Heterologous production of caffeic acid from tyrosine in *Escherichia coli*

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ABSTRACT

Caffeic acid is a plant secondary metabolite and its biological synthesis has attracted increased attention due to its beneficial effects on human health. In this study, *Escherichia coli* was engineered for the production of caffeic acid using tyrosine as the initial precursor of the pathway. The pathway design included tyrosine ammonia lyase (TAL) from *Rhodotorula glutinis* to convert tyrosine to *p*-coumaric acid and 4-coumarate 3-hydroxylase (C3H) from *Saccharothrix espanaensis* or cytochrome P450 CYP199A2 from *Rhodospseudomonas palustris* to convert *p*-coumaric acid to caffeic acid. The genes were codon-optimized and different combinations of plasmids were used to improve the titer of caffeic acid. TAL was able to efficiently convert 3 mM of tyrosine to *p*-coumaric acid with the highest production obtained being 2.62 mM (472 mg/L). CYP199A2 exhibited higher catalytic activity towards *p*-coumaric acid than C3H. The highest caffeic acid production obtained using TAL and CYP199A2 and TAL and C3H was 1.56 mM (280 mg/L) and 1 mM (180 mg/L), respectively. This is the first study that shows caffeic acid production using CYP199A2 and tyrosine as the initial precursor. This study suggests the possibility of further producing more complex plant secondary metabolites like flavonoids and curcuminoids.

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1. Introduction

Caffeic acid is a natural phenolic compound derived from the phenylpropanoid pathway in plants. Phenylpropanoic acids, especially caffeic acid, have attracted an increased attention owing to their valuable properties, including antioxidant [1], anti-inflammatory [2], anticancer [3], antiviral [4], antidiabetic [5] and antidepressive [6]. Due to its pharmaceutical applications there is an established market for caffeic acid production. Caffeic acid is mainly obtained by extraction from plants and these extraction

methods include high temperatures, treatment with petroleum and solvent extraction (methanol, ethyl acetate), thus being energy-intensive and environmentally unfriendly [7]. Moreover, the yields obtained are low because it accumulates at low levels in plant cells. To meet the phenylpropanoids market needs, it is imperative to look for new green and environmental production methods to replace the traditional extraction one. One way to achieve high levels of caffeic acid is the production via engineered microorganisms, such as *Escherichia coli* [8–10].

Natural caffeic acid production starts with the deamination of the amino acid phenylalanine which is converted to cinnamic acid by phenylalanine ammonia lyase (PAL) (Fig. 1). Then, cinnamic acid is converted to *p*-coumaric acid by cinnamate-4-hydroxylase (C4H), and caffeic acid is obtained from *p*-coumaric acid using 4-coumarate 3-hydroxylase (C3H). Tyrosine can also be used as a precursor as some PALs also have tyrosine ammonia lyase (TAL) activity [11,12]. Since tyrosine already possesses a 4-hydroxyl group, its use is advantageous as it can be directly converted to *p*-coumaric acid, thus decreasing the number of steps to produce caffeic acid. Also, using TAL, the C4H enzyme that is essential for caffeic acid production in plants and has not yet been successfully expressed in prokaryotic organisms [13] is not needed anymore.

Abbreviations: TAL, tyrosine ammonia lyase; C3H, 4-coumarate 3-hydroxylase; PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4HPA3H, hydroxyphenylacetate 3-hydroxylase; 4CL, 4-coumarate-CoA ligase; MCSs, multiple cloning sites; PCR, polymerase chain reaction; RBS, ribosome binding site; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoretic.

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Fig. 1. Artificial caffeic acid biosynthetic pathway. The dashed box represents the caffeic acid pathway in plants. Inside and outside the box some strategies used to produce caffeic acid in *E. coli* are illustrated. TAL: tyrosine ammonia lyase; C3H: 4-coumarate 3-hydroxylase; 4HPA3H: hydroxyphenylacetate 3-hydroxylase; 4CL: 4-coumarate-CoA ligase.

In the last decade several efforts have been conducted to produce caffeic acid using microorganisms. Berner et al. [14] identified *sam8* and *sam5* genes, encoding TAL and C3H involved in caffeic acid biosynthesis in the actinomycete *Saccharothrix espanaensis* and expressed them in *Streptomyces fradiae* XKS. After that, these

enzymes were used to produce caffeic acid in *E. coli* (Table 1). Other bacterial TAL (*Rhodobacter capsulatus* and *Rhodotorula glutinis*) were also used for the production of *p*-coumaric acid and hydroxyphenylacetate 3-hydroxylase (4HPA3H) from *E. coli* or *Pseudomonas aeruginosa*, and cytochrome P450 CYP199A2 from

Table 1

Caffeic acid production in *Escherichia coli*. Genes/organisms used in the caffeic acid biosynthetic pathway, fermentation conditions and yields.

Genes – organisms ^a	Fermentation conditions	Titer (mg/L) ^b	Reference
TAL – <i>S. espanaensis</i> C3H – <i>S. espanaensis</i>	First induction in LB at 37 °C. After 5 h cells were transferred to M9 medium (glucose) at 26 °C for 36 h	(not mentioned)	Choi et al. [15]
TAL – <i>R. capsulatus</i> 4HPA3H – <i>E. coli</i> $\Delta tyrR$; <i>tyrA</i> ^{tr} – <i>ppsA</i> – <i>tktA</i> – <i>aroG</i> ^{tr}	M9 medium (glucose and glycerol) at 37 °C. After induction shake flasks were transferred to 30 °C for 48 h	50.2 (TYR strain) 12.1 (wild-type)	Lin and Yan [16]
TAL and opTAL – <i>S. espanaensis</i> C3H – <i>S. espanaensis</i> $\Delta tyrR$; <i>tyrA</i> ^{tr} – <i>aroG</i> ^{tr}	First induction in LB at 37 °C. After 5 h cells were harvested and transferred to M9 medium (glucose) at 26 °C for 36 h in shake flasks	150 (TAL, TYR strain) 40 (opTAL, TYR strain) 42 (TAL, wild-type) 14 (opTAL, wild-type)	Kang et al. [17]
CYP199A2 (wild-type and mutant F185L) – <i>R. palustris</i> Pdr – <i>Pseudomonas putida</i> Pux – <i>R. palustris</i>	Potassium phosphate buffer (glucose or glycerol) at 30 °C in shake flasks for 24 h. 20 mM <i>p</i> -coumaric acid was added	510 (wild-type) 2,800 (mutant)	Furuya et al. [8]
TAL – <i>R. glutinis</i> C3H – <i>S. espanaensis</i> $\Delta pheA$ $\Delta tyrR$; <i>tyrA</i> ^{tr} – <i>aroG</i> ^{tr}	MOPS medium (glucose or xylose), synthetic medium (glucose or xylose) or LB (glucose or xylose) at 37 °C for 72 h in test tubes; synthetic medium (glucose) at 37 °C for 7 days in bioreactor	88 (LB, glucose, test tube) 106 (bioreactor)	Zhang and Stephanopoulos [18] ^c
TAL – <i>R. glutinis</i> 4HPA3H – <i>E. coli</i> $\Delta pheA$; <i>tyrA</i> ^{tr} – <i>ppsA</i> – <i>tktA</i> – <i>aroG</i> ^{tr}	M9 medium (yeast extract and glycerol, or glucose and glycerol) at 37 °C for 72 h in shake flasks. 20 mM <i>p</i> -coumaric acid fed 3 h after induction and 3 mM added afterward (wild-type case)	3,820 (wild-type) 766.68 (TYR strain)	Huang et al. [10]
4HPA3H – <i>P. aeruginosa</i>	Potassium phosphate buffer (glucose or glycerol) at 30 °C for 24 h in shake flasks. 20 mM of <i>p</i> -coumaric acid was added 4 times	10,200	Furuya and Kino [9]

^a In some studies a tyrosine overproducing strain was used in which: *tyrR* (tyrosine repressor) and *pheA* (chorismate mutase/prephenate dehydratase) were deleted ($\Delta tyrR$ and $\Delta pheA$) to direct the pathway only to tyrosine production; *ppsA* (PEP synthase) and *tktA* (transketolase) were overexpressed to increase the availability of the two main precursors of aromatic amino acids biosynthesis; and *tyrA* (chorismate mutase/prephenate dehydrogenase) and *aroG* (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) gene sequences were modified and the feedback inhibition-resistant derivatives (*tyrA*^{tr} and *aroG*^{tr}) were overexpressed to remove the transcriptional control mediated by tyrosine and phenylalanine. opTAL means codon-optimized TAL.

^b TYR strain – tyrosine overproducing strain.

^c An alternative route through coumaroyl-CoA and caffeoyl-CoA (Fig. 1) using 4CL from *P. crispus* and *E. coli* endogenous thioesterase was tested to increase the yield but it was not successful.

Rhodopseudomonas palustris was proved to convert *p*-coumaric acid to caffeic acid with a high yield.

In this study, we describe the production of caffeic acid from tyrosine or *p*-coumaric acid. To convert tyrosine to *p*-coumaric acid we used TAL from *R. glutinis*. *p*-Coumaric acid was converted to caffeic acid using C3H from *S. espanaensis* or CYP199A2 from *R. palustris*. Although all these genes have previously been used in the caffeic acid production, it is important to mention that, as far as we know, the caffeic acid production from tyrosine using CYP199A2 in the pathway has never been attempted. Additionally, in the current study, the titers of caffeic acid obtained using TAL and C3H genes were higher than the ones reported in other studies using the same genes. These high yields were obtained by using different combinations of plasmids and genetic arrangements.

Furthermore, it is important to mention that the caffeic acid pathway can be further used to produce other products of the phenylpropanoid pathway with high added-value like flavonoids and curcuminoids.

2. Materials and methods

2.1. Bacterial strains, plasmids and chemicals

E. coli ElectroMAX™ DH10B competent cells (Invitrogen/Life Technologies, Carlsbad, CA, USA) were used for molecular cloning and vector propagation. *E. coli* K-12 MG1655(DE3) [19] was used as the host for the expression of genes under the control of the T7 promoter.

Pseudomonas putida JCM 6157 strain (ATCC 17453, Manassas, VA, USA) and *R. palustris* CGA009 gDNA (ATCC BAA-98) were used to amplify the *pdr* and *pux* genes. The characteristics of all the strains and plasmids used in this study are described in Table 2.

Restriction, ligation and Q5 enzymes (NEB, Ipswich, MA, USA), QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA), DNA Clean and Concentrator and Gel DNA Recovery Kits (Zymo Research, Orange, CA, USA) and Wizard® Genomic DNA

Purification Kit (Promega, Madison, WI, USA) were used according to the instructions provided by the manufacturers.

L-Tyrosine, *p*-coumaric acid and caffeic acid were purchased from Sigma–Aldrich (Steinheim, Germany), isopropyl β-D-thiogalactopyranoside (IPTG) and Luria-Bertani (LB) medium from NZYTech (Lisbon, Portugal) and anhydrotetracycline (aTc) from Acros (Geel, Belgium). Glucose (Acros), Na₂HPO₄ (Scharlau, Sentmenat, Spain), MgSO₄, KH₂PO₄ (Riel-de Haën, Seelze, Germany), NH₄Cl, NaCl, CaCO₃ (Panreac, Barcelona, Spain) and thiamine (Fisher Scientific, Loughborough, UK) were used to prepare the M9 modified salt medium. The following mineral traces and vitamins were supplemented to the M9 medium: FeCl₃, ZnCl₂, CoCl₂, CuCl₂, nicotinic acid (Riedel-de Haën), NaMoO₄, H₂BO₃, pyridoxine, biotin, folic acid (Merck), riboflavin and pantothenic acid (Sigma Aldrich). Ampicillin (Applichem, Darmstadt, Germany), chloramphenicol, kanamycin (NZYTech) and spectinomycin (Panreac) were used when necessary.

2.2. Codon-optimization and synthesis of TAL, C3H and CYP199A2

TAL, C3H and CYP199A2 genes were codon-optimized for *E. coli*, synthesized and cloned in pUC57 vector by GenScript (Piscataway, NJ, USA). In addition to codon-optimization, the phenylalanine residue at 185 position (F185) of CYP199A2 was replaced by leucine (F185L) [8]. The DNA sequences of the codon-optimized genes are provided in Table 3.

2.3. Construction of plasmids

The genes encoding TAL and C3H were expressed in *E. coli* cells using the pETDuet-1, pCDFDuet-1, pRSFDuet-1 and pKVS45 vectors (Table 2). The gene encoding CYP199A2 mutant was cloned in pCDFDuet-1 and CYP199A2 redox partners, *pdr* and *pux* genes, were cloned in an operon in pKVS45 using restriction enzymes. All the primers used are summarized in Table 4. TAL and CYP199A2 were also cloned in an operon using Phusion DNA polymerase (NEB) and overlap extension polymerase chain reaction (PCR) [21]. The ribosome binding site (RBS) chosen was the same used in pETDuet-1, pCDFDuet-1 and pRSFDuet-1. Reverse primers of TAL gene were overlapped with forward primers of the CYP199A2 gene to introduce the RBS and spacer. Briefly, the TAL and CYP199A2 genes were amplified and the overlapping strands of these intermediate products hybridized in a subsequent PCR and were

Table 2
Bacterial strains and plasmids used in this study.

Strains	Relevant genotype	Source
<i>E. coli</i> ElectroMAX DH10B	<i>F</i> [−] <i>mcrA</i> Δ(<i>mrr</i> – <i>hsdRMS</i> – <i>mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1 endA1 araD139</i> Δ(<i>ara</i> , <i>leu</i>)7697 <i>galU galK</i> λ [−] <i>rpsL nupG</i>	Invitrogen/Life Technologies
<i>E. coli</i> K-12 MG1655(DE3) <i>P. putida</i> JCM 6157	<i>F</i> [−] λ [−] <i>ilvG</i> [−] <i>rfb</i> [−] 50 <i>rph</i> [−] 1 λ(DE3)	[19] ATCC 17453
Genomic DNA	Source	
<i>R. palustris</i> CGA009	ATCC BAA-98D-5	
Plasmids	Construct	Source
pETDuet-1	ColE1(pBR322) <i>ori</i> , <i>lacI</i> , double T7 <i>lac</i> , Amp ^R	Novagen
pCDFDuet-1	CloDF13 <i>ori</i> , <i>lacI</i> , double T7 <i>lac</i> , Strep ^R	Novagen
pRSFDuet-1	RSF <i>ori</i> , <i>lacI</i> , double T7 <i>lac</i> , Kan ^R	Novagen
pKVS45	<i>p15A ori</i> , tetR, P _{tet} , Amp ^R	[20]
pUC57_TAL	pUC57 carrying codon-optimized TAL from <i>R. glutinis</i>	GenScript
pUC57_C3H	pUC57 carrying codon-optimized C3H from <i>S. espanaensis</i>	GenScript
pUC57_CYP199A2	pUC57 carrying codon-optimized CYP199A2 from <i>R. palustris</i>	GenScript
pETDuet.TAL	pETDuet-1 carrying codon-optimized TAL from <i>R. glutinis</i>	This study
pETDuet.C3H	pETDuet-1 carrying codon-optimized C3H from <i>S. espanaensis</i>	This study
pETDuet.TAL.C3H	pETDuet.TAL carrying codon-optimized C3H from <i>S. espanaensis</i>	This study
pCDFDuet.TAL	pCDFDuet-1 carrying codon-optimized TAL from <i>R. glutinis</i>	This study
pCDFDuet.C3H	pCDFDuet-1 carrying codon-optimized C3H from <i>S. espanaensis</i>	This study
pCDFDuet.CYP	pCDFDuet-1 carrying codon-optimized CYP199A2 from <i>R. palustris</i>	This study
pCDFDuet.CYP (+7aa)	pCDFDuet-1 carrying codon-optimized CYP199A2 from <i>R. palustris</i> with the first 7 amino acids	This study
pCDFDuet.TAL.CYP	pCDFDuet.TAL carrying codon-optimized CYP199A2 from <i>R. palustris</i>	This study
pCDFDuet.TAL.CYP (+7aa)	pCDFDuet.TAL carrying CYP199A2 from <i>R. palustris</i> with the first 7 amino acids	This study
pCDFDuet.TAL.CYP_op	pCDFDuet-1 carrying codon-optimized TAL from <i>R. glutinis</i> and CYP199A2 from <i>R. palustris</i> in an operon	This study
pCDFDuet.TAL.CYP(+7aa)_op	pCDFDuet-1 carrying codon-optimized TAL from <i>R. glutinis</i> and CYP199A2 from <i>R. palustris</i> with the first 7 amino acids in an operon	This study
pKVS45.TAL	pKVS45 carrying codon-optimized TAL from <i>R. glutinis</i>	This study
pKVS45.C3H	pKVS45 carrying codon-optimized C3H from <i>S. espanaensis</i>	This study
pRSFDuet.TAL	pRSFDuet-1 carrying codon-optimized TAL from <i>R. glutinis</i>	This study
pRSFDuet.C3H	pRSFDuet-1t carrying codon-optimized C3H from <i>S. espanaensis</i>	This study
pKVS45_Pdr_Pux_op	pKVS45 carrying Pdr from <i>P. putida</i> and Pux from <i>R. palustris</i> in an operon	This study
pETDuet.Pdr_Pux_op	pETDuet-1 carrying Pdr from <i>P. putida</i> and Pux from <i>R. palustris</i> in an operon	This study

Table 3
Gene sequences of TAL, C3H and CYP199A2 with codon optimization.

Gene	Sequence
TAL (<i>Rhodotorula glutinis</i>)	ATGGCTCCGCGTCCGACCTCGCAATCCCAAGCTCGCACCTGCCCGACCACCCAAGTTACCCAAGTTGACATCGTTGAAAAAATGCTGGCGGCGCCGA- CCGATTTCGACGCTGGAAGCTGGACGGCTATAGCCTGAACCTGGGTGATGTGGTTTCTGCAGCAGCTAAAGGTCGTCGGTGCCTGTTAAAGATTGAGA- CGAAATTCGCTCGAAAATCGATAAAAGCGTGAATTTCTCGCTAGCCAGCTGAGCATGTCGTGTTACGGCGCTCACCCAGCGGTTTCGGCGGTTACGC- CGATACCCCGACCGAAGACGCCATTTCGCTGCAGAAAGCACTGCTGGAACATCAACTGTGCGGCGTGCTGCCGAGCTCTTTGATAGCTTCCGCTT- GGGCGGTGGTCTGGAAGCTTCTGCCGTGGAAGTCGTCGGTGGTGAACATGACCATCCGCTGTAATTCCTGACGCGCGGTCACTACGCTGTCCGTC- TGGTTGTCTGGAAGCGCTGACCAACTTTCTGAATCACGGTATTACGCCGATCGTGCCGCTGCGTGGTACCATTAGTGCATCCGGTGATCTGAGCCCGC- TGTTCTATATTGACGCTGCGATCTCTGCGCCACCCGGACAGTAAAGTTCATGTGGTTACGAGGGTAAAGAAAAAATCCTGTACGCCGCTGAAGCTATGG- CGCTGTTCACCTGGAACCGGTGCTGCTGGGCCGAAGAAGCGCTGGGTCTGGAATGTTGACGGCTGTTTCAGCGTCGATGGCCACCTGGCACTGCA- TGATGCCACATCTGAGCTGCTGAGCCAGTCTCTGACCGCATGACGGTGAAGCGATGTTGGGCCATGAGCGTAGCTTTTACCTCGTCTCTGACCAT- GTGACCCGTCCGCACCCGACGCAGATTGAAGTTGCAGGCAACATCCGCAAACCTGCTGGAAGGTAGCCGCTTTTGGCGTGCATCACGAAGAAGTGAAG- TGAAGATGACGAAGGCATTCTGCGCCAGGATCGTTATCCGCTGCGTACCAAGTCGCAATGGCTGGGTCCGCTGCTGCTCCGACCTGATTATGCCCCGCA- TGCTCGACCATCGAAGCGGTCAGAGTACCACGGATAACCCGCTGATTGACGTGGAATAAAACCTTCTATCACGCGCGTAACTTCAAGCCGACGCTGT- TGCCAATACGATGGAaaaaaacgcgctggcgctggcacagatcggtaaacTGAATTCACCCAACCTGACGGAAATGCTGAACGACGAGCATGAATCGTGGT- TGCCGAGCTGCCTGGCAGCAGAAGATCCGAGTCTGTCTATCATTGTAAGGCTCGACATGTCAGCTGCGGCCCTACACCTCTGAACCTGGGTATCTGCGCA- ACCCGGTTACACGACGCTCCAGCCGCTGAATGGCGAACCAAGCGTGAAATTCCTGGCATGATCTCAGCTCAGCTGACACACGACGGAATCGAATGATTC- TGAGCCTGCTGTGGCGACCCATCTGTATGTGTTCTGCAGGCTATTGACCTGCGCGCGATCGAAATTTGAATTCAAAAAGCTTTGGCCCGCATTTGTG- AGCCTGATCGATCAACACTTCGGCTCTGCCATGACCGGTAGTAACCTGCGTGACGAACCTGGTGAaaaaagTGAATAAAACGCTGGCCAAACGCTGGAAAC- GACCAACAGTTACGATCTGGTGGCGGTTGGCATGACGCATTTTCTTCGCGACCTGGTACGGTGTGTCGAAGTTCTGAGTTCACCTCACTGTCGCTGGCG- CCGTCAATGCTGGAAGTGGCAGCTGCGAAAGTGCAATTTCCCTGACCCGCAAGTGCGGTGAACAGCTTTTGTGACGAGCATGACGTCATGCGCGGCA- CTGAGCTATCTGTCTCCGCGCACCAAAATCTGTACGCTTTTGTTCGTGAAGAACTGGGCGTCAAAGCGCGTCGCGCGGATGTTTTCTGGGTAACACGGA- AGTGACCATCGGTAGTAATGTTTCAAAATCTATGAAGCTATCAAAGCGGTGCTATCAATAATGTGCTGCTGAAAAATGCTGGCATAA
C3H (<i>Saccharothrix espanaensis</i>)	ATGACGATTACCTCTCCGGCACCGGCTGGTCGCTGAACAATGTCCGCCCGATGACGGGTGAAGAATACCTGGAATCCCTGCGTGACGGCCGTGAAG- TGATATTTACGGTGAACCGCTCGATGACGTGACCACGCATCTGGCGTTCCGCAACAGCGCTTCGTTCTATCGCCCCGCTGTATGATGCTCTGCACGCC- GGCATCCGAAGGTGTTCTCGGTTCCGACCCGATACGGGTAAATGCGTGTGTTTACCCATCCGTTTCTTCAAAAGCGCGGTAGCTGTGAAGCATCGTGGCGG- CCGTGAAGCCATTGTCGGCTGGCAACGCGCTGGTGTATGGCTGGATGGTCTGATCCCGGATTACAAGACGCGTTTTTCGGTACGCTGGACGCTAACCGGGA- ATTTTATGCGCGTTCGAAGCCAATGCACGTGCTGCTGATCGTGATGCACAGGAACCGGTTCTGTACTTCAACCATGTATCGTTACCCCGCGCTCGATCG- TGACCGTCCGGTGTATGCTACCGCAGACATTTCGCTCCATGTGGAAGAAGAACCGGATTACGCGCTGATGCTGTGCGGTGCCAAATGTTGTCAACCGGTTT- TGCTATGACGAACGCGAATCTGATTGCCCACTATGGTCTGCCGTTTCGCGATAAAAAAATTTGGCCTGGTGTTCACCGTTCCGATGAACAGTCCGGGTCTGAA- ACTGATCTGTCGTACCTCTATGAACATGATGTGGCCACGCAGGGCTACCGCTTGATTACCCGCTGAGTTTCCCGCTGGATGAAAATGACACGATTATGAT- CTTTGATCGTGTCTGCTCCGCTGGGAAAAACGTTTATCATGTACGACGCGACCGCGCGCAATAGCTTTGTCTACCGGCTCTGGTTCTCTGGAACGCTTTACCTT- CCATGGTCTGCAGCGCTGTCGCAGTGAACCTGATTTTATTCAGGCTGTGTTATGAAGCTGTGGAAGTACCCGCAACGACGCATCTCCGCGTCTTCAGG- GCAAGTCGGCAAGTGCTGAACCTGGCGTGATGCTTTTTGGGCTCTGTCGACGCTATGCGCAAAAGTCCGAATTCCTGGTGGGCGGTAGCGTTCAGCCGA- ACCTGAATTAAGCGCTGGCTACCCGACCTTTATGGGCTGGGTTATCCCGCTATTAAAGAAATATCCAGCAAAACGCTGGGCTCTGGTCTGATCTCACTGA- ACTCATCGCAGCTGATTGAAAAATCCGACGTTTCGCCGTATCTGGATCGTTACTGCTGCGCGCAATCGTGGTATTTCAGGCAATCGATCGTGTCTCAACTG- CTGAAACTGCTGTGGGACGCAGTGGGTACCGAATTCGCAGGTCGTCATGAACCTGTATGAACGCAACTACGGCGGTGATCACGAAGGTATTCTGTGTCAGA- CCCTGCAAGCCTATCAGGCAAAATGGTCAAGCGGCGCACTGAAAGGCTTTGCGGAACAGTGTATGTCGGAATATGACCTGGATGGCTGGACCCGCCCGGA- CCTGATTAACCCGGGACGTTAA
CYP199A2 (<i>Rhodopseudomonas palustris</i>)	ATGACGACCCGCTCCGAGCTGATGCCGTTACGACGCCGTCTCAACATGGTGTGGTGTGCCGATCTGGGTATCGACCCGTTCCGACTGGATTAT- TTTGACAGCCCGTACCCGGAACAGGAACGCTGCGTGAAAGCGGTCGGGTGGTTTATCTGGAATAATGAACGTTTACGGCGTCGCCGCTATGCAGA- AGTGTACGCGGTTCTGAATGATCCGCTGACCTTTTGCAGCTCTCGTGGCGTGGGTCTGTCAGACTTCAAAAAAGAAAAACCGTGGCGCCCGCTGCG- TGATTCTGGAAGCTGATCGCGCGCACATACCGGTACCCGTGCTGTCTCTCAAAAGTGTGTGCGCGGCGACCATGAAACGCTGTGCGCGATGTGTTT- GCGGCGCAGCTGATGCCAAATCGACGAAGCTGCGACGTGGCGGTAACATTTATGATGTCGCGGACCTGGCCAGCATATCCGCTGTGAGTTTT- TCGGATGCGATGGGTCTGAAAACGAAGGCGCGGAAAAATCTGTCGCGTACGCTGGTGTGTTCTGCAAGCAATTCGGTCCGCGAATGAACGT- CGTCAGAGCCCATGGAACGCTGACCCGCATCAGCGGTATGTTGCGGAACAGTGCACAGTCCGAACCTGGCACCGGGCGGTTTTGTGTGATGTA- TTACACGATCTCCGATCGCGGGAATACCCCGGAAGAAGCTCCGCTGCTGGTGGTGTGCTGCTGCGCGCTGTGACACCCAGCGTGAACGGT- ATCGCAGCAGCTGTTACTGCTGGCCGCTTTCCGGATGAATTCGCTGCTGCGTGCGGACCCGCACTGGCCGCTAATGCAATGAAGAAGCAGT- TCGCTTCGAATCTCCGTTCCAGACGTTTTTCCGTACCACGACCCGCGATGTCGAACCTGGCTGGTGGCACCATTGGCGAAGGTGAAAAAGTGTGATGTT- TCTGGGCAGCGCAAATCGTAGCCGCGCTGCGGTGAGTACCCGATCGTTATGACATACCGCGCAAAACCAAGTGTGTCATGTTGGCTTCGGTTCGCGG- TTCACATGTGTGTGCGTCAACTGGTGGCGGCTGGAAGGTGAAGTCGTTGCTGCTGCACTGGCACGTAAAGTGGCAGCAATTGAAATCGCAGGCCCG- TGAAACCGCGTTTTAAACAATACCTCGGTGGTCTGGAAGGCTGCGGATCACTGACCTGACCCGCGCTGA

extended to generate the full-length product amplified by flanking primers that included restriction enzyme sites for inserting the operon into the plasmid.

All construction plasmids described were verified by colony PCR or digestion and sequenced by Macrogen (Amsterdam, The Netherlands) or Genewiz (Cambridge, MA, USA).

2.4. Growth conditions – *p*-coumaric and caffeic acid production

E. coli cells for gene cloning, plasmid propagation, and inoculum preparation were grown in LB medium at 37°C and 200 rpm.

For p-coumaric acid and/or caffeic acid production, cultures were grown at 37 °C in 50 mL LB to an optical density at 600 nm (OD₆₀₀) of 0.4. IPTG and/or aTc were added at the same time (unless otherwise specified) at a final concentration of 1 mM and 100 ng/mL, respectively, and the culture was incubated for 5 h at 26 °C. The cells were harvested by centrifugation, resuspended in 50 mL of modified M9 minimal salt medium containing (per liter): glucose (40 g), Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NH₄Cl (1 g), NaCl (0.5 g), CaCl₂ (17 mg), MgSO₄ (58 mg), thiamine (340 mg) and CaCO₃ (5 g) (to control the pH), and incubated at 26 °C for 63 h. Trace elements [FeCl₃ (54 mg), ZnCl₂ (4 mg), CoCl₂ (4 mg), NaMoO₄ (4 mg), CuCl₂ (2 mg) and H₂BO₃ (1 mg)] and vitamins [riboflavin (0.84 mg), folic acid (0.084 mg), nicotinic acid (12.2 mg), pyridoxine (2.8 mg), biotin (0.12 mg) and pantothenic acid (10.8 mg)] were also supplemented to the M9 medium. Depending on the plasmid(s) present in the strain, 100 µg/mL of ampicillin, 100 µg/mL of spectinomycin and/or 50 µg/mL of kanamycin were added.

PTG and/or aTc and substrates were added at time 0 of induction in M9 medium (unless otherwise stated): tyrosine, 3 mM or *p*-coumaric acid, 2 mM. Samples of the supernatant (1.5 mL) were collected at time 0 and after 15, 24, 43 and 63 h. All the experiments were done in triplicate and analyzed by high-performance liquid chromatography (HPLC).

2.5. HPLC analysis of the products

HPLC analysis was used to quantify *p*-coumaric acid and caffeic acid using a HPLC system from Jasco (Easton, MD, USA) (PU-2080 Plus Pump unit, LG-2080-02 Ternary Gradient unit, a DG-2080-53 3-Line Degasser unit, a UV-2075 Plus Intelligent UV/VIS Detector unit and AS-2057 Plus Intelligent Sampler unit) and a Grace Alltech Platinum EPS C18 column (3 μ m, 150 mm $\times$ 4.6 mm) (Grace, Columbia, MD, USA). Mobile phases A and B were composed of water (0.1% trifluoroacetic acid) and acetonitrile, respectively. The following gradient was used at a flow rate of 1 mL/min: 10–20% acetonitrile (mobile phase B) for 17 min. Quantification was based on the peak areas of absorbance at 275 nm (tyrosine) and 310 nm (*p*-coumaric acid and caffeic acid). The retention times of tyrosine, *p*-coumaric acid and caffeic acid were 3.3, 8.0 and 11.8 min, respectively.

2.6. Protein analysis

E. coli K-12 MG1655(DE3) cells harboring pETDuet-1, pETDuet.TAL, pETDuet.C3H and pETDuet.TAL.C3H were grown in LB at 37°C to an OD₆₀₀ of 0.6.

Table 4
Set of primers for PCR amplification (forward and reverse primers – FW and REV).

Primer name	Primer sequence ^a	Restriction enzyme
TAL_pET_pCDF_pRSF_FW	<i>GGCGCGCCAAATGGCTCCGCTCCG</i>	<i>AscI</i>
TAL_pET_pCDF_pRSF_REV	<i>GCGGCCGCTTATGCCAGCATTTTCAGCAG</i>	<i>NotI</i>
TAL_pKVS45_FW	<i>CCTAGGAAGGAGATATACCATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGCTCCGCGTCCG</i>	<i>AvrII</i>
TAL_pKVS45_REV	<i>GGATCCTTATGCCAGCATTTTC</i>	<i>BamHI</i>
C3H_pET_FW	<i>AGATCTCATGACGATTACCTCTCCGGC</i>	<i>BglII</i>
C3H_pET_REV	<i>CTCGAGCGTGCCCGGGTTAATCAG</i>	<i>XhoI</i>
C3H_pKVS45_FW	<i>CCTAGGAAGGAGATATACCATGGGCAGCAGCCATCACCATCATCACCACAGCCAGCAGATTACCTCTCCGGCA</i>	<i>AvrII</i>
C3H_pKVS45_REV	<i>GGATCCTTACGTGCCCGGGTTAATCAG</i>	<i>BamHI</i>
C3H_pCDF_pRSF_FW	<i>GGCGCGCCAAATGACGATTACCTCTCCGG</i>	<i>AscI</i>
C3H_pCDF_pRSF_REV	<i>AAGCTTTTACGTGCCCGGGTTAATC</i>	<i>HindIII</i>
CYP(+7aa)_pCDF_FW	<i>CATATGCATCACCATCATCACCACATGACGACCGCTCCGAGCCT</i>	<i>NdeI</i>
CYP_pCDF_FW	<i>CATATGCATCACCATCATCACCACATGCCGGTTACGACG</i>	<i>NdeI</i>
CYP_pCDF_REV	<i>CTCGAGTCAGGCCGGGGTC</i>	<i>XhoI</i>
TAL_op_FW	<i>GGATCCAATGGCTCCGCGTC</i>	<i>BamHI</i>
TAL_op_REV	<i>CGTCATGGTATATCTCCTTTTATGCCAGCATTTTCAGC</i>	-
CYP(+7aa)_op_FW	<i>ATAAAAGGAGATATACCATGACGACCGCTC</i>	-
CYP_op_REV	<i>AAGCTTTTACGCCGGGGTC</i>	<i>HindIII</i>
TAL_op2_REV	<i>GGCATGGTATATCTCCTTTTATGCCAGCATTTTCAGC</i>	-
CYP_op2_FW	<i>ATAAAAGGAGATATACCATGCCGGTTACGACG</i>	-
Pdr_pKVS45_FW	<i>CCTAGGAATAATTTTGTGTTAACTTTAAGAAGGAGATATAATGAACGCAAACGAC</i>	<i>NdeI</i>
Pdr_pKVS45_REV	<i>GAGCTCTCAGGCACTACTCAGTTCAGC</i>	<i>SacI</i>
Pux_pKVS45_FW	<i>GGATCCATAATTTTGTGTTAACTTTAAGAAGGAGATATAATGCCAGTATCAGTTTCATTCTT</i>	<i>BamHI</i>
Pux_pKVS45_REV	<i>GCATGCTCAGACCTGACGATCCGGAAT</i>	<i>SphI</i>
Pdr_Pux_op_pET_FW	<i>GAATTCAATGAACGCAAACGACAAC</i>	<i>EcoRI</i>
Pdr_Pux_op_pET_REV	<i>GATATCTCAGACCTGACGATCCG</i>	<i>EcoRV</i>

^a Start and stop codons in **bold**, occasionally the start codon is placed upstream of the His₆-tag sequence and no stop codon is included because of the presence of a Strep-tag; restriction sites in *italic*; His₆-tag underlined; the Ribosome Binding Site (RBS) and spacer are double underlined. In order for the sequence to remain in frame one or two bases were occasionally added between the restriction site and the gene start codon.

IPTG was added at a final concentration of 1 mM, and the culture was incubated for 24 h. Samples (10 mL culture medium) were taken at times 0, 5 and 24 h. Samples were centrifuged and the cells were resuspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 1.8 mM KH₂PO₄, pH 7.4) and further disrupted by sonication on ice for 3 min. After centrifugation the protein concentration from the resulting supernatant was determined using Protein Assay Dye Reagent Concentrate (BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) (NEB) as a standard. The expression levels of TAL and C3H were evaluated through sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS–PAGE). Fifteen to 20 µg of total protein were loaded onto a 4–20% Mini-PROTEAN® TGX™ Precast Gels (BioRad). The protein marker used was Precision Plus Protein™ Unstained (BioRad). For gel staining, Bio-Safe Coomassie Stain (BioRad) was used.

3. Results and discussion

3.1. Selection of the appropriate enzyme sources

TAL was chosen from the red yeast *R. glutinis*, since it was reported to have the highest TAL activity and a low PAL/TAL catalytic activity ratio [22–24]. TAL prefers tyrosine and is therefore favored over PAL and C4H, both of which are required to begin the pathway from phenylalanine (Fig. 1). This decreases the pathway number of steps and at the same time eliminates the issues associated with the use of C4H. C4H is a P450-dependent monooxygenase and the functional expression of plant P450 enzymes is always hard to achieve in bacteria, which is mainly due to the absence of cytochrome P450 reductases (CPRs)/redox partners in *E. coli* needed for electron transfer, and to the absence of endoplasmatic reticulum which prevents the efficient translational of the membrane signal modules of microsomal P450 enzymes [25]. C3H from *S. espanaensis* was chosen as it is one of the rare cytochrome P450 enzymes that was successfully expressed in *E. coli* [15] (Table 1). CYP199A2 from *R. palustris* was chosen as it was effectively used in previous studies to achieve one of the highest caffeic acid production from *p*-coumaric acid [8].

3.2. Production of *p*-coumaric acid from tyrosine using TAL

In order to study *p*-coumaric acid production from tyrosine, codon-optimized TAL was cloned in pETDuet-1, pCDFDuet-1, pRSFDuet-1 and pKVS45. Fig. 2 illustrates *p*-coumaric acid production from 3 mM of tyrosine. *p*-Coumaric acid was also produced from endogenous tyrosine in the presence of pCDFDuet.TAL, but in a very low quantity compared to conditions with tyrosine

Fig. 2. Production of *p*-coumaric acid using TAL from *Rhodotorula glutinis* using different plasmids after 63 h (A) and using pRSFDuet.TAL during 63 h in M9 medium (B). (*) no substrate added. TAL: tyrosine ammonia lyase.

Fig. 3. Production of caffeic acid using opC3H from *Saccharothrix espanaensis*. C3H: 4-coumarate 3-hydroxylase.

supplemented. Since the endogenous tyrosine is not enough to produce *p*-coumaric acid in high concentrations, two strategies can be used, namely supplement the medium with tyrosine or engineer *E. coli* to overproduce tyrosine from glucose. *p*-Coumaric acid production using TAL was found to be highly dependent on the plasmid chosen. The highest production obtained was 2.62 mM (472 mg/L) and it was achieved with pRSFDuet-1, which is a high copy number plasmid. In this case, the product yield was 0.87 (mol *p*-coumaric acid/mol tyrosine) and the remaining tyrosine was presumably used for growth and primary metabolism since no tyrosine was detected by HPLC. Santos et al. [26] used a codon-optimized *R. glutinis* TAL with a 80% similarity to the one used in this study. Although both plasmids (pETDuet-1 and pTrcHis2B) have a pBR322 origin, they have different promoters, with the T7 system being stronger than the *trc* promoter. The *p*-coumaric acid production obtained using pETDuet.TAL (the lowest production in the current study) was higher than that reported by Santos et al. [26] after 72 h using pTrcHis2B (104 mg/L – 0.61 mM). They obtained a product yield of 0.23 (mol *p*-coumaric acid/mol tyrosine) in a *E. coli* K12 MG1655(DE3) strain. From analyzing these results it can be concluded that the *R. glutinis* TAL used in our study and the expression systems chosen are a very good option to produce *p*-coumaric acid.

Fig. 2B shows the production of *p*-coumaric acid in the strain harboring pRSFDuet.TAL over time (63 h). The results showed that the *p*-coumaric concentration increases along time, and this trend was also observed when using other plasmid constructions (*data not shown*). Although 63 h of incubation is a long time, it is not unusual to prolong the fermentations more than 24 h in the caffeic acid production to obtain higher titers and yields, as can be observed in Table 1. Albeit the incubations can be performed at higher temperatures, the production would be lower since our preliminary results showed that using TAL and the other enzymes used in this study at 30 °C and 37 °C leads to lower titers than 26 °C (*data not shown*).

3.3. Production of caffeic acid from *p*-coumaric acid using C3H

Caffeic acid was produced from *p*-coumaric acid using *S. espanaensis* C3H (Fig. 3). C3H, although being a plant cytochrome P450 enzyme, has been successfully expressed in *E. coli* [15,17,18]. As previously shown in the *p*-coumaric acid production from tyrosine (Fig. 2), the highest caffeic acid titer was also obtained with the high copy number plasmid pRSFDuet-1. The titer obtained was around 0.93 mM (168 mg/L), corresponding to a product yield of 0.47 (mol caffeic acid/mol *p*-coumaric acid).

3.4. Production of caffeic acid from tyrosine using TAL and C3H

In a second phase of the current study, TAL and C3H were combined to produce caffeic acid from 3 mM of tyrosine (Fig. 4). In

Fig. 4. Production of caffeic acid using opTAL from *Rhodotorula glutinis* and opC3H from *Saccharothrix espanaensis*. TAL: tyrosine ammonia lyase; C3H: 4-coumarate 3-hydroxylase.

a first approach, the genes were cloned together in pETDuet-1. When these genes were combined, the production of caffeic acid increased 170%. This increase is probably due to the fact that in this case the *p*-coumaric concentration in the medium is lower than when it is added directly, thus reducing the toxic effect to the cells as previously described in the literature [9,10,27–30]. To confirm that the addition of *p*-coumaric acid had a toxic effect to the cells, *p*-coumaric acid and tyrosine were added separately to *E. coli* harboring pETDuet-1 and pETDuet.TAL.C3H. When *p*-coumaric acid was added, the *E. coli* cultures had a lower growth rate and the OD₆₀₀ was 10–11% lower than when tyrosine was added (*data not shown*). Moreover, the protein expression was found to be lower. When only TAL is cloned in the pETDuet-1 plasmid the protein band in the SDS gel could be clearly visualized (76.34 kDa) (*data not shown*). However, when TAL was combined with C3H, protein production was not observed maybe due to the metabolic burden imposed on the host cells by the expression of the two proteins in the same plasmid. Although in small amounts, a band around 56.33 kDa corresponding to C3H production could be observed.

Cloning TAL in pCDFDuet-1 and C3H in pETDuet-1 led to results very similar to the experiments in which these enzymes were used alone to produce *p*-coumaric acid or caffeic acid, respectively (Fig. 2A and Fig. 3). This occurs because the expression of TAL when alone in the plasmid is very high, leading to high production of *p*-coumaric acid. Consequently, caffeic acid amounts are comparable to the ones obtained when *p*-coumaric acid is added as substrate. Similar results were observed with the combination pETDuet.TAL and pCDFDuet.C3H. The production of *p*-coumaric acid was 1.28 mM and caffeic acid was 0.77 mM. Since caffeic acid is produced from *p*-coumaric, this suggests that in total more than 2 mM of *p*-coumaric acid was produced. When only pETDuet.TAL was used (Fig. 2A), the maximum *p*-coumaric acid production obtained was 0.9 mM, which demonstrates that tyrosine can be converted faster if *p*-coumaric acid is being converted to the next product. Again we observed that caffeic acid production does not increase (compared to the cases when only C3H is used and the substrate is *p*-coumaric acid) since the *p*-coumaric acid concentration in the medium is still very high. The combinations pKVS45.TAL/pCDFDuet.C3H, pCDFDuet.TAL/pKVS45.C3H and pRSFDuet.TAL/pCDFDuet.C3H showed very similar results.

The combination pCDFDuet.TAL/pRSFDuet.C3H is very interesting as almost all the *p*-coumaric acid produced is converted to caffeic acid. In the end, only around 3 μM of *p*-coumaric acid is detected by HPLC and the concentration in time was never higher than 0.15 mM, meaning that almost all the *p*-coumaric acid being

Fig. 5. Production of caffeic acid from *p*-coumaric acid using CYP199A2 from *Rhodotorula palustris*. CYP: cytochrome P450 CYP199A2.

produced was being converted by C3H to caffeic acid. A final caffeic acid concentration of around 1 mM (180 mg/L) was obtained. This combination enabled the highest yield from tyrosine with an additional benefit of having in the end a very low amount of the intermediate *p*-coumaric acid, contrary to what was observed in the other cases.

Zhang and Stephanopoulos [18] used codon optimized TAL from *R. glutinis* and C3H from *S. espanaensis* to produce caffeic acid. Also, the authors used a tyrosine over producing strain and no tyrosine limitation was observed during the caffeic acid production. After several medium optimizations, the highest titer they reported after 72 h was 106 mg/L (Table 1), which is 1.7 times lower than the maximum titer obtained in the current study after 63 h. Although those authors used the same plasmid to carry C3H (pRSFDuet-1), they used the pTrcHis2B plasmid to carry TAL, which was previously described by Santos et al. [26] and that we concluded it is not the best plasmid for this gene and to produce *p*-coumaric acid (see Section 3.2). Our study reports the highest titer of caffeic acid produced so far using the combination of TAL and C3H genes.

3.5. Production of caffeic acid from *p*-coumaric acid using CYP199A2

Until the recent studies published using 4HPA3H [9,10], CYP199A2 gene from *R. palustris* was reported to produce the highest amounts of caffeic acid from *p*-coumaric acid [8]. The results obtained in our study with CYP199A2 are summarized in Fig. 5. CYP199A2 was cloned in pCDFDuet-1 and its redox partners, Pdr and Pux, were expressed in another plasmid (pKVS45 or pETDuet-1) as part of an operon as described before [31,32]. The first results obtained with CYP199A2 in the caffeic acid production were surprisingly low as compared to the Furuya et al. [8] report. After analyzing the CYP199A2 DNA sequence it was verified that those authors did not use the first 21 bp of CYP199A2 to clone the gene. This decision was based on software results that annotated the 8th CYP199A2 amino acid (GTG) as a start codon before the sequence was published (T. Furuya, personal communication). We also confirmed that, for example, EasyGene 1.2b Server [33,34] identified the 8th amino acid as the start codon. Based on this, we cloned CYP199A2 without the first 7 amino acids. The production using this new CYP199A2 increased considerably the caffeic acid production compared to the original clone (CYP199A2(+7aa)). Additionally, it was found that the production was more than 2.7 times higher when the inducer of pKVS45_Pdr.Pux.op, aTc, was added 2.5 h after addition of IPTG (to induce CYP199A2 expression). The delay of induction can alleviate the metabolic burden of several plasmids [26,35]. Since pKVS45 has CYP199A2 redox partners (Pdr and Pux)

Fig. 6. Production of caffeic acid using TAL from *Rhodotorula glutinis* and CYP199A2 from *Rhodopseudomonas palustris*. TAL: tyrosine ammonia lyase; CYP: cytochrome P450 CYP199A2.

and these proteins are only needed after CYP199A2 is present in a significant concentration to support its catalytic activity (NADPH- and O₂-dependent hydroxylation reactions), their expression can be delayed. The results obtained with CYP199A2 in this case were 1.8 times better than the one obtained with C3H in pCDFDuet-1 adding *p*-coumaric acid (Fig. 3). Nevertheless, to improve the yield and taking into consideration that the addition of a high concentration of *p*-coumaric acid can have a detrimental effect on the caffeic acid production, as discussed before, a different feeding system was tested: 1 mM of *p*-coumaric acid was added at time 0 of induction in M9 medium and 0.5 mM added at 5 h and 24 h. Caffeic acid production increased to 1.72 mM (310 mg/L) using this three step feeding. This approach was successfully demonstrated before for the production of caffeic acid [9,10] and allowed us to obtain a product yield of 0.86 (mol caffeic acid/mol *p*-coumaric acid) after 63 h. Furuya et al. [8] obtained a maximum yield of 0.75 (mol caffeic acid/mol *p*-coumaric acid) after 24 h using glycerol as energy source. When the authors used glucose, the maximum yield obtained after 24 h was around 0.46 (mol caffeic acid/mol *p*-coumaric acid), thus very similar to the one obtained in the current study after 24 h–0.47 (mol caffeic acid/mol *p*-coumaric acid), which suggests that glycerol is a more effective energy source to regenerate NADH from NAD⁺ [8].

Since pKVS45 time of induction with aTc seems to limit caffeic acid production, pETDuet-1, that is induced using IPTG similarly to pCDFDuet-1, was chosen to clone the CYP199A2 redox partners and evaluate if the caffeic acid production could be increased. However, the results obtained with pETDuet-1 were very similar to the ones obtained with pKVS45 induced at time 0. This result is not surprising since pKVS45 alone, or combined with pCDFDuet-1, gave better overall results using TAL and C3H than the pETDuet-1 plasmid (Figs. 2–4).

3.6. Production of caffeic acid from tyrosine using TAL and CYP199A2

To produce caffeic acid from tyrosine, TAL and CYP199A2 were combined using different approaches, namely together in the same plasmid but in different MCSs (TAL in MCS1 and CYP199A2 in MCS2), or in an operon in MCS1 (Fig. 6). Both CYP199A2 sequences were tested and again it was concluded that CYP199A2 starting at the 8th amino acid provides better results. The low caffeic acid concentration obtained was due to the addition of aTc at time zero of induction. However, even with a low concentration, it is possible

to conclude that the genes work better when cloned in different MCSs than when cloned in an operon. Thus, this approach was chosen to proceed with other tests including pKVS45 later induction with aTc; three phases of *p*-coumaric acid addition; and the use of another plasmid to carry CYP199A2 redox partners (pETDuet-1). The results obtained were very similar to the ones found with only CYP199A2 in the plasmid (Fig. 5). The expression of TAL in a different plasmid (pRSFDuet-1) improved caffeic acid production. The highest caffeic acid production was 1.56 mM (280 mg/L). According to these results, CYP199A2 with its redox partners seems to be a better option than C3H to produce caffeic acid from tyrosine or *p*-coumaric acid.

Based on the above discussion, we believe that it would be advisable to, in the future, clone CYP199A2 with its redox partners in different plasmids and combine them with TAL to confirm if caffeic acid production can be improved without a high accumulation of the intermediate as its accumulation may result in suboptimal production titers. Although no kinetic parameters were determined in our study, it was possible to observe that over time the conversion of tyrosine to *p*-coumaric acid was faster than the conversion of *p*-coumaric acid to caffeic acid. The faster conversion of tyrosine to *p*-coumaric acid leads to the accumulation of *p*-coumaric acid which represents a drawback to the caffeic acid production since its toxicity leads to an even more pronounced decrease of the production. The kinetic parameters of the enzymes used in this study should be determined, especially regarding the C3H, since TAL and CYP199A2 kinetic parameters were characterized by Xue et al. [22] and Furuya et al. [8], respectively. Furthermore, it will be important to evaluate a combination of plasmids that allows a fine-tuned production like the one observed when TAL was cloned in pCDFDuet-1 and C3H in pRSFDuet-1, where no *p*-coumaric acid was accumulated (Fig. 4). Also, to improve the yield and to avoid the need of two separate stages of cultivation for biomass/protein generation and caffeic acid production, the use of potassium phosphate buffer [8,9] or MOPS [18,26] with glucose or glycerol should be tested. The use of M9 minimal medium without the production of biomass and protein in LB does not allow obtaining productions as high as the ones obtained by first using LB and then transferring the cells to M9 (data not shown).

Caffeic acid has for a long time been recognized for its therapeutic properties, which makes it an attractive target for metabolic engineering and synthetic biology. We have successfully designed a pathway for the production of caffeic acid via metabolic engineering approaches in *E. coli*. We tested different genetic arrangements with two (or four) genes to balance the expression of the enzymes and achieve an optimized performance, and we obtained significantly different levels of caffeic acid productions. The layout of genes and operons in the plasmid, as well as the use of different plasmids had an enormous impact on gene expression. In addition, codon-optimization when expressing heterologous genes in *E. coli* was considered to improve gene expression. The repeated addition of the substrate and the delay in the induction of protein expression also led to an increase of the titers by decreasing the toxicity of *p*-coumaric acid and the metabolic burden of heterologous protein expression.

In conclusion, the caffeic acid is a phenylpropanoic acid and this pathway can be further used to produce other products of the phenylpropanoid pathway from tyrosine like flavonoids, stilbenoids, isoflavonoids and curcuminoids. Until now the biosynthesis of these compounds is in the range of the titers obtained in this study for *p*-coumaric acid and caffeic acid or much lower. Therefore, the strategy of adding *p*-coumaric acid at concentrations as high as 20 mM, concentrations used in some studies for the caffeic acid production (Table 1), was considered unreasonable, especially knowing that high concentrations of *p*-coumaric acid are toxic to the cells. For the production system to be even more

economically viable, the use of a tyrosine overproducing strain should be considered.

Competing interests

The authors declare no competing interests.

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