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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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## 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; **TAL**, tyrosine ammonia lyase

## Abstract

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 (4CL1) 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 (TAL) from *Rhodotorula glutinis* and 4-coumarate 3-hydroxylase (C3H) from *Saccharothrix espanaensis* were used. Caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) 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 mg/L, 0.3 mg/L 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 through caffeic acid. This alternative pathway represents a step forward in the heterologous production of curcumin using *E. coli*.

## 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 anticancer, anti-inflammatory, anti-oxidant, anti-Alzheimer'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 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 produce 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 curcuminoids production in *E. coli* using an artificial pathway (Fig. 1). We tested 4CL1 from *Arabidopsis thaliana* (*At4CL1*) and different PKSs for curcuminoids 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 *Rhodotorula 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 (*manuscript 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* NZY5 $\alpha$  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, CURS, 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 Supplementary Material (Table S1). pAC\_*At4CL1* 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<sup>®</sup> 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  $\beta$ -D-thiogalactopyranoside (IPTG) and Luria-Bertani (LB) medium from NZYTech (Lisbon, Portugal) and anhydrotetracycline (aTc) from Acros. Glucose (Acros), Na<sub>2</sub>HPO<sub>4</sub> (Scharlau, Sentmenat, Spain), MgSO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub> (Riel-deHaën, Seelze, Germany), NH<sub>4</sub>Cl, NaCl, CaCO<sub>3</sub> (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<sub>3</sub>, ZnCl<sub>2</sub>, CoCl<sub>2</sub>, CuCl<sub>2</sub>, nicotinic acid (Riedel-deHaën), NaMoO<sub>4</sub>, H<sub>2</sub>BO<sub>3</sub>, 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, CURS, 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 (Table S2).

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

## **2.3 Curcuminoids 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<sub>2</sub>HPO<sub>4</sub> (6g), KH<sub>2</sub>PO<sub>4</sub> (3g), NH<sub>4</sub>Cl (1g), NaCl (0.5 g), CaCl<sub>2</sub> (17 mg), MgSO<sub>4</sub> (58 mg), thiamine (340 mg) and CaCO<sub>3</sub> (5 g) (to control the pH). Trace elements [FeCl<sub>3</sub> (54 mg), ZnCl<sub>2</sub> (4 mg), CoCl<sub>2</sub> (4 mg), NaMoO<sub>4</sub> (4 mg), CuCl<sub>2</sub> (2 mg) and H<sub>2</sub>BO<sub>3</sub> (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 (2 mM). All the experiments were performed in triplicate unless otherwise specified.

## **2.4 Curcuminoids 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 the products**

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 $\mu$ 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. 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 curcuminoids 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**

*E. coli* K-12 MG1655(DE3) cells harbouring pRSFDuet\_CUS, pRSFDuet\_CURS, 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<sub>2</sub>PO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 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  $\mu$ g of total protein were loaded in

4-20 % Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Gels (BioRad). The protein marker used was Precision Plus Protein<sup>™</sup> Unstained (BioRad) and/or ColorPlus<sup>™</sup> 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)

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 (*LeAt4CL1*, *At4CL2* and *At4CL1*) and only *At4CL1* 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 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) (Fig. S1). After that, CUS was tested in *E. coli* with *At4CL1* (pRSFDuet\_CUS + pAC\_*At4CL1*) (Fig. 2). These enzymes produced bisdemethoxycurcumin and curcumin when *p*-coumaric acid and ferulic acid,

respectively, were added to the culture medium. Curcumin was produced in higher amount (6.7  $\mu\text{M}$ ) than bisdemethoxycurcumin (0.9  $\mu\text{M}$ ).

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  $\mu\text{M}$ ) could be easily detected after 63 h (Fig. S2, A and B) by the observation of some cells that adhered to the walls of the shake flasks.

To improve curcuminoids 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), *At4CL1* 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  $\mu\text{M}$ , thus suggesting that the TAL enzyme was highly functional.

### **3.3 Production of curcuminoids using DCS and CURS1 from *C. longa***

Codon-optimized DCS and CURS1 were cloned in pRSFDuet-1 and their protein expression was also confirmed on a protein gel (Fig. S1). The protein expression of DCS and CURS (42.9 kDa) was very high; hence *in vivo* production was further tested.

Fig. 3A illustrates the curcumin production from ferulic acid using pAC\_At4CL1, pCDFDuet\_DCS and pRSFDuet\_CURS1. The production with these enzymes was very high, 187.9  $\mu\text{M}$  (70 mg/L) after 63 h. Besides, the culture medium showed a dark orange color (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), the curcuminoids 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 ( $\text{OD}_{600}$ ) at the time of induction in LB influenced the production of curcuminoids, we induced the cultures at different  $\text{OD}_{600}$  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  $\text{OD}_{600}$  of 0.4 yielded the highest production titer (187.9  $\mu\text{M}$ ), thus suggesting that induction should be performed at an  $\text{OD}_{600}$  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 curcuminoids 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  $\mu\text{M}$ ) compared to curcumin (187.9  $\mu\text{M}$ ), 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  $\mu\text{M}$ ), 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), i.e. almost 10 times lower than when *p*-coumaric acid is added. To improve the curcuminoids production we cloned *At4CL1* in pCDFDuet-1 with DCS, however the curcumin production was extremely low ( $\sim 0.1 \mu\text{M}$ ) compared to that obtained when *At4CL1* was in pAC plasmid (187.9  $\mu\text{M}$ ).

### **3.4 Production of curcuminoids using caffeic acid as a precursor or intermediate**

CCoAOMT ( $\sim 27.3 \text{ kDa}$ ) was successfully expressed in *E. coli* (Fig. S3) and was used to produce curcumin from caffeic acid. *At4CL1* 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  $\mu\text{M}$ ) as compared with the production from ferulic acid (187.9  $\mu\text{M}$ ). This lower production is probably due to the *At4CL1* specificity. The enzyme shows a much higher preference for ferulic acid than for *p*-coumaric acid and caffeic acid. However, *At4CL1* 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 production is residual. Consequently, the first approach was chosen to attempt the production of curcuminoids from tyrosine.

Curcuminoids 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  $\mu\text{M}$ ), followed by demethoxycurcumin. This occurs probably because, due to the high concentration of *p*-coumaric acid added, *p*-coumaroyl-CoA is present 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 (*manuscript submitted*). Consequently, a higher caffeic acid concentration (299.5  $\mu\text{M}$ ) allowed the production of more curcumin (0.3  $\mu\text{M}$ ). When tyrosine is used as substrate, the caffeic acid concentration obtained is lower (79.9 - 174.4  $\mu\text{M}$ ), and the *p*-coumaric acid concentration produced is also lower (354.6 – 568.0  $\mu\text{M}$ ) than that maintained when it is added as a precursor (1392.6 – 1571.3  $\mu\text{M}$ ). Although *p*-coumaric is present in a higher concentration than caffeic acid (Fig.4C, (5) and (6)), At4CL1 seems to prefer caffeic acid over *p*-coumaric acid and consequently, curcumin (0.1 – 0.2  $\mu\text{M}$ ) is produced in higher amounts than bisdemethoxycurcumin (0.03 – 0.07  $\mu\text{M}$ ). 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  $\mu\text{M}$ ) and consequently the caffeic acid production was also low (79.9  $\mu\text{M}$ ), 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  $\beta$ -keto acid; and (c) decarboxylative condensation of the  $\beta$ -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  $\mu\text{M}$  of bisdemethoxycurcumin and 6.7  $\mu\text{M}$  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, *At4CL1* 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 *Le4CL* 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 *Le4CL* substrate specificity, since it is unknown.

DCS and CURS1 were also chosen to test the curcuminoids 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 feruloyl-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  $\mu$ M), 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 *Le4CL*, 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, *Le4CL*, 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, *At4CL1* and CUS, and adding phenylalanine obtained 360 mg/L of dicinnamoylmethane – i.e. 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 curcuminoids 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 (*manuscript submitted*). However, in the current study the two-step feeding was shown to be counterproductive, i.e. the production decreased which means that the slow process of curcuminoids 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 *At4CL1* 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]. *At4CL1* 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 curcuminoids production ratios, and more information about the catalytic properties of all the enzymes involved in the curcuminoids biosynthetic pathway is required. Although up to now the production of curcuminoids from tyrosine is low (up to 0.6  $\mu\text{M}$ ) and the curcuminoids 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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The authors declare no commercial or financial conflict of interest.

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**Table 1.** Bacterial strains and plasmids used in this study

Strains	Relevant Genotype	Source
<i>E. coli</i> NZY5 $\alpha$	<i>fhuA2</i> $\Delta$ ( <i>argF-lacZ</i> )U169 <i>phoA glnV44</i> $\Phi$ 80 $\Delta$ ( <i>lacZ</i> )M15 <i>gyrA96 recA1 relA1 endA1 thi-1</i> <i>hsdR17</i>	NZYTech
<i>E. coli</i> K-12 MG1655 DE3	<i>F' <math>\lambda</math> ilvG<sup>-</sup> rfb<sup>-</sup>50 rph<sup>-</sup>1</i> $\lambda$ (DE3)	[11]
<b>Plasmids</b>	<b>Construct</b>	<b>Source</b>
pETDuet-1	ColE1(pBR322) <i>ori, lacI</i> , double T7 <i>lac</i> , Amp <sup>R</sup>	Novagen
pCDFDuet-1	CloDF13 <i>ori, lacI</i> , double T7 <i>lac</i> , Strep <sup>R</sup>	Novagen
pRSFDuet-1	RSF <i>ori, lacI</i> , double T7 <i>lac</i> , Kan <sup>R</sup>	Novagen
pKVS45	<i>p15A ori</i> , tetR, P <sub>tet</sub> , Amp <sup>R</sup>	[24]
pUC57_CCoAOMT	pUC57 carrying codon-optimized CCoAOMT from <i>M. sativa</i>	GenScript
pUC57_CUS	pUC57 carrying codon-optimized CUS from <i>O. sativa</i>	NZYTech
pUC57_DCS	pUC57 carrying codon-optimized DCS from <i>C. longa</i>	NZYTech
pUC57_CURS1	pUC57 carrying codon-optimized CURS1 from <i>C. longa</i>	NZYTech
pAC_At4CL1	pAC carrying not codon-optimized 4CL1 from <i>A. thaliana</i>	Addgene (35947)
pETDuet_TAL	pETduet-1 carrying codon-optimized TAL from <i>R. glutinis</i>	unpublished data
pETDuet_C3H	pETduet-1 carrying codon-optimized C3H from <i>S. espanaensis</i>	unpublished data
pETDuet_TAL_C3H	pETduet-1 carrying codon-optimized TAL from <i>R. glutinis</i> and C3H from <i>S. espanaensis</i>	unpublished data
pKVS45_C3H	pKVS45 carrying codon-optimized C3H from <i>S. espanaensis</i>	unpublished data
pCDFDuet_TAL	pCDFDuet-1 carrying codon-optimized TAL from <i>R. glutinis</i>	unpublished data
pRSFDuet_CUS	pRSFDuet-1 carrying codon-optimized CUS from <i>O. sativa</i>	this study
pRSFDuet_CURS1	pRSFDuet-1 carrying codon-optimized CURS1 from <i>C. longa</i>	this study
pCDFDuet_DCS	pCDFDuet-1 carrying codon-optimized DCS from <i>C. longa</i>	this study
pRSFDuet_CCoAOMT	pRSFDuet-1 carrying codon-optimized CCoAOMT from <i>M. sativa</i>	this study
pRSFDuet_At4CL1_CUS	pRSFDuet_At4CL1 carrying codon-optimized CUS from <i>O. sativa</i>	this study
pCDFDuet_DCS_CCoAOMT	pCDFDuet_DCS carrying codon-optimized CCoAOMT from <i>M. sativa</i>	this study
pRSFDuet_CURS1_CCoAOMT	pRSFDuet_CURS carrying codon-optimized	this study

pCDFDuet_DCS_TAL	CCoAOMT from <i>M. sativa</i> pCDFDuet_DCS carrying codon-optimized TAL from <i>R. glutinis</i>	this study
pCDFDuet_DCS_A4CL1	pCDFDuet_DCS carrying 4CL1 from <i>A. thaliana</i>	this study

### Figure Legends:

**Figure 1.** Proposed curcuminoids 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.

**Figure 2.** Curcuminoids production from *p*-coumaric acid and ferulic acid using 4-coumarate-CoA ligase (4CL1) from *Arabidopsis thaliana* and curcuminoid synthase (CUS) from *Oryza sativa*. Bisdemethoxycurcumin was also produced from tyrosine using tyrosine ammonia lyase (TAL) from *Rhodotorula glutinis*. Error bars are standard deviations from triplicate experiments.

**Figure 3.** Curcuminoids production using 4-coumarate-CoA ligase (4CL1) from *Arabidopsis thaliana* and diketide-CoA synthase (DCS) and curcumin synthase 1 (CURS1) from *Curcuma longa*. Curcumin production from ferulic acid (A). Effect of IPTG induction at different OD<sub>600</sub> values and effect of induction temperature on the production titers of curcumin using ferulic acid as substrate (B). Curcuminoids 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 *Rhodotorula glutinis* and curcumin production from ferulic acid using a different plasmid configuration (D). Error bars are standard deviations from triplicate experiments.

**Figure 4.** Curcumin production from caffeic acid using caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) from *Medicago sativa*, 4-coumarate-CoA ligase (4CL1) from *Arabidopsis thaliana* and diketide-CoA synthase (DCS) and curcumin synthase 1 (CURS1) from *Curcuma longa* (A). Curcuminoid production from *p*-coumaric acid and tyrosine using caffeic acid as an intermediate and using tyrosine ammonia lyase (TAL) from *Rhodotorula 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 are reported. Error bars are standard deviations from triplicate experiments.



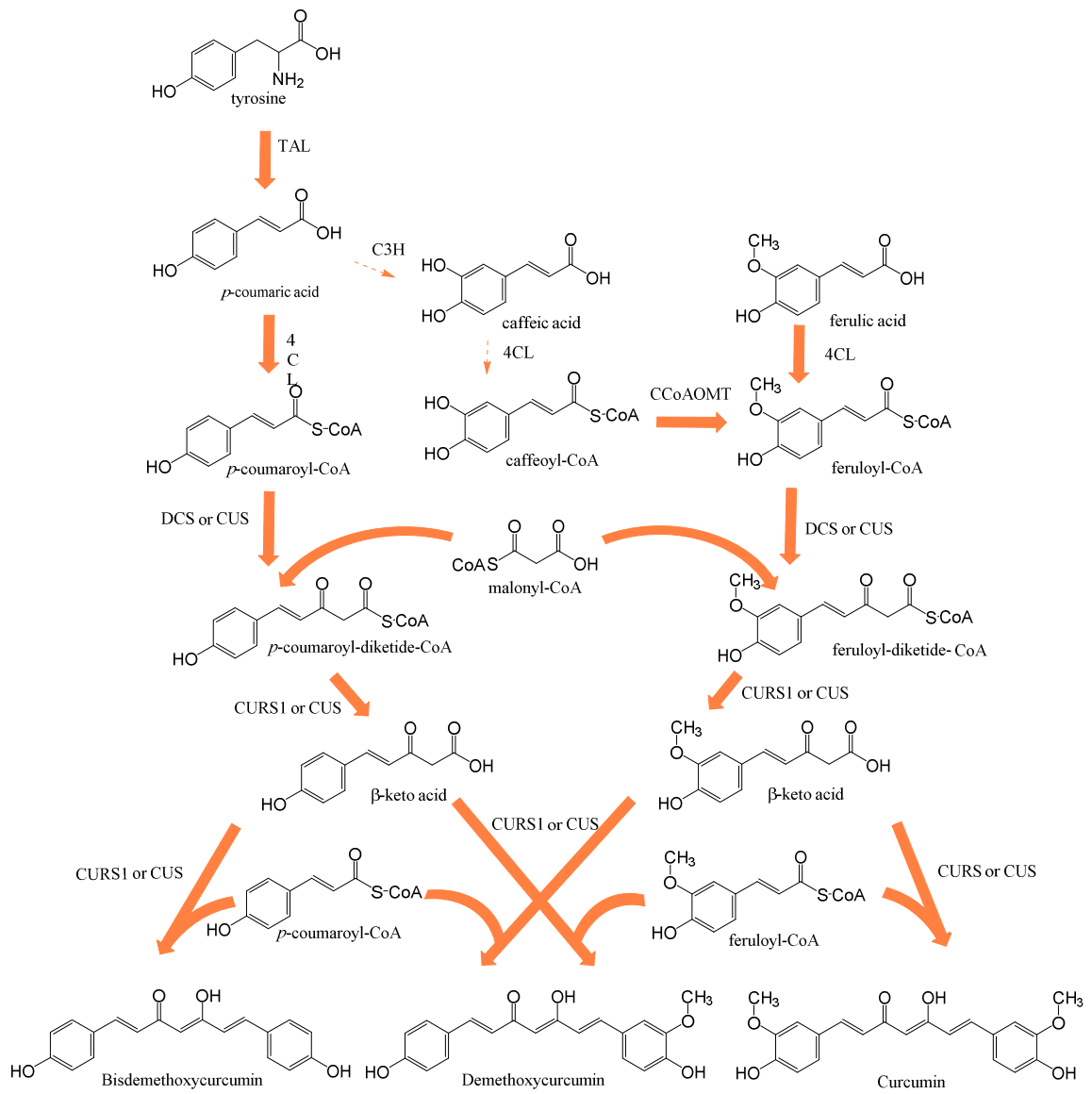
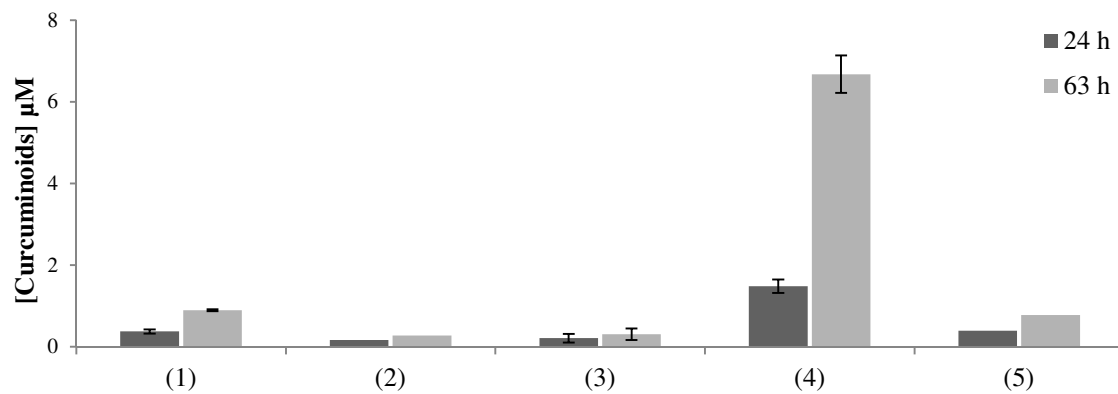
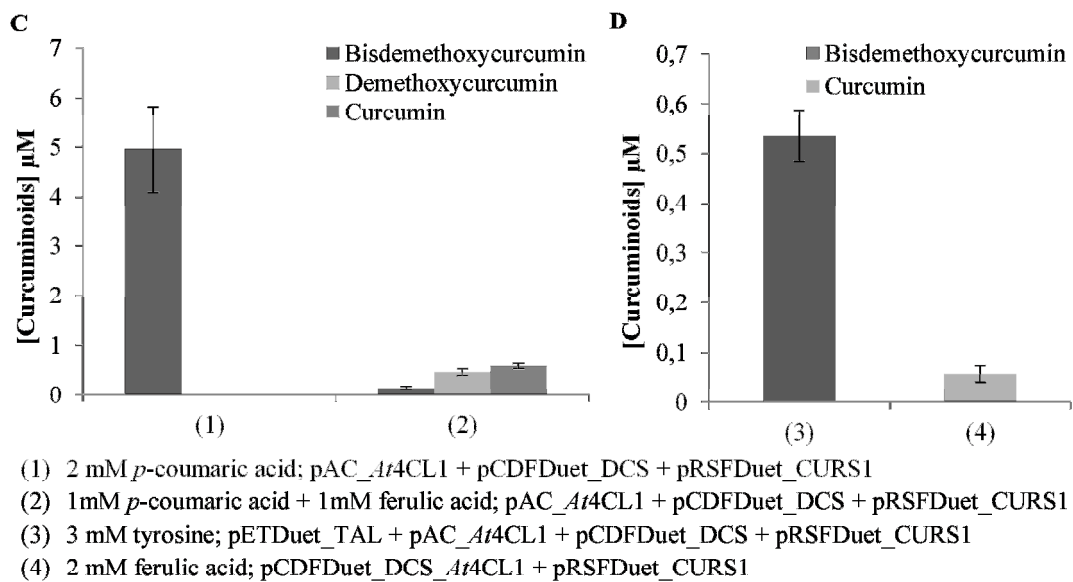
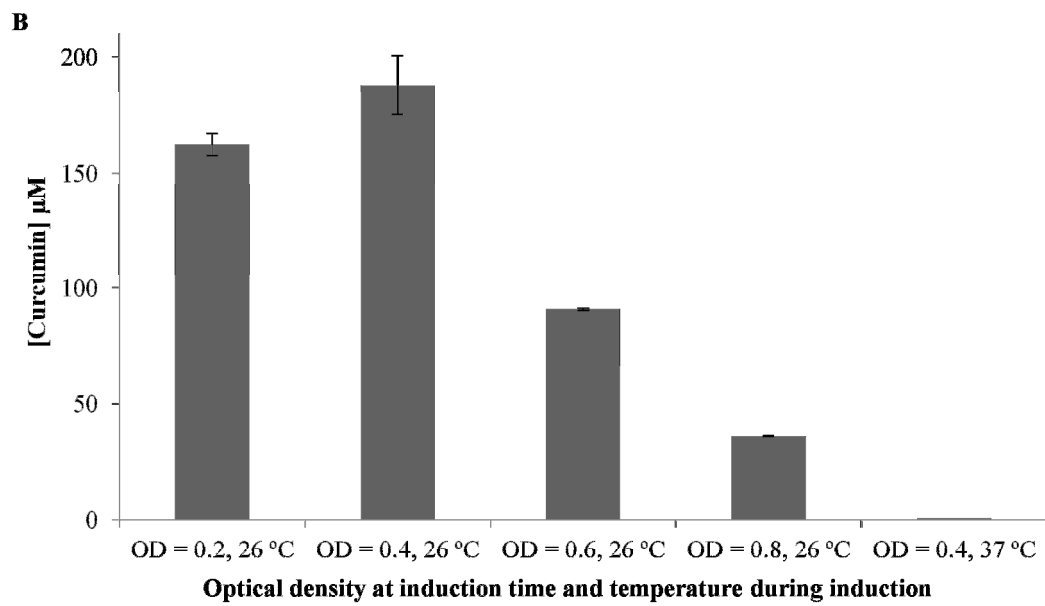
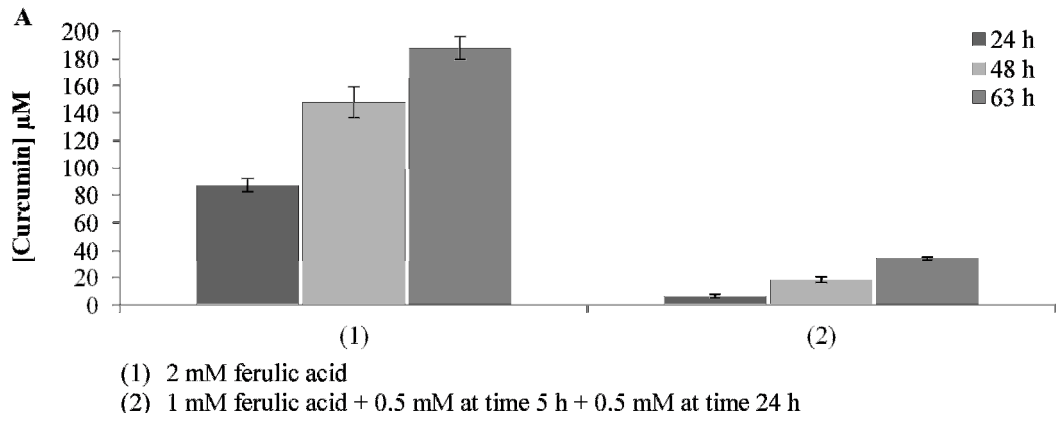


Fig1



- (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

Fig2



1

Fig3

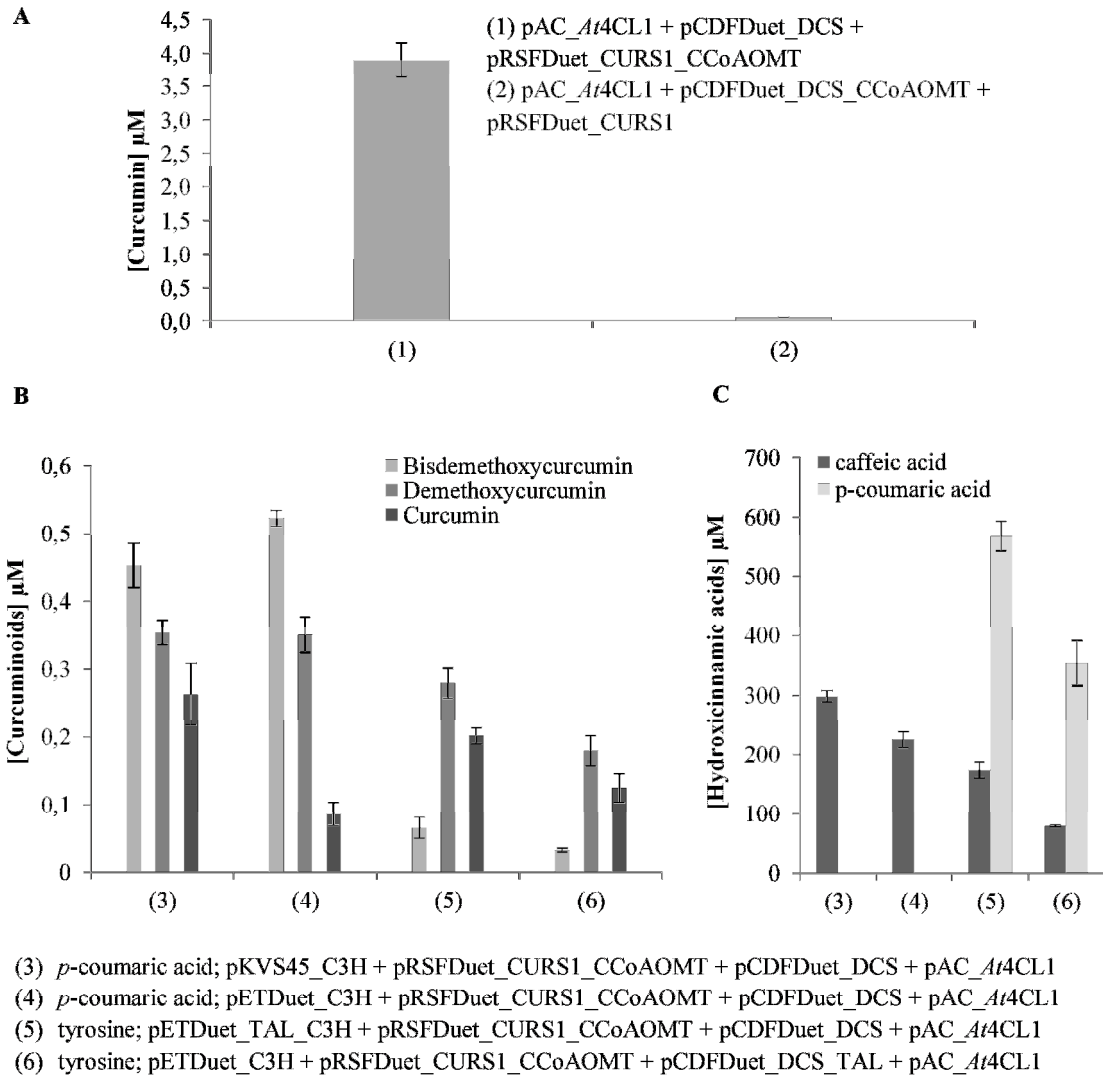


Fig4

# Supplementary Material

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

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**Table S1.** Gene sequences of TAL, C3H, CCoAOMT, CUS, DCS and CURS1 with codon optimization

Gene	Sequence
<p style="text-align: center;">TAL (<i>Rhodotorula glutinis</i>)</p>	<p>ATGGCTCCGCGTCCGACCTCGCAATCCCAAGCTCGCACCTGCCCGACCACCCAAGTTACCCAAGTTGACATCGTTGAAAAAATGCTG  GCGGCGCCGACCGATTGACGCTGGAAGTGGACGGCTATAGCCTGAACCTGGGTGATGTGGTTTCTGCAGCACGTAAAGGTCGTCCG  GTGCGTGTTAAAGATTGACGACGAAATTCGCTCGAAAATCGATAAAAGCGTGGAATTTCTGCGTAGCCAGCTGAGCATGTCTGTTTAC  GGCGTACCACGGGTTTCGGCGGTTACGCCGATACCCGCACGGAAGACGCCATTTCTGCTGCAGAAAGCACTGCTGGAACATCAACTG  TGCGGCGTGCTGCCGAGCTCTTTTGATAGCTTCCGCCTGGGCCGTGGTCTGGAAAACCTCTGCGCGTGGAAAGTCGTGCGTGGTGCAA  TGACCATCCGTGTTAATTCCTGACGCGCGGTCATTCAGCTGTCCGTCTGGTTGTCCTGGAAGCGCTGACCAACTTTCTGAATCACGG  TATTACGCCGATCGTGCCGCTGCGTGGTACCATTAGTGCATCCGGTGATCTGAGCCCGCTGTCTTATATTGCAGCTGCGATCTCTGGC  CACCCGGACAGTAAAGTTCATGTGGTTCACGAGGGTAAAGAAAAAATCCTGTACGCCCGTGAAGCTATGGCGCTGTTCAACCTGGAA  CCGGTCTGTGCTGGGCCCGAAAAGAAGGCCTGGGTCTGGTGAATGGTACGGCTGTTTCAGCGTCGATGGCCACCCTGGCACTGCATGAT  GCCCCATGCTGAGCCTGCTGAGCCAGTCTCTGACCCGCGATGACGGTCAAGCGATGGTGGGCCATGCAGGTAGCTTTCATCCGTTT  CTGCACGATGTGACCCGTCGACCCGACGCAGATTGAAGTTGCAGGCAACATCCGAAACTGCTGGAAGGTAGCCGTTTTGCGGTG  CATCACGAAGAAGAAGTGAAGTGAAGATGACGAAGGCATTCTGCGCCAGGATCGTTATCCGCTGCGTACCAGTCCGCAATGGCT  GGGTCCGCTGGTCTCCGACCTGATTCATGCCACGCAGTGTGACCATCGAAGCGGGTCAAGTACCACGGATAACCCGCTGATTGA  CGTGGAATAAAACCTCTCATCACGGCGGTAACTTTCAAGCCGAGCTGTTGCCAATACGATGGAAAAAACGCGCCTGGGCCCTGGC  ACAGATCCGTAAACTGAATTTACCCAACTGACGGAAATGCTGAACGCAGGCATGAATCGTGGTCTGCCGAGCTGCCTGGCAGCAGA  AGATCCGAGTCTGTCTATCATTGTAAGGCCTGGACATTGCAGCTGCGGCCCTACACCTCTGAACTGGGTATCTGGCGAACCCGTT  ACCACGCACGTCCAGCCGGCTGAAATGGCGAACCAAGCCGTGAATTCCTGGCACTGATCTCAGCTCGTCCGACCACGGAATCGAAT  GATGTCTGAGCCTGCTGCTGGCGACCCATCTGTATTGTGTTCTGCAGGCTATTGACCTGCGCGCGATCGAATTTGAATTCAAAAAC  AGTTTGGCCCGCTATTGTGAGCCTGATCGATCAACACTTCGGCTCTGCCATGACCCGGTAGTAACCTGCGTGACGAACTGGTGGAAA  AAGTTAATAAAACGCTGGCCAAACGCCTGGAACAGACCAACAGTTACGATCTGGTGCCGCGTTGGCATGACGCATTTTCCTTCGAG  CTGGTACGGTTGTGGAAGTTCTGAGTTCCACCTCACTGTGCTGGCGGCCGTCAATGCCTGGAAGTGGCAGCTGCGGAAAGTGCAA  TTTCCCTGACCCGCCAAGTGCCTGAAACGTTTGGTTCAGCAGCATCGACGTCATCGCCGGCACTGAGCTATCTGTCTCCGCGCACCCA  AATTCTGTACGTTTTTGTTCGTGAAGAACTGGGCGTCAAAGCGCGTCGCGGCGATGTTTTCTGGGTAAACAGGAAGTGACCATCGG  TAGTAATGTTTCCAAAATCTATGAAGCTATCAAAGCGGTCTGATCAATAATGTGCTGCTGAAAATGCTGGCATAA</p>
<p style="text-align: center;">C3H (<i>Saccharothrix espanaensis</i>)</p>	<p>ATGACGATTACCTCTCCGGCACCCGGCTGGTGCCTGAACAATGTCCGCCCGATGACGGGTGAAGAATACCTGGAATCCCTGCGTGAC  GGCCGTGAAGTGTATATTTACGGTGAACGCGTCGATGACGTGACCACGCATCTGGCGTTCCGCAACAGCGTTTCGTTCTATCGCCCGCC  TGTATGATGTCCTGCACGACCCGGCATCCGAAGGTGTTCTGCGTGTCCCGACCGATACGGGTAATGGTGGTTTTACCCATCCGTTTTT  CAAAACGGCGCGTAGCTCTGAAGACCTGGTGGCGGCCCGTGAAGCCATTGTCGGCTGGCAACGCCTGGTGTATGGCTGGATGGGTGCG  TACCCCGGATTACAAAGCAGCGTTTTTTCGGTACGCTGGACGCTAACGCGGAATTTTATGGCCCGTTTCAAGCCAATGCACGTCGCTG  GTATCGTATGCACAGGAACGCGTTCTGTACTTCAACCATGCTATCGTTACCCGCGGTCGATCGTGACCGTCCGGCTGATCGTACC  GCAGACATTTGCGTCCATGTGGAAGAAGAAACGGATTGAGGCTGATCGTGTGCGGTGCCAAAGTGGTTGCAACCGGTTCTGCTATG  ACGAACGCGAATCTGATTGCCACTATGGTCTGCCGTTTCGCGATAAAAAATTTGGCCTGGTGTTCACCGTTCCGATGAACAGTCCGG  GTCTGAAACTGATCTGTGCTACCTCCTATGAACTGATGGTGGCCACGCAGGGCTCACCGTTGATTACCCGCTGAGTTCCCGCCTGGA</p>

	TGAAAATGACAGCATTATGATCTTTGATCGTGTCTGGTCCCCTGGGAAAACGTTTTTCATGTACGACGCAGGCGCGGCCAATAGCTTT GCTACCGGCTCTGGTTTCCTGGAACGCTTTACCTTCCATGGTTGCACGCGTCTGGCAGTGAAACTGGATTTTATTGCAGGCTGTGTTAT GAAAGCTGTGGAAGTTACCGGCACCACGCACTTCCGCGGTGTTACGGCGCAAGTCGGCGAAGTGCTGAACTGGCGTGATGTCTTTTG GGGTCTGTCCGACGCTATGGCGAAAAGTCCGAATTCCTGGGTGGGCGGTAGCGTTCAGCCGAACCTGAATTATGGCCTGGCCTACCG CACCTTTATGGGCGTGGGTTATCCGCGTATTAAGAAATTATCCAGCAAACGCTGGGCTCTGGTCTGATCTACCTGAACTCATCGGCA GCTGATTGGA AAAATCCGGACGTTCCGCCGATCTGGATCGTTACCTGCGCGGCAGTCGTGGTATTAGGCAATCGATCGTGTCAA CTGCTGAAACTGCTGTGGGACGCAAGTGGGTACCGAATTCGAGGTCGTCATGAACTGTATGAACGCAACTACGGCGGTGATCACGAA GGTATTCGTGTGCAGACCCTGCAAGCCTATCAGGCAAATGGTCAAGCGGCCGCACTGAAAGGCTTTGCGGAACAGTGTATGTCCGAA TATGACCTGGATGGCTGGACCCGCCGGACCTGATTAACCCGGGCACGTAA
CCoAOMT ( <i>Medicago sativa</i> )	ATGGCTACGAATGAAGACCAGAAACAGACGGAATCAGGCCGCCACCAAGAAGTCGGTCAAAAATCACTGCTGCAATCAGATGCGCT GTATCAGTACATTCTGGAAACGAGCGTCTTTCCGCGTGAACATGAAGCTATGAAAGAACTGCGCGAAGTGACCGCGAAACACCCGCTG GAACATCATGACCACGAGCGCTGATGAAGGCCAATTCCTGTCTATGCTGCTGAAACTGATTAACCGGAAAAATACCATGGAAATCGG CGTGTATACGGTTACAGCCTGCTGGCAACCGCACTGGCAATTCGGAAAGACGGTAAAATTCTGGCCATGGATATCAACAAAGAAAA CTACGAACTGGGCCTGCCGTTATCAAAAAAGCCGGTGTGACCATAAAAATCGATTTTCGTGAAGGCCCGGCACTGCCGTTCTGGA CGAAATGATCAAAGATGAGAAAAACCATGGTAGTTACGATTTTCATCTTCGTCGATGCGGACAAAGATAACTATCTGAATTACCACAA ACGCCTGATTGACCTGGTCAAAGTGGGCGGTGTGATCGGCTATGATAACACGCTGTGGAATGGTTCCGTGGTTGCACCGCCGGACGC ACCGCTGCGTAAATACGTGCGTTATTACCGCGATTTTCGTTCTGGAAGTGAATAAAGCTCTGGCGGTTGATCCGCGCATGAAATCTGT ATGCTGCCGGTGGGTGACGGTATTACCATCTGTGCTCGTATTAATGA
CUS ( <i>Oryza sativa</i> )	ATGGCTCCGACCACGACGATGGGTTCCGCTCTGTATCCGCTGGGTGAAATGCGCCGTAGTCAACGCGCCGATGGCCTGGCAGCAGTC CTGGCCATTGGCACCGCAAACCCGCCGAATTGCGTTACGCAGGAAGAAATCCCGGATTTTTATTTCCGCGTCACCAACAGCGACCAT CTGACGGCCCTGAAAGACAAGTTCAAACGTATTTGTCAGGAAATGGGTGTGCAACGTCGCTACCTGCATCACACCGAAGAAATGCTG AGCGCGCACCCGGAATTTGTCGATCGTGACGCCCCGCTCTGGATGCACGTCTGGACATTGCCGCGGATGCCGTTCCGGAACCTGGCA GCTGAAGCGGCCAAAAAGGCCATTGCAGAATGGGGCCGTCGCGCAGCTGATATCACCATCTGGTGGTTACCACGAATAGTGGCGC GCACGTGCCGGGTGTTGATTTTCGCTGGTGCCGCTGCTGGGTCTGCGCCCGTCCGTTTCGTCGCACGATGCTGCATCTGAATGGTTGC TTTGCGGGTTGTGCCGCCCTGCGTCTGGCTAAAGATCTGGCGGAAATTCCTGTTGGCGCACGCGTGTGGTCTGTTGATGGTGCGGCGGCACTG ACCCTGATGATTTTTACGGGCCCGGATGAAGGTTGCTTCCGCACCCTGCTGGTGCAGGGTCTGTTTGGTATGGTGCGGCGGCACTGTA TTGTCGGTGTGATGCGGATGACGTTGAACGTCGCTGTTCCGAAATCGTCTCAGCTGCGCAGACCATTATCCCGGAATCGGACCATGC ACTGAACATGCGTTTTACGGAACGTCGCTGGATGGTGTCTGGTCCGCAAGTGCCGGTCTGATTGGTGACAACGTGGAACGTTG TCTGCTGGATATGTTTGGTCCGCTGCTGGGCGGTGACGGCGGTGGCGGTTGGAATGACCTGTTTTGGGCGGTCCACCGGGTAGCTCT ACCATTATGGATCAGGTGGATGCGGCACTGGGCTGGAACCGGGCAAGCTGGTGTGCGAGTCGTCGCGTGCTGTCCGACTACGGCAAT ATGAGCGGTGCTACGGTTATCTTTGCGCTGGATGAACTGCGTCGCCAACGCAAAGAAGCCGAGCTGCGGGTGAATGGCCGGAACCTG GGTGTATGATGGCCTTTGGTCCGGGTATGACGGTTGATGCGATGCTGCTGCACGCCACGAGCCATGTGAACTAA
DCS ( <i>Curcuma longa</i> )	ATGGAAGCTAACGGTTACCGTATCACCCACTCTGCTGACGGTCCAGCTACCATCCTGGCTATCGGTACCGCTAACCCGACCAACGTTG TTGACCAGAACGCTTACCCGGACTTCTACTTCCGTGTTACCAACTCTGAATACCTGCAGGAACTGAAAGCTAAATTCCGTCTGATCTG CGAAAAAGCTGCTATCCGTAAACGTCACCTGTACCTGACCGAAGAAATCCTGCGTGAAAACCCGCTCTCTGCTGGCTCCGATGGCTCC GTCTTTTCGACGCTCGTCAGGCTATCGTTGTTGAAGCTGTTCCGAAACTGGCTAAAGAAGCTGCTGAAAAAGCTATCAAAGAATGGGG

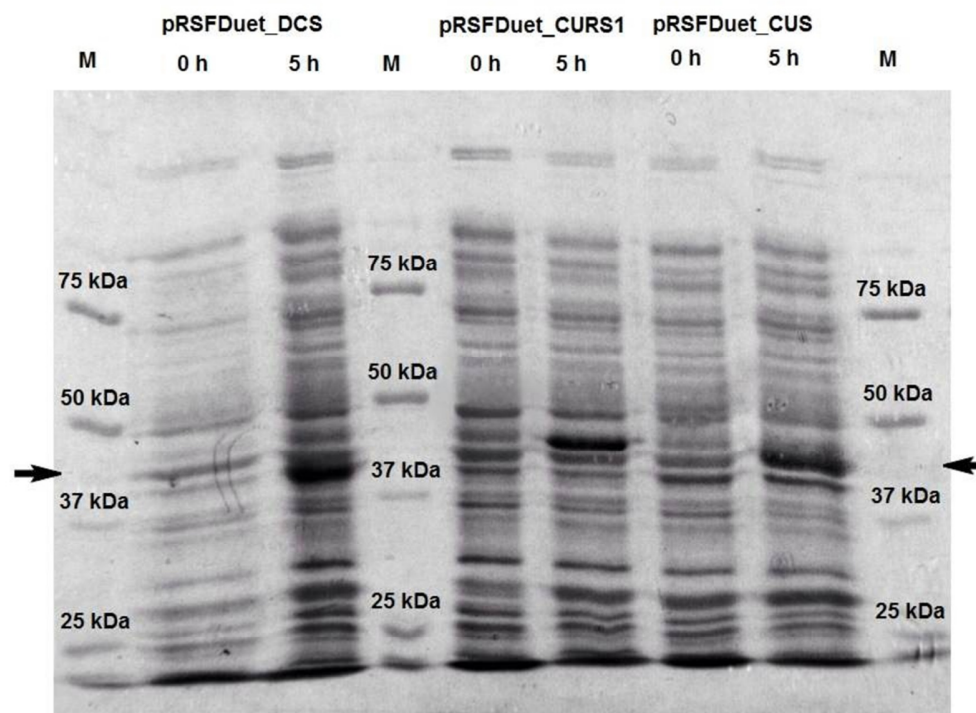
	<p>TCGTCCGAAATCTGACATCACCCACCTGGTTTTCTGCTCTGCTTCTGGTATCGACATGCCGGGTTCTGACCTGCAGCTGCTGAAACTGC  TGGGTCTGCCGCCGTCTGTTAACCGTGTTATGCTGTACAACGTTGGTTGCCACGCTGGTGGTACCGCTCTGCGTGTGCTAAAGACCT  GGCTGAAAACAACCGTGGTGCTCGTGTCTGGCTGTTTCTGCTCTGAAGTTACCGTTCTGTCTTACCGTGGTCCGCACCCGGCTCACATC  GAATCTCTGTTTCGTTACAGGCTCTGTTTCGGTGACGGTGCTGCTGCTGCTGGTTGTTGGTTCTGACCCGGTTGACGGTGTGAACGTCCGAT  CTTCGAAATCGCTTCTGCTTCTCAGGTTATGCTGCCGGAATCTGCTGAAGCTGTTGGTGGTACCTGCGTGAAATCGGTCTGACCTTCC  ACCTGAAATCTCAGCTGCCGTCTATCATCGCTTCTAACATCGAACAGTCTCTGACCACCGCTTGTCTCCGCTGGGTCTGTCTGACTGG  AACCAGCTGTTCTGGGCTGTTACCCGGGTGGTCTGCTATCCTGGACCAGGTTGAAGCTCGTCTGGGTCTGGAAAAAGACCGTCTG  GCTGCTACCCGTCACGTTCTGTCTGAATACGGTAACATGCAGTCTGCTACCGTTCTGTTTCATCCTGGACGAAATGCGTAACCGTTCTG  CTGCTGAAGGTCACGCTACCACCGGCGAAGGTCTGGACTGGGGTGTCTGCTGGGTTTCGGTCCAGGTCTGTCTATCGAAACCGTTGT  TCTGCACTCTTGCCGTCTGAACTAA</p>
<p>CURS1  (<i>Curcuma longa</i>)</p>	<p>ATGGCTAACCTGCACGCTCTGCGTCGTGAACAGCGTGCTCAGGGTCCGGCTACCATCATGGCTATCGGTACCGCTACCCCGCCGAAC  CTGTACGAACAGTCTACCTTCCCGACTTCTACTTCCGTGTTACCAACTCTGACGACAAACAGGAACTGAAAAAAAATTCCGTCGTA  TGTGCGAAAAAACCATGGTTAAAAACGTTACCTGCACCTGACCGAAGAAATCCTGAAAGAACGTCCGAAACTGTGCTTTACAAAG  AAGTTCTTTCGACGACCGTCAGGACATCGTTGTTGAAGAAATCCCGCGTCTGGCTAAAGAAGCTGCTGAAAAAGCTATCAAAGAAT  GGGGTCGTCCGAAATCTGAAATCACCCACCTGGTTTTCTGCTCTATCTCTGGTATCGACATGCCGGGTGCTGACTACCGTCTGGCTAC  CCTGCTGGGTCTGCCGCTGACCGTTAACCGTCTGATGATCTACTCTCAGGCTTGCCACATGGGTGCTGCTATGCTGCGTATCGCTAAA  GACCTGGCTGAAAACAACCGTGGTGCTCGTGTCTGGTTGTTGCTTTCGAAATCACCGTTCTGTCTTTCGTTGGTCCGAACGAAGGTG  ACTTCGAAGCTCTGGCTGGTCAGGCTGGTTTTCGGTGACGGTGCTGGTGCTGTTGTTGTTGGTGGTACCGCTGGAAGGTATCGAAAA  ACCGATCTACGAAATCGCTGCTGCTATGCAGGAAACCGTTGCTGAATCTCAGGGTGTGTTGGTGGTACCTGCGTGCTTTCGGTTGG  ACTTTCTACTTCTGAACCAGCTGCCGGCTATCATCGCTGACAACTGGGTGCTTCTGGAACGTGCTCTGGCTCCGCTGGGTGTTT  GTGAATGGAACGACGTTTTCTGGGTTGCTCACCCGGGTAACCTGGGCTATCATCGACGCTATCGAAGCTAAACTGCAGCTGTCTCCGG  ACAAACTGTCTACCGCTCGTCACGTTTTACCGAATACGGTAACATGCAGTCGGCGACCGTCTACTTCGTGATGGACGAACTGCGTAA  ACGTTCTGCTGTTGAAGGTCGTTCTACCACCGGCGACGGTCTGCAGTGGGGTGTCTGCTGGGTTTCGGTCCAGGTCTGTCTATCGAA  ACCGTTGTTCTGCGTTCTATGCCGCTGTA</p>

1 **Table S2.** Set of primers for PCR amplification (forward (FW) and reverse (REV) primers)

Primer name	Primer sequence <sup>[a]</sup>	Restriction enzyme site
CUS_FW	<i>GGATCC</i> <b>CA</b> TGGCTCCGACCACG	<i>Bam</i> HI
CUS_REV	AAG <b>CTTT</b> TA <b>GT</b> TCACATGGCTCGTGG	<i>Hind</i> III
At4CL1_FW	<i>CATATG</i> <b>AT</b> GGCGCCACAAGAACA	<i>Nde</i> I
At4CL1_REV	<i>CTCGAG</i> <b>TC</b> ACAATCCATTTGCTAGTTTTGC	<i>Xho</i> I
CURS1_FW	<i>GGATCC</i> <b>TA</b> TGGCTAACCTGCACGCTC	<i>Bam</i> HI
CURS1_REV	<i>GGCGCG</i> <b>CCTT</b> ACAGCGGCATAGAACGC	<i>Asc</i> I
DCS_FW	<i>GGATCC</i> <b>GA</b> TGGAAGCTAACGGTTACCG	<i>Bam</i> HI
DCS_REV	AAG <b>CTTT</b> TA <b>GT</b> TCAGACGGCAAGAGTG	<i>Hind</i> III
TAL_FW	<i>CATATG</i> <b>GG</b> CTCCGCGTCC	<i>Nde</i> I
TAL_REV	<i>CTCGAG</i> <b>TT</b> ATGCCAGCATTTTCA	<i>Xho</i> I
CCoAOMT_FW	<i>CATATG</i> <b>CATCACC</b> ATCATCACC <b>ACAT</b> GGCTACGAATGAA	<i>Nde</i> I
CCoAOMT_REV	GACCAG <i>CTCGAG</i> <b>TC</b> ATTTAATACGACGACAGATGGT	<i>Xho</i> I

[a] Start and stop codons in **bold**; restriction sites in *italic*; His<sub>6</sub>-Tag 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.

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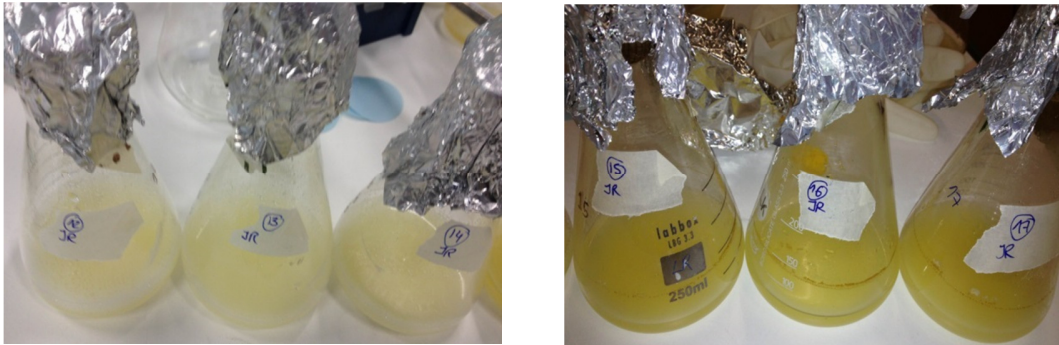


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4 **Figure S1.** Protein gel showing DCS, CURS1 and CUS expression in cell-free extracts at  
5 time zero of induction and after 5 hours of induction. DCS and CURS1 have 42.90 kDa and

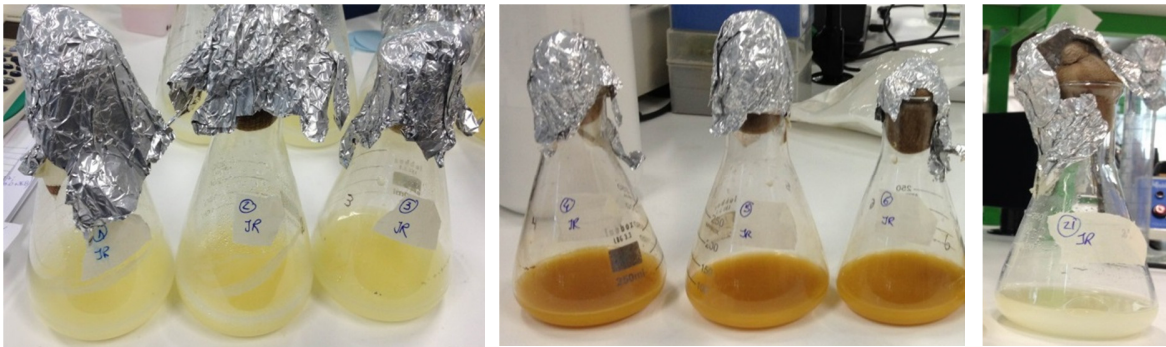
6 CUS has 44.33 kDa. DCS: diketide-CoA synthase; CURS1: curcumin synthase; CUS:  
7 curcuminoid synthase; M: Marker.

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(A)

(B)

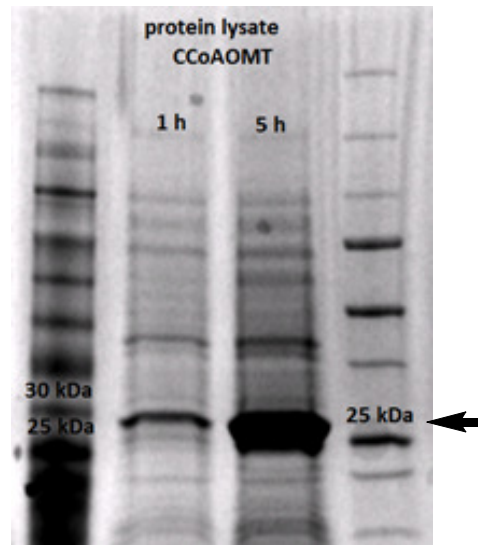


(C)

(D)

(E)

**Figure S2.** Curcuminoid production can be observed by the yellow / orange color of the cells. Bisdemethoxycurcumin (A, C) and curcumin (B, D) were produced using *p*-coumaric and ferulic acid as substrates, respectively. Two different combinations of enzymes were tested: CUS (A, B) and CURS1 and DCS (C, D). When curcuminoids are not produced the culture medium remains white (E). CUS: curcuminoid synthase; CURS1: curcumin synthase 1; DCS: diketide-CoA synthase.



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33 **Figure S3.** Protein gel showing caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT) enzyme  
34 present in pRSFDuet-1 in cell-free extract. CCoAOMT is expected around 27.28 kDa.

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