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Production of curcuminoids from tyrosine by a metabolically engineered *Escherichia coli* using caffeic acid as an intermediate

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Curcuminoids are phenylpropanoids with high pharmaceutical potential. Herein, we report an engineered artificial pathway in *Escherichia coli* to produce natural curcuminoids through caffeic acid. *Arabidopsis thaliana* 4-coumaroyl-CoA ligase and *Curcuma longa* diketide-CoA synthase (DCS) and curcumin synthase (CURS1) were used to produce curcuminoids and 70 mg/L of curcumin was obtained from ferulic acid. Bisdemethoxycurcumin and demethoxycurcumin were also produced, but in lower concentrations, by feeding *p*-coumaric acid or a mixture of *p*-coumaric acid and ferulic acid, respectively. Additionally, curcuminoids were produced from tyrosine through the caffeic acid pathway. To produce caffeic acid, tyrosine ammonia lyase from *Rhodotorula glutinis* and 4-coumarate 3-hydroxylase from *Saccharothrix espanaensis* were used. Caffeoyl-CoA 3-*O*-methyltransferase from *Medicago sativa* was used to convert caffeoyl-CoA to feruloyl-CoA. Using caffeic acid, *p*-coumaric acid or tyrosine as a substrate, 3.9, 0.3, and 0.2 mg/L of curcumin were produced, respectively. This is the first time DCS and CURS1 were used in vivo to produce curcuminoids and that curcumin was produced by feeding tyrosine. We have shown that curcumin can be produced using a pathway involvoing caffeic acid. This alternative pathway represents a step forward in the heterologous production of curcumin using *E. coli*.

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1 Introduction

Curcuminoids are natural phenylpropanoids from the plant *Curcuma longa* Linn. Its rhizome contains a mixture of curcuminoids, with curcumin, demethoxycurcumin, and bisdemethoxycurcumin present in higher amounts. These compounds present in turmeric have long been used in traditional Asian food and medicine. Their therapeutic properties include anti-cancer, anti-inflammatory, anti-oxidant, anti-Alzeimer's, anti-HIV, and anti-Parkinson [1–4]. Despite their numerous benefits to human health, curcuminoids have poor bioavailability and their natural abundance is low, thus making their heterologous biosynthetic production very interesting.

Recently, curcuminoids were found to be synthesized by type III polyketide synthases (PKSs) and additional enzymes from the phenylpropanoid pathway in plants [5]. Katsuyama et al. [6] were the first to produce curcuminoids using an artificial pathway in *E. coli*. They used phenylalanine ammonia lyase (PAL) from *Rhodotorula rubra* with tyrosine ammonia lyase (TAL) activity to convert the amino acids phenylalanine and tyrosine directly to cinnamic acid and *p*-coumaric acid. 4-coumarate-CoA ligase (4CL) from *Lithospermum erythrorhizon* was used to convert cinnamic acid and *p*-coumaric acid to cinnamoyl-CoA and *p*-coumaroyl-CoA, respectively, and

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Abbreviations: 4CL, 4-coumaroyl-CoA ligase; **ACC,** acetyl-CoA carboxylase; **C3H,** 4-coumarate 3-hydroxylase; **CCoAOMT,** caffeoyl-CoA 3-*O*-methyltransferase; **CURS,** curcumin synthase; **CUS,** curcuminoid synthase; **DCS,** diketide-CoA synthase; **PAL,** phenylalanine ammonia lyase; **TAL,** tyrosine ammonia lyase

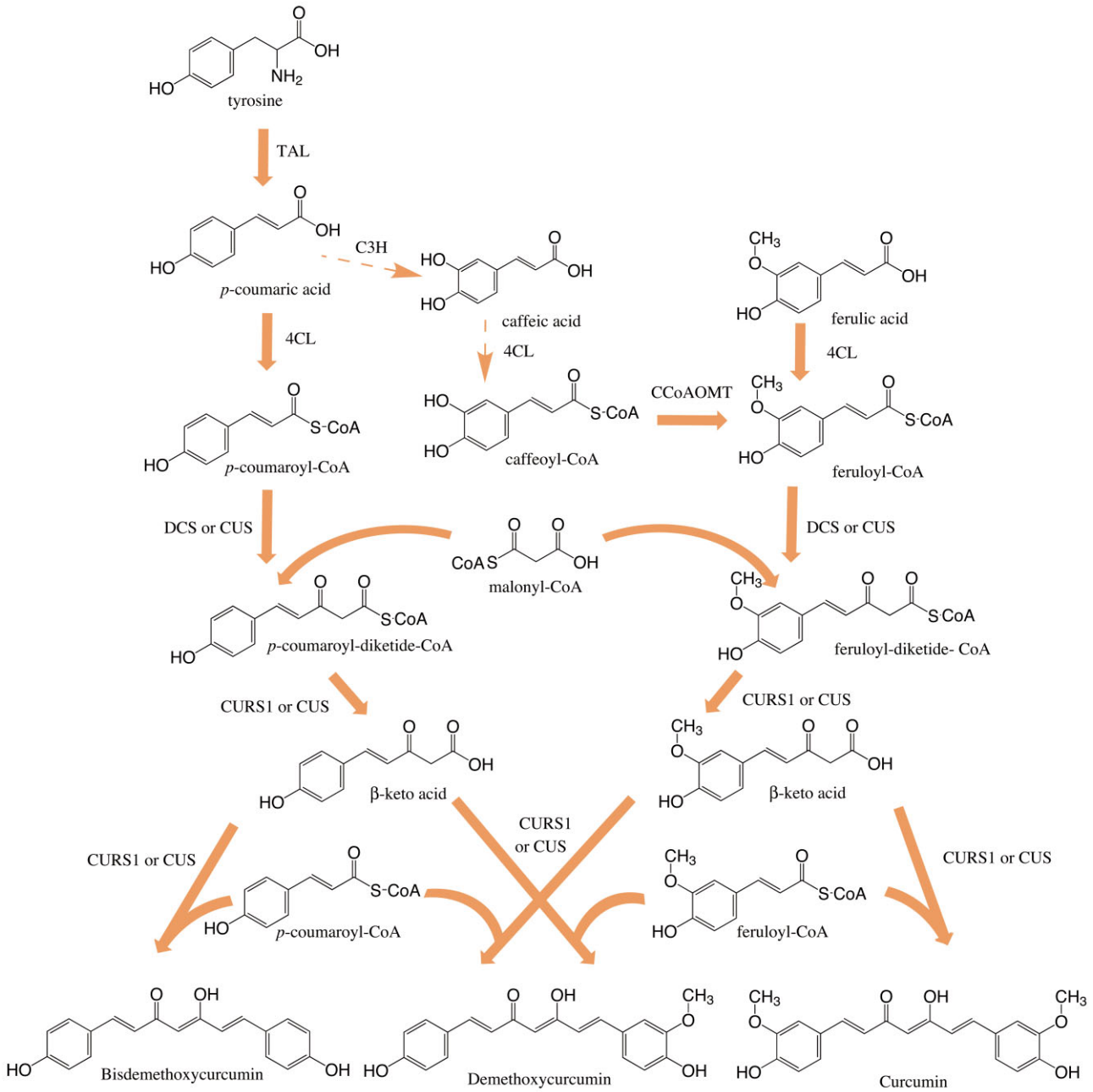


Figure 1. Proposed curcuminoid biosynthetic pathway in *E. coli* and the reactions catalyzed by the enzymes in this study. TAL, tyrosine ammonia lyase; C3H, 4-coumarate 3-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCoAOMT, caffeoyl-CoA 3-*O*-methyltransferase; DCS, diketide-CoA synthase; CURS1, curcumin synthase; CUS, curcuminoid synthase.

then to curcuminoids by curcuminoid synthase (CUS) from *Oryza sativa*. Acetyl-CoA carboxylase (ACC) from *Corynebacterium glutamicum* was also overexpressed to increase the intracellular pool of malonyl-CoA. The supplementation of amino acids to the medium led to the production of bisdemethoxycurcumin and other two curcuminoids, cinnamoyl-*p*-coumaroylmethane, and dicinnamoylmethane. The direct supplementation of carboxylic acids, as ferulic acid for instance, led to other curcuminoids including curcumin and demethoxycurcumin [6]. By adding two different unnatural carboxylic acids simultaneously (analogs of *p*-coumaric acid), Katsuyama and coworkers also produced unnatural curcuminoids [7]. Moreover, Wang et al. [8] produced the curcuminoid dicinnamoylmethane by using PALs from *Trifolium pratense*, 4CL1 from *Arabidopsis thaliana* and CUS from *O. sativa*. After CUS was discovered, Katsuyama et al. [9] reported that in the *C. longa* plant, the PKSs used to pro-

duce curcuminoids were diketide-CoA synthase (DCS) and curcumin synthase (CURS1). They also identified other CURS enzymes (CURS2 and CURS3) with different substrate specificities [10]. It is important to bear in mind that CUS catalyzes both steps that are catalyzed separately by DCS and CURS [9] (Fig. 1).

In this work, we describe curcuminoid production in *E. coli* using an artificial pathway (Fig. 1). We tested 4CL1 from *A. thaliana* (*At*4CL1) and different PKSs for curcuminoid production (CUS, DCS, and CURS1). Curcumin, demethoxycurcumin, and bisdemethoxycurcumin were produced by adding ferulic acid and/or *p*-coumaric acid as precursors. To produce curcuminoids, including curcumin, from the amino acid tyrosine, caffeic acid had to be produced as an intermediate in the pathway. TAL from *R. glutinis* and 4-coumarate 3-hydroxylase (C3H) from *S. espanaensis* were selected to produce caffeic acid based on the results obtained in our previous work (J. Rodrigues et al., submitted). Caffeoyl-CoA was converted to feruloyl-CoA by CCoAOMT from *Medicago sativa*. This alternative pathway through caffeic acid allowed, for the first time, the production of curcumin, the most studied curcuminoid for therapeutic purposes and considered in many studies as the most potent and active [3, 4], from the amino acid tyrosine, thus representing an advance in the heterologous production of curcumin by *E. coli*.

2 Materials and methods

2.1 Bacterial strains, plasmids, and chemicals

E. coli NZY5α competent cells (NZYTech, Lisbon, Portugal) were used for molecular cloning and vector propagation and *E. coli* K-12 MG1655(DE3) [11] was used as the host for the expression of genes under T7 promoter control. The characteristics of all the strains and plasmids used in this study are described in Table 1. TAL, C3H, DCS, CURS1, CUS, and CCoAOMT genes were codon-optimized for *E. coli*, synthesized and cloned in the plasmid vector pUC57 by GenScript (Piscataway, NJ, USA) or NZYTech (Lisbon, Portugal). The DNA sequences of the codon-optimized genes are provided in Supporting Information, Table S1. pAC_*At*4CL1 was purchased from Addgene (Cambridge, MA, USA).

Restriction and ligation enzymes (NEB, Ipswich, MA, USA), KAPA HiFi DNA polymerase enzyme (Kapa Biosystems, Wilmington, MA, USA), NucleoSpin® Plasmid Miniprep Kit (Macherey-Nagel, Düren, Germany) and DNA Clean and Concentrator and Gel DNA Recovery Kits (Zymo Research, Orange, CA, USA) were used according to the instructions provided by the manufacturers.

L-tyrosine, *p*-coumaric, caffeic acid, demethoxycurcumin, and bisdemethoxycurcumin were purchased from Sigma–Aldrich (Steinheim, Germany), ferulic acid from Acros (Geel, Belgium), curcumin from Fisher Scientific (Loughborough, UK), isopropyl β-D-thiogalactopyranoside (IPTG) and Luria-Bertani (LB) medium from NZYTech (Lisbon, Portugal) and anhydrotetracycline (aTc) from Acros. Glucose (Acros), Na_2HPO_4 (Scharlau, Sentmenat, Spain), $MgSO_4$, KH_2PO_4 (Riel-deHaën, Seelze, Germany), NH_4Cl, NaCl, $CaCO_3$ (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: $FeCl_3$, $ZnCl_2$, $CoCl_2$, $CuCl_2$, nicotinic acid (Riedel-deHaën), $NaMoO_4$, H_2BO_3, 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 Construction of plasmids

The genes encoding CUS, CURS1, DCS, CCoAOMT, TAL, and C3H were expressed in *E. coli* cells using the pETDuet-1, pCDFDuet-1, pRSFDuet-1 and pKVS45 vectors (Table 1) and were cloned using the appropriate restriction enzymes (Supporting information, Table S2).

All constructed plasmids herein described were verified by colony PCR or digestion and confirmed by sequencing (Macrogen, Amsterdam, The Netherlands).

2.3 Curcuminoid production

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

For the production of curcuminoids, the cultures were grown at 37°C in LB (50 mL) to an optical density at 600 nm of 0.3–0.4. IPTG and/or aTc were added (final concentration of 1 mM and 100 ng/mL, respectively) to induce protein expression. The culture was then incubated for 5 h at 26°C. Next, the cells were harvested by centrifugation, suspended, and incubated at 26°C for 63 h in modified M9 minimal salt medium (50 mL) containing (per liter): glucose (40 g), Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), NaCl (0.5 g), $CaCl_2$ (17 mg), $MgSO_4$ (58 mg), thiamine (340 mg), and $CaCO_3$ (5 g) (to control the pH). Trace elements [$FeCl_3$ (54 mg), $ZnCl_2$ (4 mg), $CoCl_2$ (4 mg), $NaMoO_4$ (4 mg), $CuCl_2$ (2 mg), and H_2BO_3 (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, ampicillin (100 μg/mL), spectinomycin (100 μg/mL), chloramphenicol (30 μg/mL), and/or kanamycin (50 μg/mL) were added. aTc and IPTG were added at the same time. Substrates were added at time 0 of induction in M9 medium (unless otherwise stated): tyrosine (3 mM), *p*-coumaric acid (2 mM), caffeic acid (1 mM) and ferulic acid

Table 1. Bacterial strains and plasmids used in this study

Strains	**Relevant Genotype**	**Source**
E. coli NZY5a	*fhuA2Δ(argF-lacZ)*U169 *phoA glnV44* Φ80 Δ*(lacZ)*M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NZYTech
E. coli K-12 MG1655 DE3	*F⁻ λ⁻ ilvG⁻ rfb*-50 *rph*-1 λ(DE3)	[11]
Plasmids	**Construct**	**Source**
pETDuet-1	ColE1(pBR322) *ori*, *lacI*, double T7*lac*, Amp^R	Novagen
pCDFDuet-1	CloDF13 *ori*, *lacI*, double T7*lac*, $Strep^R$	Novagen
pRSFDuet-1	RSF *ori*, *lacI*, double T7*lac*, Kan^R	Novagen
pKVS45	*p15A ori*, tetR, P_{tet}, Amp^R	[24]
pUC57_CCoAOMT	pUC57 carrying codon-optimized CCoAOMT from *M. sativa*	GenScript
pUC57_CUS	pUC57 carrying codon-optimized CUS from *O. sativa*	NZYTech
pUC57_DCS	pUC57 carrying codon-optimized DCS from *C. longa*	NZYTech
pUC57_CURS1	pUC57 carrying codon-optimized CURS1 from *C. longa*	NZYTech
pAC_*At*4CL1	pAC carrying not codon-optimized 4CL1 from *A. thaliana*	Addgene (35947)
pETDuet_TAL	pETduet-1 carrying codon-optimized TAL from *R. glutinis*	*unpublished data*
pETDuet_C3H	pETduet-1 carrying codon-optimized C3H from *S. espanaensis*	*unpublished data*
pETDuet_TAL_C3H	pETduet-1 carrying codon-optimized TAL from *R. glutinis* and C3H from *S. espanaensis*	*unpublished data*
pKVS45_C3H	pKVS45 carrying codon-optimized C3H from *S. espanaensis*	*unpublished data*
pCDFDuet_TAL	pCDFDuet-1 carrying codon-optimized TAL from *R. glutinis*	*unpublished data*
pRSFDuet_CUS	pRSFDuet-1 carrying codon-optimized CUS from *O. sativa*	this study
pRSFDuet_CURS1	pRSFDuet-1 carrying codon-optimized CURS1 from *C. longa*	this study
pCDFDuet_DCS	pCDFDuet-1 carrying codon-optimized DCS from *C. longa*	this study
pRSFDuet_CCoAOMT	pRSFDuet-1 carrying codon-optimized CCoAOMT from *M. sativa*	this study
pRSFDuet_*At*4CL1_CUS	pRSFDuet_*At*4CL1 carrying codon-optimized CUS from *O. sativa*	this study
pCDFDuet_DCS_CCoAOMT	pCDFDuet_DCS carrying codon-optimized CCoAOMT from *M. sativa*	this study
pRSFDuet_CURS1_CCoAOMT	pRSFDuet_CURS carrying codon-optimized CCoAOMT from *M. sativa*	this study
pCDFDuet_DCS_TAL	pCDFDuet_DCS carrying codon-optimized TAL from *R. glutinis*	this study
pCDFDuet_DCS_*At*4CL1	pCDFDuet_DCS carrying 4CL1 from *A. thaliana*	this study

(2 mM). All the experiments were performed in triplicate unless otherwise specified.

2.4 Curcuminoid extraction

For posterior analysis, 2 mL of culture broth were taken at several points during the fermentation and adjusted to pH 3.0 with HCl (6 M). Then, the curcuminoids present in the samples were extracted with an equal volume of ethyl acetate. The extraction procedure was performed more than once for the cases in which the presence of curcuminoids inside the cells after the first extraction was still visible to the naked eye (yellow coloration). The extracts were concentrated by solvent evaporation in a fume hood, suspended in at least 200 μL of acetonitrile and then subjected to product analysis by high-performance liquid chromatography (HPLC).

2.5 HPLC analysis of hydroxycinnamic acids and curcuminoids

HPLC analysis was used to quantify *p*-coumaric acid, caffeic acid, ferulic acid, curcumin, demethoxycurcumin, and bisdemethoxycurcumin using a 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 μm, 150 mm × 4.6 mm) (Grace, Columbia, MD, USA). Mobile phases A and B were composed of water (0.1% trifluoroacetic acid) and acetonitrile, respectively. For the hydroxycinnamic acids quantification, the following gradient was used at a constant flow rate (1 mL/min): 10–20% acetonitrile (mobile phase B) for 16 min. Quantification was based on the peak areas obtained at 310 nm for *p*-coumaric acid, caffeic acid and ferulic acid. The retention times of *p*-coumaric acid, caffeic acid and ferulic acid were 8.0, 11.8, and 13.8 min, respectively. For the curcuminoid quantification, a gradient of 40–43% acetonitrile (mobile phase B) for 15 min and 43% acetonitrile for an additional 5 min was used. The curcuminoids were detected at 425 nm and the retention times of bisdemethoxycurcumin, demethoxycurcumin and curcumin were 12.4, 13.5, and 14.5 min, respectively.

2.6 Protein analysis (CUS, CURS1, DCS, and CCoAOMT)

E. coli K-12 MG1655(DE3) cells harboring pRSFDuet_CUS, pRSFDuet_CURS1, pRSFDuet_DCS and pRSFDuet_CCoAOMT were grown in LB at 37°C to an optical density at 600 nm of 0.6. IPTG was added (at a final concentration of 1 mM) to induce protein expression, and the culture was incubated for 5 h. Samples (10 mL culture medium) were taken at time 0 and 5 h of induction. Samples were centrifuged and the cells were suspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4) and 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) and bovine gamma globulin (BSA) (NEB) as a standard. The expression levels of the enzymes were examined using sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS–PAGE) analysis. Fifteen to 20 μg of total protein were loaded in 4–20% Mini-PROTEAN® TGX™ Precast Gels (BioRad). The protein marker used was Precision Plus Protein™ Unstained (BioRad) and/or ColorPlus™ Prestained Protein Ladder Broad Range (NEB). For gel staining, Bio-Safe Coomassie Stain (BioRad) was used.

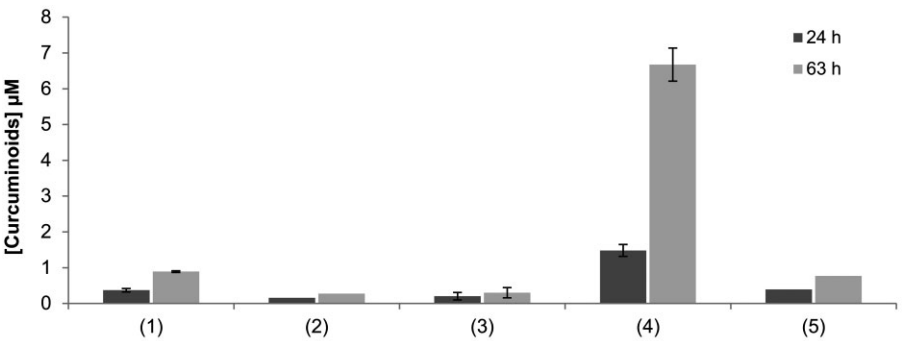
3 Results

3.1 The 4-coumarate-CoA ligase (4CL) limiting step

4CL enzymes convert the hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, and ferulic acid) to their corresponding CoA esters (*p*-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA) (Fig. 1) with different substrate preferences and specificities. We studied three different 4CL enzymes (*Le*At4CL1, *At*4CL2, and *At*4CL1) and only *At*4CL1 was found to be functionally expressed. Recently, this enzyme was successfully used in the production of curcuminoids [8] and in stilbene and flavonoid biosynthesis [12, 13]. In the current work, this enzyme was tested in vivo with CUS and CURS1/DCS. Since the results obtained regarding the production of curcuminoids were positive, all the further optimization tests were performed using this 4CL enzyme.

3.2 Very low production of curcuminoids using CUS from *O. sativa*

Codon-optimized CUS was cloned in pRSFDuet-1 and its expression was confirmed on a protein gel (44.3 kDa) (Supporting information, Fig. S1). After that, CUS was tested in *E. coli* with *At*4CL1 (pRSFDuet_CUS + pAC_*At*4CL1) (Fig. 2). These enzymes produced bis-



(1) Bisdemethoxycurcumin; 2 mM *p*-coumaric acid
(2) Bisdemethoxycurcumin; 1 mM *p*-coumaric acid + 1 mM *p*-coumaric acid at 24 h
(3) Bisdemethoxycurcumin; 3 mM tyrosine
(4) Curcumin; 2 mM ferulic acid
(5) Curcumin; 1 mM ferulic acid + 1 mM ferulic acid at 24 h

Figure 2. Curcuminoid production from *p*-coumaric acid and ferulic acid using 4-coumarate-CoA ligase (4CL1) from *A. thaliana* and curcuminoid synthase (CUS) from *O. sativa*. Bisdemethoxycurcumin was also produced from tyrosine using TAL from *R. glutinis*. Error bars are standard deviations from triplicate experiments.

demethoxycurcumin and curcumin when *p*-coumaric acid and ferulic acid, respectively, were added to the culture medium. Curcumin was produced in higher amount (6.7 μM) than bisdemethoxycurcumin (0.9 μM).

Additionally, we found that the production of curcuminoids at 24 h is lower compared to 63 h which is in agreement with the results reported by Katsuyama et al. [6]. This difference is significant (4.5 times lower) when curcumin is produced compared to bisdemethoxycurcumin production. Also, a yellow color of the culture medium, due to the increase of curcuminoids inside the cells, could only be observed after 24 h. The production of curcuminoids, even at low concentrations (0.9 μM) could be easily detected after 63 h (Supporting information, Fig. S2A and B) by the observation of some cells that adhered to the walls of the shake flasks.

To improve curcuminoid production, *p*-coumaric acid and ferulic acid were supplemented to the culture medium in two steps: 1 mM at time 0 of induction and 1 mM after 24 h of induction. However, the production decreased up to around 8.6 times. Bisdemethoxycurcumin was also produced from tyrosine using TAL from *R. glutinis* (cloned in pCDFDuet_TAL), *At*4CL1 and CUS. The production was very similar to that obtained in the experiment in which *p*-coumaric acid was added in two steps, which probably means that the production/concentration of *p*-coumaric acid in the first 24 h affects the production of bisdemethoxycurcumin. The production of *p*-coumaric acid at 63 h was around 1500 μM, thus suggesting that the TAL enzyme was highly functional.

3.3 DCS and CURS1 from *C. longa* allowed high production levels of curcumin

Codon-optimized DCS and CURS1 were cloned in pRSFDuet-1 and their protein expression was also confirmed on a protein gel (Supporting information, Fig. S1). The protein expression of DCS and CURS1 (42.9 kDa) was very high; hence in vivo production was further tested. Figure 3A illustrates the curcumin production from ferulic acid using pAC_*At*4CL1, pCDFDuet_DCS, and pRSFDuet_CURS1. The production with these enzymes was very high, 187.9 μM (70 mg/L) after 63 h. Besides, the culture medium showed a dark orange color (Supporting information, Fig. S2D), thus suggesting the production of curcuminoids.

The addition of substrate (ferulic acid) was also tested at three different time points, namely 1 mM at time zero of induction in M9 medium, 0.5 mM at 5 h and an additional 0.5 mM 24 h after induction. As previously observed (Fig. 3A), curcuminoid production was also significantly lower (5.5 times) with multiple additions compared to adding all the ferulic acid at time zero of induction.

To understand if the amount of cells (OD_{600}) at the time of induction in LB influenced the production of curcuminoids, we induced the cultures at different OD_{600} values (Fig. 3B). We found that, although the cells should be induced early, induction should not be too early since it can impose a metabolic burden on the host strain associated with protein overexpression. The addition of IPTG at an OD_{600} of 0.4 yielded the highest production titer (187.9 μM), thus suggesting that induction should be performed at an OD_{600} of 0.3–0.4. Moreover, we tested the production by inducing the cells in LB and then in M9 at 37°C instead of 26°C. As expected the production of curcumin at 37°C was residual. Low temperatures, around 26–30°C, should be used for optimal enzyme synthesis and curcuminoid production.

Additionally, the effect of adding *p*-coumaric acid as a substrate, or a mixture of *p*-coumaric acid and ferulic acid in the production of other curcuminoids was also evaluated (Fig. 3C). The amount of bisdemethoxycurcumin produced was very low (5.0 μM) compared to curcumin (187.9 μM), albeit 5 times higher than that obtained when CUS was used instead of DCS and CURS1. The addition of both substrates enabled the production of bisdemethoxycurcumin, demethoxycurcumin and curcumin. Curcumin was produced at the highest concentration (0.6 μM), followed by demethoxycucumin, due to the preference of feruloy-CoA as a substrate, compared to *p*-coumaroyl-CoA.

Bisdemethoxycurcumin could also be produced from tyrosine, although in very small amounts (Fig. 3D), that is almost 10 times lower than when *p*-coumaric acid is added. To improve the curcuminoid production we cloned *At*4CL1 in pCDFDuet-1 with DCS, however the curcumin production was extremely low (~0.1 μM) compared to that obtained when *At*4CL1 was in pAC plasmid (187.9 μM).

3.4 Caffeic acid can be successfully used as a precursor or intermediate in the production of curcuminoids

CCoAOMT (~27.3 kDa) was successfully expressed in *E. coli* (Supporting information, Fig. S3) and was used to produce curcumin from caffeic acid. *At*4CL1 showed some specificity for caffeic acid and CCoAOMT was found to be functional since curcumin was produced in vivo using these enzymes (Fig. 4A). However, the curcumin concentration obtained was low (0.1–3.9 μM) as compared with the production from ferulic acid (187.9 μM). This lower production is probably due to the *At*4CL1 specificity. The enzyme shows a much higher preference for ferulic acid than for *p*-coumaric acid and caffeic acid. However, *At*4CL1 seems to have a higher preference for caffeic acid than for *p*-coumaric acid since the production of curcumin from caffeic acid and bisdemethoxycurcumin from *p*-coumaric acid (Fig. 3C) was very similar although more steps are required to obtain curcumin. We tested different approaches to clone CCoAOMT. CCoAOMT cloned in pRSFDuet_CURS1 leads to a significant production of curcuminoids, while if cloned in pCDFDuet_DCS the pro-

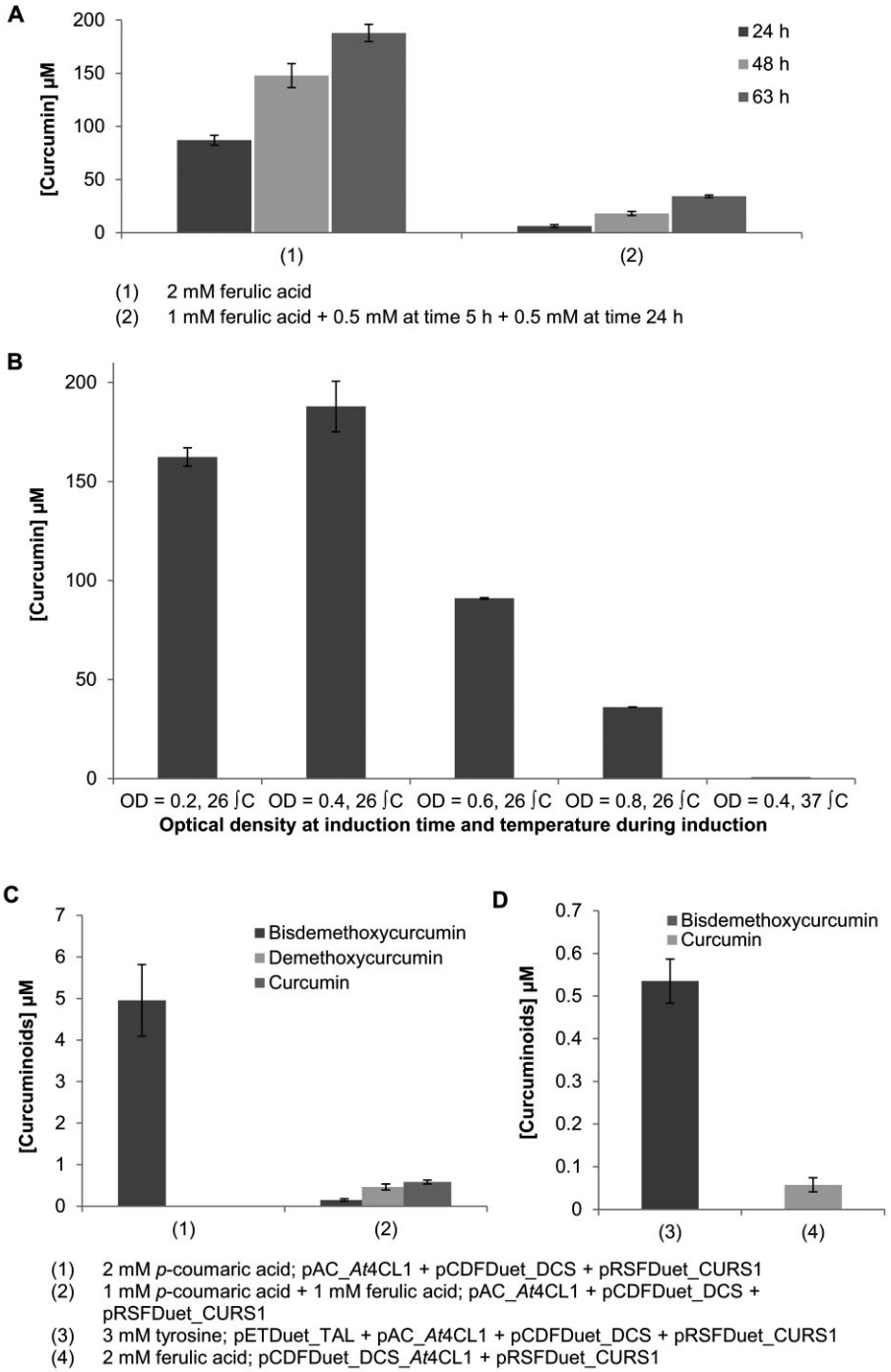


Figure 3. Curcuminoid production using 4-coumarate-CoA ligase (4CL1) from *A. thaliana* and diketide-CoA synthase (DCS) and curcumin synthase 1 (CURS1) from *C. longa*. Curcumin production from ferulic acid (**A**). Effect of IPTG induction at different OD_{600} values and effect of induction temperature on the production titers of curcumin using ferulic acid as substrate (**B**). Curcuminoid production from *p*-coumaric acid and a mixture of *p*-coumaric acid and ferulic acid (**C**). Bisdemethoxycurcumin production from tyrosine using tyrosine ammonia lyase (TAL) from *R. glutinis* and curcumin production from ferulic acid using a different plasmid configuration (**D**). Error bars are standard deviations from triplicate experiments.

duction is residual. Consequently, the first approach was chosen to attempt the production of curcuminoids from tyrosine.

Curcuminoid production, including curcumin, was obtained from *p*-coumaric acid and tyrosine by using caffeic acid as an intermediate (Fig. 4B and C). When *p*-coumaric is used as a precursor, the production of curcuminoids is higher than when using tyrosine. Indeed, this was expected since, as the number of intermediates increases, the production of curcuminoids decreases, due to the loss of product in each step of the pathway. When *p*-coumaric acid is used as a substrate, bisdemethoxycurcumin is the curcuminoid produced in higher concentrations (~0.5 µM), followed by demethoxycurcumin. This occurs probably because, due to the high concentration of *p*-coumaric acid added, *p*-coumaroyl-CoA is present

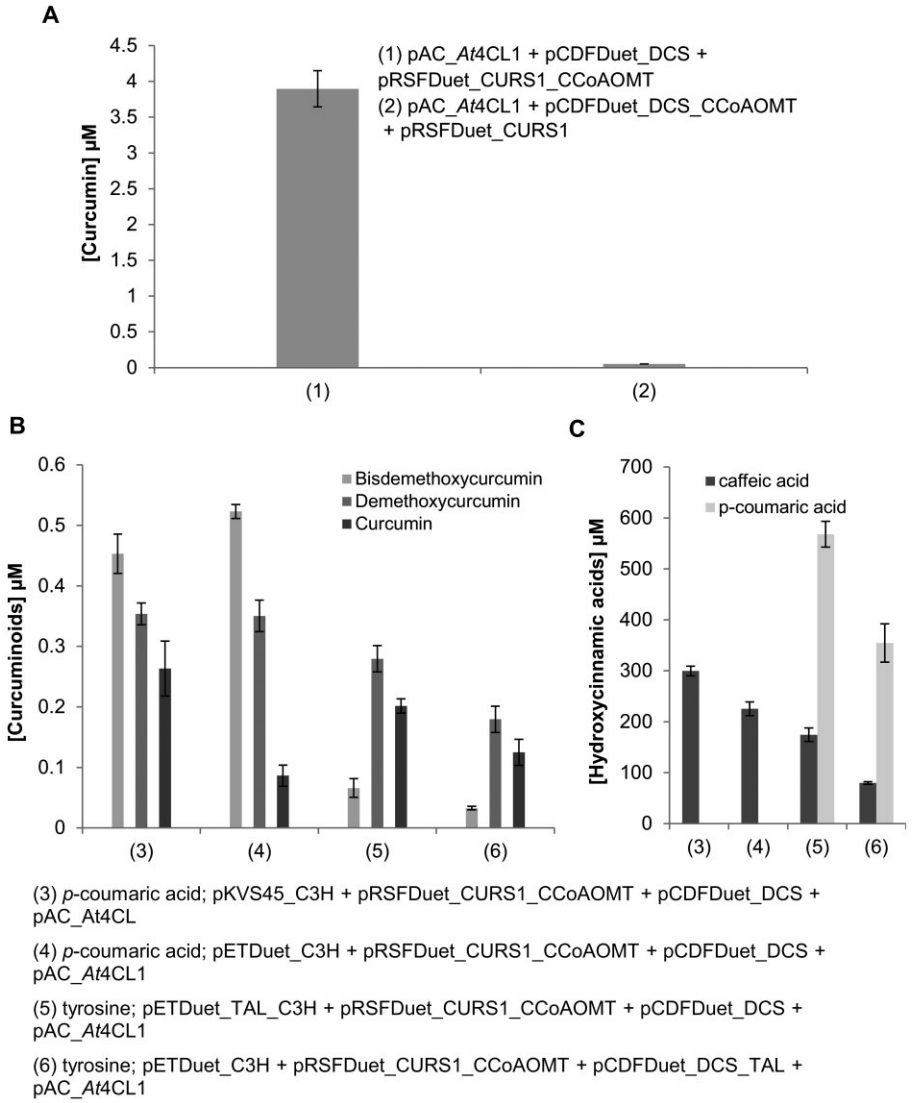


Figure 4. Curcumin production from caffeic acid using caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) from *M. sativa*, 4-coumarate-CoA ligase (4CL1) from *A. thaliana* and diketide-CoA synthase (DCS) and curcumin synthase 1 (CURS1) from *C. longa* (**A**). Curcuminoid production from *p*-coumaric acid and tyrosine using caffeic acid as an intermediate and using TAL from *R. glutinis*, 4-coumarate 3-hydroxylase (C3H) from *Saccharothrix espanaensis*, CCoAOMT, 4CL1, DCS, and CURS1. In (**B**) bisdemethoxycurcumin, demethoxycurcumin, and curcumin production are shown while in (**C**) *p*-coumaric acid and caffeic acid production are reported. Error bars are standard deviations from triplicate experiments.

in a higher concentration than feruloyl-CoA, thus being more available to produce a higher concentration of bisdemethoxycurcumin and demethoxycurcumin. Also, pKVS45_C3H enabled a higher concentration of caffeic acid than pETDuet_C3H (Fig. 4C), as we have observed previously (J. Rodrigues et al., submitted). Consequently, a higher caffeic acid concentration (299.5 µM) allowed the production of more curcumin (0.3 µM). When tyrosine is used as substrate, the caffeic acid concentration obtained is lower (79.9–174.4 µM), and the *p*-coumaric acid concentration produced is also lower (354.6–568.0 µM) than that maintained when it is added as a precursor (1392.6–1571.3 µM). Although *p*-coumaric is present in a higher concentration than caffeic acid (Fig. 4C), *At*4CL1 seems to prefer caffeic acid over *p*-coumaric acid and consequently, curcumin (0.1–0.2 µM) is produced in higher amounts than bisdemethoxycurcumin (0.03–0.07 µM). The concentration of the asymmetric curcuminoid (demethoxycurcumin) is higher than the concentration obtained of the symmetric curcuminoids (curcumin and bisdemethoxycurcumin). The system using TAL and C3H in the same plasmid (pETDuet_TAL_C3H) works better than cloning TAL in pCDFDuet_DCS. In the latter case, low expression levels of TAL seems to be the cause, as was found for CCoAOMT (Fig. 4A), since the *p*-coumaric acid production was low (354.6 µM) and consequently the caffeic acid production was also low (79.9 µM), thus leading to a lower production of curcuminoids.

4 Discussion

Since there is an increasing interest in curcumin and other curcuminoids due to their several recognized beneficial effects, synthetic biology and metabolic engineering constitute good approaches to improve their availability. The

discovery of CUS from *O. sativa* allowed the production of curcuminoids in *E. coli* [6, 7] and in vitro [14], which represented an important step forward in the production of curcuminoids using the above mentioned strategies. CUS has the advantage of catalyzing the three steps that are catalyzed by different PKSs (CURS and DCS) in *C. longa*: (a) condensation of malonyl-CoA with feruloyl-CoA (or other CoA ester) to produce diketide-CoA; (b) hydrolysis of diketide-CoA to its corresponding β-keto acid; and (c) decarboxylative condensation of the β-keto acid to the second molecule of feruloyl-CoA (Fig. 1). This enzyme would make the production of curcuminoids from tyrosine simpler by decreasing the number of enzymes needed for the pathway to function. In this study, we used CUS to produce the three main curcuminoids in *C. longa*: bisdemethoxycurcumin, demethoxycurcumin and curcumin. However, the amount of curcuminoids produced with CUS was lower than the expected. The low titers (0.9 μM of bisdemethoxycurcumin and 6.7 μM of curcumin) may be due to use of plant enzymes in the pathway, which usually are poorly stable in prokaryotes even with codon-optimization owing to the lack of specific protein folding chaperones and post-translational modifications. Ferulic acid and/or feruloyl-CoA seem to be the preferred substrates since curcumin was produced in higher amount than bisdemethoxycurcumin. However, *At*4CL1 was reported to prefer *p*-coumaric acid to ferulic acid [15] and Katsuyama et al. [14] also concluded by in vitro experiments that CUS prefers *p*-coumaroyl-CoA as a substrate. Therefore, the reason behind the higher curcumin production in this study is not clear. However, in vivo experiments by the same authors [6] revealed that the CUS enzyme together with *Le*4CL produces very similar amounts of bisdemethoxycurcumin and curcumin from *p*-coumaric acid and ferulic acid, respectively, although the curcumin concentration obtained was slightly higher. It cannot be concluded if the higher concentration of curcumin obtained in vivo was due to *Le*4CL substrate specificity, since it is unknown.

DCS and *CURS1* were also chosen to test the curcuminoid production due to the promising results previously reported in vitro [9, 10, 16]. Although these two genes were successfully expressed in *E. coli* and the enzymes were used to produce several curcuminoids, including curcumin, *DCS* and *CURS1* were never tested in vivo. *CURS1* was named curcumin synthase due to its pronounced preference for feruloyl-CoA [9]. Moreover, other *CURS* were identified. *CURS2* was also reported to prefer feruloyl-CoA and *CURS3* favored both feruloy-CoA and *p*-coumaroyl-CoA [10]. Since curcumin has been reported to possess, in some systems, a higher therapeutic value than the other curcuminoids [17], *CURS1* was chosen as it has the highest turnover rate towards feruloyl-CoA, as well as the highest difference between the catalytic efficiency towards feruloyl-CoA and *p*-coumaroyl-CoA [10]. The production of curcumin using CURS1 and DCS was surprisingly higher than we expected (187.9 μM), especially taking into account the results obtained with CUS. This is the first report that demonstrates that these two enzymes can be used efficiently to produce curcuminoids in *E. coli* and provide high production titers. A higher concentration (113 mg/L) was reported using *Le*4CL, CUS, and ACC [6]. It is important to note that ACC can be very important to obtain high curcuminoids titers, in particular if there is a limitation of malonyl-CoA in the cells. However, comparing the results from Katsuyama et al. [6] and Wang et al. [8] we can conclude that a high production of curcuminoids can also be obtained without ACC overexpression. Katsuyama et al. [6] when using *R. rubra* PAL, *Le*4CL, CUS, and ACC, and adding phenylalanine or cinnamic acid were able to obtain around 107 mg/L or 84 mg/L of dicinnamoylmethane, respectively; while Wang et al. [8] by using *T. pratense* PAL, *At*4CL1, and CUS, and adding phenylalanine obtained 360 mg/L of dicinnamoylmethane – that is a dicinnamoylmethane concentration 3.4 times higher without the need of using overexpressed ACC to increase the malonyl-CoA pool in *E. coli*. Even though Wang et al. [8] obtained high production of dicinnamoylmethane and we were able to achieve a high production of curcumin, the ACC requirements should be further evaluated to confirm if malonyl-CoA is limiting the production of curcuminoids.

To improve curcuminoid production, the substrates were supplemented to the culture medium in two steps. This stepwise approach was tested due to the possible toxicity of *p*-coumaric and ferulic acids to the cells [12, 18–22], and because we found that this strategy improved the production of caffeic acid in our previous work (*submitted*). However, in the current study the two-step feeding was shown to be counterproductive, that is the production decreased which means that the slow process of curcuminoid production requires all the substrate available at the beginning of the fermentation to achieve a high production.

The previous report on the heterologous production of curcumin involves the direct supplementation of ferulic acid in the culture medium [6]. To our knowledge, this is the first report on the production of curcumin from tyrosine through the production of caffeic acid as an intermediate. CCoAOMT from *M. sativa* was used to catalyze the methylation of caffeoyl-CoA, its preferred substrate, to feruloyl-CoA and it was chosen since it has already been expressed in *E. coli* [23]. After *At*4CL1 converts caffeic acid to caffeoyl-CoA, CCoAOMT converts caffeoyl-CoA to feruloyl-CoA which DCS and CURS1 can convert to curcumin. To date, the levels of production are not very high; however these can be improved by balancing different expression levels. Imbalances can lead to over- or underproduction of enzymes and accumulation of intermediate metabolites which may result in suboptimal titers. CURS1 has a higher preference for feruloyl-CoA than for *p*-coumaroyl-CoA. However, the DCS preference for

p-coumaric acid or ferulic acid is unknown, although it is established that its affinity for malonyl-CoA is high [9]. *At*4CL1 was reported to have a high affinity for *p*-coumaric acid, followed by caffeic acid and a very low specificity for ferulic acid [15], which seems to be contradicted by our results. All these issues make it difficult to control the curcuminoid production ratios, and more information about the catalytic properties of all the enzymes involved in the curcuminoid biosynthetic pathway is required. Although up to now the production of curcuminoids from tyrosine is low (up to 0.6 μM) and the curcuminoid ratio difficult to control, this alternative pathway successfully led to the production of curcumin from tyrosine. Curcumin, the most studied curcuminoid for therapeutic purposes and in many studies considered as the most potent and active [3, 4], was only shown to be produced in *E. coli* using ferulic acid. This represents a disadvantage when the production cost of curcumin is relevant, but also for the cases in which curcumin is used as a drug that needs to be produced and delivered in situ. By producing curcumin using tyrosine (endogenous amino acid) and using (in the future) a tyrosine overproducing strain, the need to add expensive precursors disappears and the production process is also simplified. Although it is clear that the pathway herein described, depending on the application, has to be further refined and optimized this is the first step towards the production of curcumin from an amino acid in *E. coli* and it is also the first time that caffeic acid is used as an intermediate to produce curcuminoids. Therefore, this alternative pathway represents a significant progress in the heterologous production of curcumin using *E. coli*.

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