cdot 6H_2O$, 5 mg/L thiamine-HCL, 0.015 mg/L $CuSO_4 \cdot 5H_2O$, 0.02 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg/L $ZnCl_2$ and 0.05 mg/L H_3BO_3) at a ratio of 1:100 and at 37 °C, 250 rpm for 48 h. All the inorganic salt reagents are purchased from Sinopharm Chemical Reagent Co., Shanghai, China.

For CA or PCA production through 5 L fed-batch fermentation, the single colony of *E. coli* AAA01 or AAA03 was cultured in tubes containing 2 mL of LB medium from their plate at 37 °C, 250 rpm. The overnight seeds were then transferred to 1 L shake flasks containing 200 mL LB and cultured for 10–12 h at 37 °C, 250 rpm. After this, the seed culture was totally transferred into a 5 L bioreactor (BIOTECH-5BG, Bxbio, Shanghai, China) containing 1.8 L fermentation medium (30 g/L glucose, 1.6 g/L $(NH_4)_2SO_4$, 7.5 g/L $K_2HPO_4 \cdot 3H_2O$, 2 g/L citric acid, 2 g/L $MgSO_4 \cdot 7H_2O$, 4 mg/L $CoCl_2 \cdot 6H_2O$, 0.0075 g/L $FeSO_4 \cdot 7H_2O$, 20 mg/L Na_2SO_4, 0.6 mg/L $Cu_2SO_4 \cdot 5H_2O$, 6.4 mg/L $ZnSO_4$ and 4.525 mg/L $MnSO_4 \cdot H_2O$) at 37 °C. The agitation was changed to maintain the dissolved oxygen (DO) concentration above 30% saturation. The pH was controlled at 7.0 using 25% (*w/v*) $NH_3 \cdot H_2O$. The DO-stat feeding strategy was adopted to supply 600 g/L glucose to the bioreactor, keeping the residual glucose below 5 g/L.

2.5. Tolerance Assays of E. coli DSM 1576 to CA and PCA

The single colony of *E. coli* DSM 1576 was cultured in tubes containing 2 mL of LB medium from their plate at 37 °C, 250 rpm. Then, the overnight seeds were transferred to 100 mL shake flasks containing 10 mL of NBS media and different final concentrations of

CA and PCA (0 g/L, 1 g/L, 2 g/L, and 5 g/L), with an initial OD_{600} of 0.1 at 37 °C and 250 rpm for 24 h. Samples were collected every 6 h to determine OD_{600}.

2.6. Whole-Cell Biocatalysis Conditions

For whole-cell biocatalysis, the harvested cells expressing protein were added to the PCA fermentative broth, which was collected via centrifugation at 10,000 g for 15 min at room temperature (Avanti JXN-26, Beckman, Brea, CA, USA). The 10 mL reaction mixture consisting of 10–40 OD_{600} of whole-cell biocatalysts, the final titer of 9 g/L, or 45 g/L PCA (from PCA fermentative broth) were incubated at 20–37 °C and 3–12 h under resting or shaking conditions. Finally, the concentrations of CA and PCA were determined.

2.7. Crude-Extract Biocatalysis Conditions

For crude-extract biocatalysis, the 20 OD_{600} whole cells were resuspended in 0.9% (*w/v*) NaCl solution and crushed via sonication (25 W power, 10 s on, 10 s off for 10 minutes). Then, the supernatant was collected after centrifugation at 10,000 g for 15 min at 4 °C. The 10 mL reaction mixture consisting of crude extracts and the final titer of 45 g/L PCA (from PCA fermentative broth) were incubated at 30 °C and stewed at 12 h. Finally, the concentrations of CA and PCA were determined.

2.8. Analysis of OD_{600} and CA or PCA Accumulation

OD_{600} was measured using a spectrophotometer. The fermentation or biocatalysis broth of CA or PCA was diluted to the appropriate concentration. Additionally, 1 mL of the supernatant obtained via centrifugation at 12,000 rpm for 10 min at 4 °C was analyzed using HPLC (1200 series, Agilent Technologies, CA, USA) equipped with an Innoval C18 column (4.6 mm × 250 mm, 5 µm, Bonna-Agela, CA, USA). The ratio of phosphoric acid 0.1% (*v/v*) in phase A was 80% and methanol in phase B was 20%. The flow rate was 0.8 mL/min. The detection wavelength was 210 nm, and the column temperature was 30 °C.

3. Results

3.1. The Toxicity of CA Significantly Affects the CA Production via One-Step Fermentation

To achieve one-step fermentation for CA production in engineered *E .coli*, we first constructed an engineered *E. coli* AAA03 strain (Figure 2A) by integrating a strong artificial regulatory element M1-93 [28] and expressing *aroY* from *Klebsiella pneumoniae* at the *ptsI* locus on the chromosome of the engineered *E. coli* AAA01 strain. After 48 h of shake-flask fermentation, the engineered *E. coli* AAA01 strain produced 8.8 g/L PCA, accumulated 0.5 g/L DHS, and OD_{600} was 11.7, while the engineered *E. coli* AAA03 strain produced 2.2 g/L CA and accumulated 2.3 g/L PCA, but the OD_{600} was 50% lower than that of the engineered *E. coli* AAA01 strain (Figure 2B–E). After 42 h of fed-batch fermentation, a titer of PCA reached 53.6 g/L, and the highest OD_{600} was 41.9 (Figure 3A), while a titer of CA achieved 3.1 g/L, and OD_{600} was only 32.1 (Figure 3B). There was a significant decrease in the cell growth of the engineered *E. coli* AAA03 strain, compared with that of the engineered *E. coli* AAA01 strain using either fermentation method. These results suggested that, although the synthesis of CA was achieved in the engineered *E. coli* strains, the toxicity of CA significantly affects CA production through one-step fermentation.

To evaluate the effects of PCA and CA on cell growth, we investigated the tolerance of *E. coli* strain to different concentrations of PCA and CA. We found that when the concentration of PCA reached 5 g/L, the cell growth decreased by almost 50%, compared with that without PCA addition, while the cell growth was affected with the addition of 2 g/L PCA at 0–12 h (Figure 4A). For catechol, the cell growth of the *E. coli* strain was significantly inhibited (a 93.8% decrease compared with that without CA addition) when the concentration of CA in the culture medium was more than 1 g/L (Figure 4B). These results showed that CA was more toxic to *E. coli* cells than PCA, which led us to the conclusion that the engineered *E. coli* AAA01 strain could produce higher PCA, while the

engineered *E. coli* AAA03 strain could only produce lower CA. Therefore, how to avoid the toxicity of CA should be addressed for improving CA production.

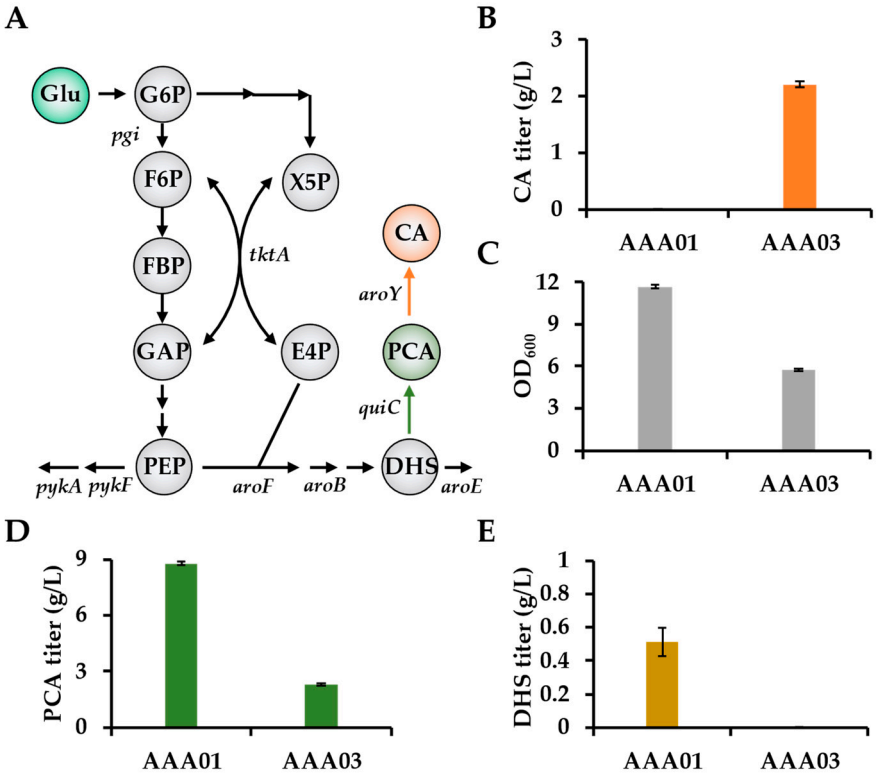

Figure 2. One-step fermentative production of catechol from glucose in engineered *E. coli* strain by shaking flask: (**A**) the synthetic pathway of catechol in engineered *E. coli* AAA03 using glucose as a carbon source. Catechol production (**B**), cell growth (**C**), PCA titer (**D**), and DHS titer (**E**) in shake-flask fermentation. Glu, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; DHS, 3-dehydroshikimic acid; X5P, xylulose-5-phosphate; PCA, protocatechuic acid; CA, catechol; *pgi*, phosphohexose isomerase; *pykF/A*, pyruvate kinase; *tktA*, transketolase; *aroF*, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase; *aroB*, 3-dehydroquinate synthase; *aroE*, shikimate dehydrogenase; *quiC*, 3-dehydroshikimic acid dehydratase; *aroY*, protocatechuate decarboxylase.

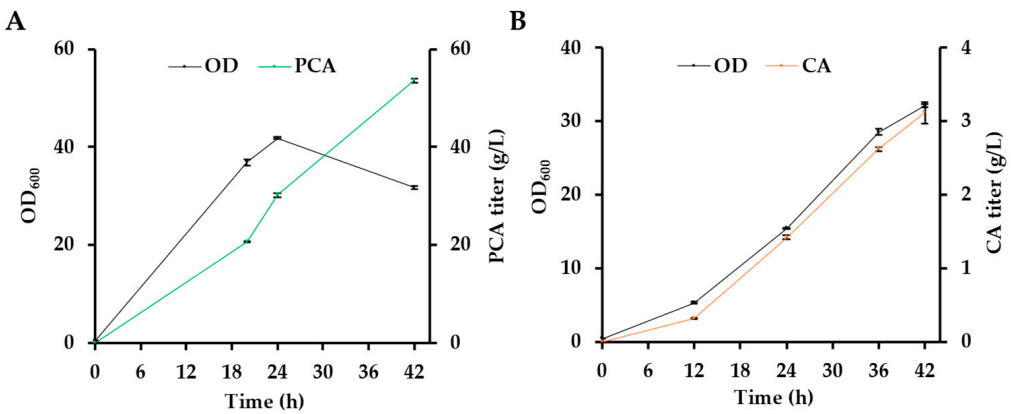

Figure 3. One-step fermentative production of PCA and CA from glucose using engineered *E. coli* AAA01 (**A**) and AAA03 (**B**) in 5 L bioreactor. Data are the average mean of triplicate samples, and the error bars represent standard deviations.

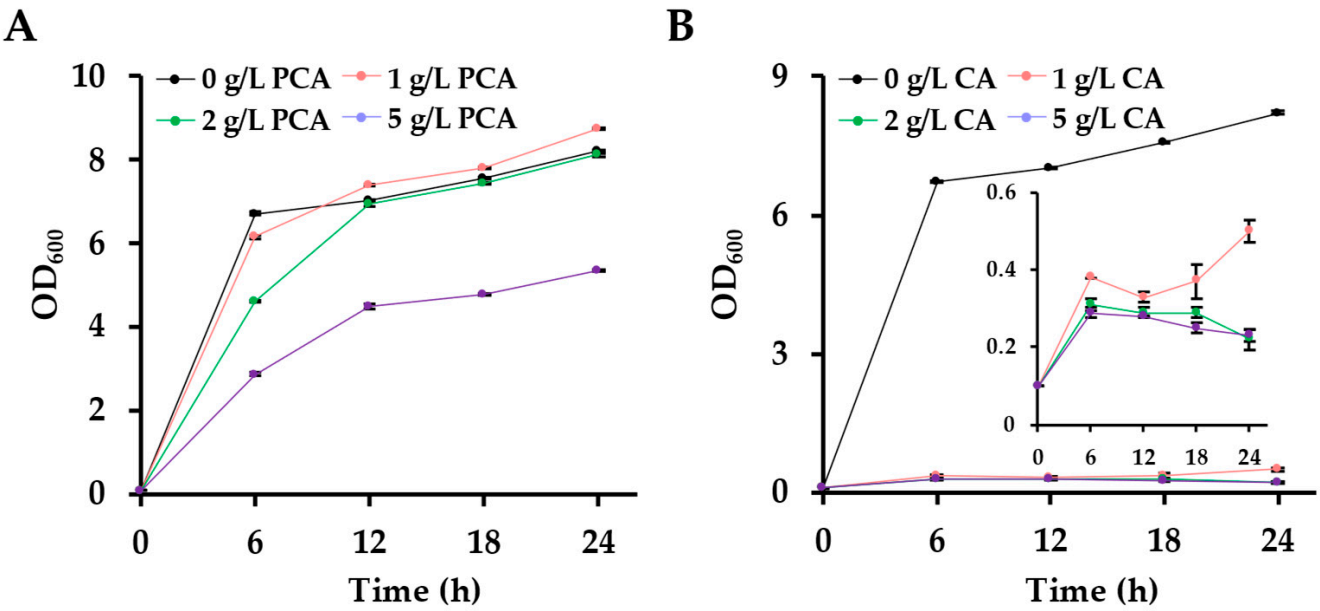

Figure 4. Growth response of engineered *E. coli* strain to the exogenous addition of PCA (**A**) and CA (**B**) at 0 g/L (black), 1 g/L (pink), 2 g/L (green), and 5 g/L (purple). Data are the average mean of triplicate samples, and the error bars represent standard deviations.

3.2. Introducing Flavin Isoprenyl Transferase Could Efficiently Improve the Conversion of PCA to CA

To address the toxicity of CA to microbial cells, we designed a two-step biosynthetic method for high-level production of CA. In detail, PCA was first efficiently produced by the engineered *E. coli* AAA03 strain in a 5 L bioreactor and then converted into CA using a whole-cell biocatalyst expressing PCA decarboxylase. As indicated in previous reports [19,20], the isoprenyl riboflavin cofactor (prFMN) is required to catalyze the PCA decarboxylation reaction. Based on this, we investigated the effects of different sources of flavin isoprenyl transferases (encoded by *ecdB*/*kpdB*/*ubiX*) on whole-cell biocatalysis for CA synthesis. The best engineered *E. coli* AAA09 co-expressing *aroY* and *kpdB* produced 5.42 g/L CA, a 70% increase, compared with engineered *E. coli* AAA04 expressing *aroY* alone, while both the engineered *E. coli* AAA10 and AAA11 strains produced 5.4 g/L CA. The PCA concentrations accumulated to about 0.1 g/L in engineered *E. coli* AAA09, AAA10, and AAA11, while it reached 3.8 g/L in engineered *E. coli* AAA04 (Figure 5 and Supplement Figure S1). To verify the maximum productive performance of the whole-cell catalytic system with different flavin isoprenyl transferases, the fermentative broth with 45 g/L PCA was used for the biocatalytic reaction. After 12 h of biocatalysis, the titer of CA reached 11.4 g/L in engineered *E. coli* AAA09, with a 235% increase compared with the CA titer in engineered *E. coli* AAA04 (Figure 5), and there was no significant difference between the three flavin isoprenyl transferases. These results indicated that the overexpression of *kpdB* or *ecdB* or ubiX increased the activity of PCA decarboxylase and enhanced the production of CA, and the three flavin isoprenyl transferases had similar enzyme activity. However, there was still about 11 g/L PCA in engineered *E. coli* AAA09 fermentative broth that could not be converted into CA, suggesting that high-level CA production was limited in whole-cell biocatalysis systems.

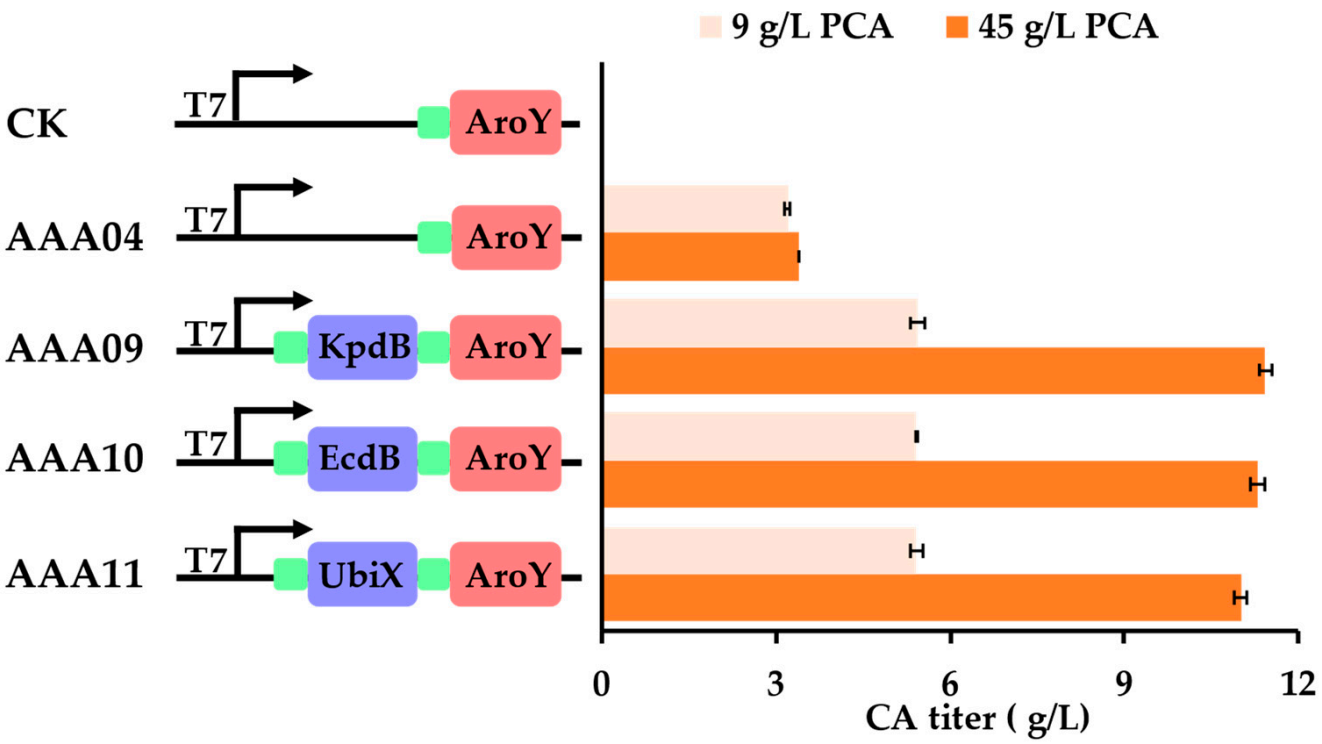

Figure 5. The effects of different flavin isoprenyl transferases on catechol production with 9 g/L or 45 g/L PCA via whole-cell biocatalysis. *E. coli* BL21 (DE3) with pET30a-T7 plasmid was used as the blank control (CK). Biocatalytic conditions: Diluted PCA fermentative broth (final concentrations of 9 g/L and 45 g/L), 20 OD_{600} whole cells, 10 mL of total volume, at 30 °C and pH 7.0. Data are the average mean of triplicate samples, and the error bars represent standard deviations.

3.3. Optimization of Whole-Cell Biocatalysis Systems Could Improve the CA Production

To completely convert PCA into CA, we optimized and characterized the whole-cell and enzymatic biocatalysis. Through whole-cell biocatalysis, the engineered *E. coli* AAA12 constructed with a higher activity of *EcaroY* from *Enterobacter cloacae*, compared with *aroY* [21], produced 15.8 g/L CA, with a 38.6% increase compared with engineered *E. coli* AAA09, which produced 11.4 g/L CA (Figures 5 and 6), with a 31.7% increase compared with engineered *E. coli* AAA08, indicating that flavin isoprenyl transferase promoted the decarboxylation reaction of PCA, which is also consistent with the previous results (Figures 5 and 6). To verify the effect of increasing the concentrations of EcaroY and KpdB on CA synthesis, we further investigated the production of CA in crude extracts. The crude extracts of engineered *E. coli* AAA12 produced 14.5 g/L CA, which was 8.2% lower compared with that of whole-cell biocatalysts, probably due to enzyme loss during the crude extract preparation. Then, the titer of CA increased by 6.9% and 11% when the concentration of flavin isoprenyl transferase or PCA decarboxylase was further increased, indicating that both enzymes could promote the conversion of PCA into CA (Figure 6). However, compared with whole-cell biocatalysis, biocatalysis with crude extracts is more complex, and the enhancement of CA titer is not significant. Therefore, the strategy of whole-cell biocatalysis is more favorable for the large-scale production of CA.

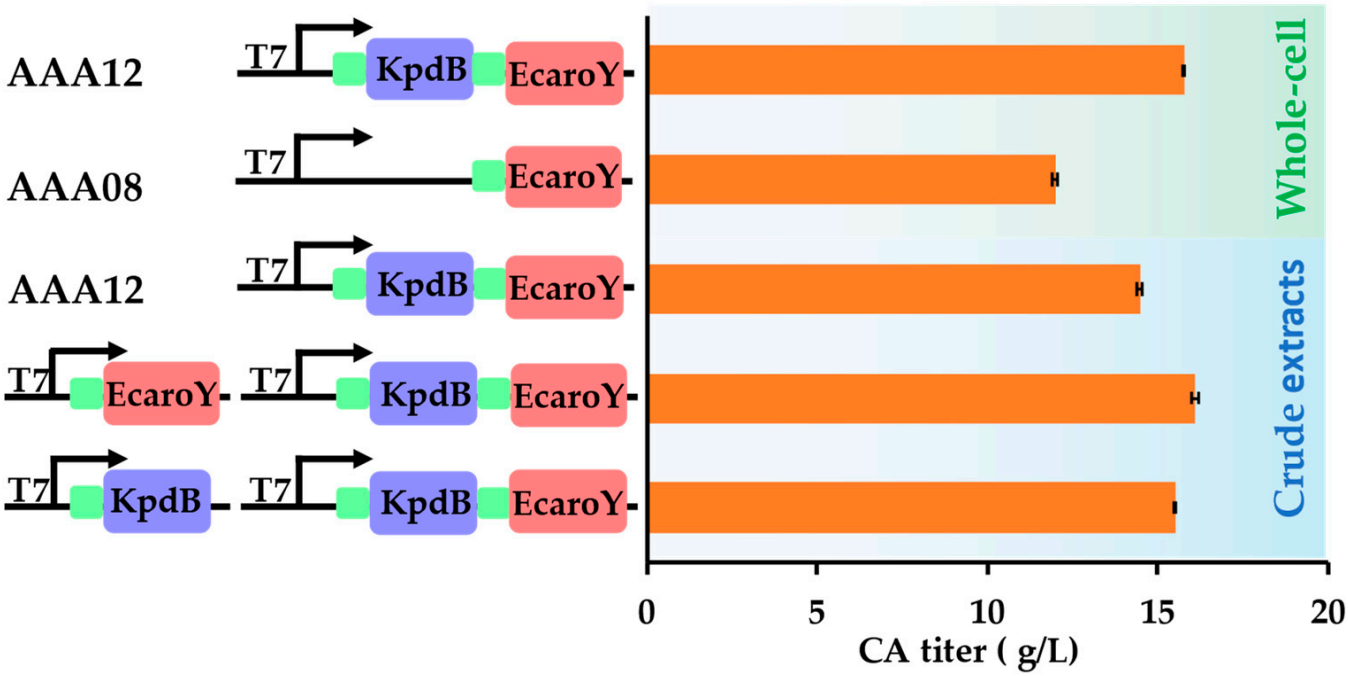

Figure 6. The effects of flavin isoprenyl transferases, protocatechuate decarboxylase, and the different ways of biocatalysts on catechol production. Whole-cell biocatalytic conditions: 20 OD_{600} whole cells; crude extracts biocatalytic conditions: 20 OD_{600} prepared for crude extracts. All reactions were performed under 45 g/L PCA fermentative broth, total volume of 10 mL, at 30 °C, and pH 7.0. Data are the average mean of triplicate samples, and the error bars represent standard deviations.

3.4. Optimization of Whole-Cell Biocatalysis Reaction Conditions Could Further Improve the CA Production

To further improve CA production, we optimized the whole-cell biocatalytic conditions, such as time, cell concentration, temperature, and dissolved oxygen using engineered *E. coli* AAA12 as the biocatalyst. We found that the higher titer (17.0 g/L) of CA was achieved at 3 h, probably due to the degradation of CA after 3 h (Figure 7A). To reduce the CA toxicity on cells by increasing the cell concentration, the highest titer of CA achieved 17.7 g/L with 40 OD_{600} for 3 h, a 4.1% and 9.3% increase compared with 20 OD_{600} and 10 OD_{600}, respectively (Figure 7B). The temperature had no significant effect on whole-cell biocatalysis, and the higher CA titer achieved 17.0 g/L at 30 °C (Figure 7C). The effect of different dissolved oxygen (DO) levels on CA production was investigated using resting or shaking biocatalysis. Only 7.3 g/L CA was produced under high DO level conditions (via shaking biocatalysis), a 57% reduction compared with that observed at low DO level (via resting biocatalysis) (Figure 7D). These results indicated that the high DO levels significantly affected whole-cell biocatalytic reactions. In summary, a titer of 17.7 g/L CA was produced by the AAA12 whole-cell biocatalyst under optimum conditions, which was the highest titer reported so far (Table 2).

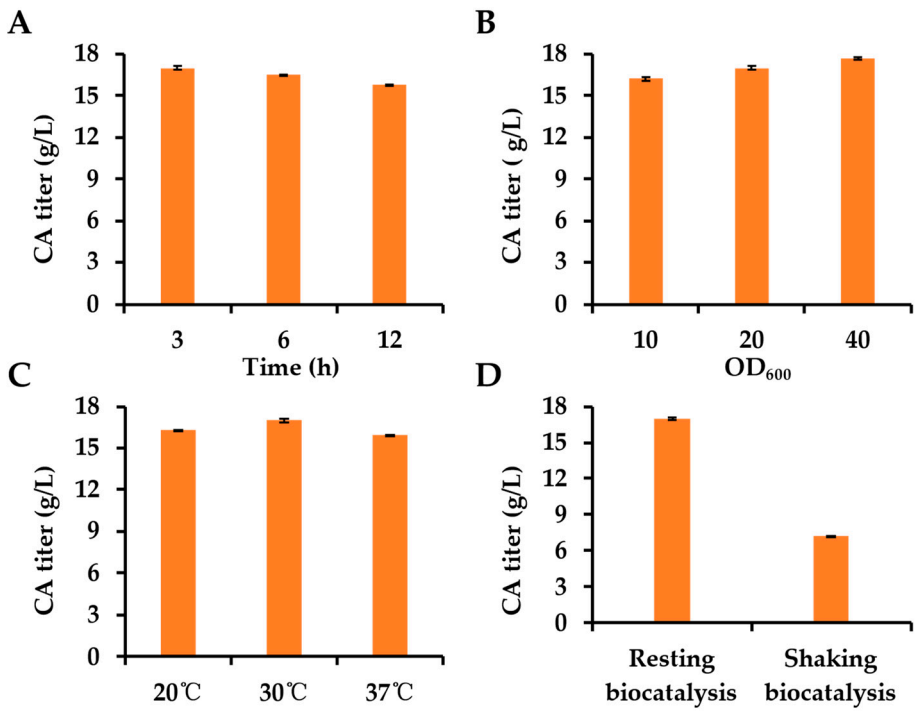

Figure 7. Effects of time (**A**), OD_{600} (**B**), temperature (**C**), and dissolved oxygen (**D**) on CA production. All reactions were performed under 45 g/L PCA fermentative broth, total volume of 10 mL, and pH 7.0. Data are the average mean of triplicate samples, and the error bars represent standard deviations.

Table 2. Production of CA by engineered microbial cell factories.

Strain	Carbon Source	Synthetic Method	Time (h)	Titer (g/L)	Reference
E. coli	Glucose	Resin-based extraction	36	8.50	[3]
E. coli	Glucose	Fed-batch fermentation	48	2.00	[6]
S. cerevisiae	Glucose	Shake-flask fermentation	120	0.58	[17]
E. coli	Glucose	Batch fermentation	84	4.47	[2]
E. coli	PCA	Whole-cell biocatalysis	3	0.08	[18]
E. coli	Glucose	Batch fermentation	86	0.63	[1]
S. cerevisiae	PCA	Whole-cell biocatalysis	168	0.09	[30]
S. cerevisiae	Glucose	Shake-flask fermentation	96	0.62	[31]
E. coli	Glucose	Fed-batch fermentation	42	3.10	This work
E. coli	Glucose	Whole-cell biocatalysis	3	17.70	This work

4. Discussion

As we mentioned in this study, only 2.2 g/L (20 mmol/L) CA and 2.3 g/L PCA were produced by the engineered *E. coli* AAA03 strain via one-step fermentation. If there was no degradation of CA, only 5.4 g/L PCA (20 mmol/L PCA plus 2.3 g/L PCA) was theoretically produced by the engineered *E. coli* AAA03 strain, a 38.6% decrease compared with the engineered *E. coli* AAA01 strain (8.8 g/L PCA), indicating that CA toxicity inhibited cell growth (50% decrease in OD_{600}) and thus affected PCA synthesis (Figure 2B–D). These results suggested that the toxicity of CA to cells is a key factor limiting CA bioproduction. According to a previous report [32], during the oxidation of CA to form quinone, large amounts of hydrogen peroxide are generated, which leads to cell death by oxidizing proteins as well as fragmenting DNA. In addition, more hydroxyl radicals can be generated by the intracellular Fenton reaction in the presence of CA [33]. Hydroxyl radical has a reduction potential greater than H_2O_2 and fragments DNA and proteins, resulting in cell death [34] (Supplementary Figure S2). These results explain the low titer of CA in

one-step fermentation (aerobic fermentation) (Figure 3B) or two-step shaking biocatalysis (Figure 7D).

To reduce the toxicity of CA to microbial cells, we designed a two-step whole-cell biocatalytic approach to overproduce CA in a resting biocatalytic way and then optimized and characterized the key enzymes, flavin isoprenyl transferase, and PCA decarboxylase. We found that the co-expression of flavin isoprenyl transferases with PCA decarboxylase facilitated the efficient production of CA (Figure 6), but the effects of different sources of them on CA were marginal (Figures 5 and 6). However, only the supply of isoprenyl riboflavin cofactor (prFMN) was enhanced in our work, and the recycling of prFMN was not actually achieved, which could be the reason for the highest titer of CA at 3 h via two-step whole-cell biocatalysis.

For PCA decarboxylase, we utilized *EcaroY* from *Enterobacter cloacae* instead of *aroY* from *Klebsiella pneumoniae* according to the literature [21], and the production of CA was increased by 38.6% (from 11.4 g/L to 15.8 g/L) using the two-step whole-cell biocatalysis (Figures 5 and 6). However, we only characterized a reported PCA decarboxylase (*EcaorY*) of which the enzymatic activity is higher than *aroY*, and it might be very necessary to test more PCA decarboxylases.

Finally, we efficiently produced CA with a titer of 17.7 g/L based on the designed two-step whole-cell biocatalysis approach (Figure 7B), which is the highest titer reported so far. Furthermore, in-depth research will help to enhance the titer of CA. Further studies may include adding more antioxidants (Vitamin C) or biocatalysis under micro-oxygenated conditions to further reduce the toxicity of CA; constructing an isoprenyl riboflavin cofactor recycling system to maintain the efficiency of the whole-cell biocatalytic process; and screening PCA decarboxylase with higher enzyme activity from a larger database or modifying PCA decarboxylase based on protein structure/machine learning. In summary, using different strategies to solve the bottlenecks of CA production through two-step whole-cell biocatalysis will facilitate the large-scale production of bio-based CA and also provide effective guidance for the green biomanufacturing of high-value-added toxic chemicals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8070344/s1, Figure S1: The residual PCA after producing catechol via two-step whole-cell biocatalysis; Figure S2: The mechanism of CA toxicity to microbial cells.

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