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Author for correspondence:
Joana L. Rodrigues
e-mail: joana.joanalucia@deb.uminho.pt

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Optimization of fermentation conditions for the production of curcumin by engineered *Escherichia coli*

Márcia R. Couto, Joana L. Rodrigues and Lígia R. Rodrigues

Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

MRC, 0000-0001-8154-0292; JLR, 0000-0002-3217-2320; LRR, 0000-0001-9265-0630

Curcumin is a plant secondary metabolite with outstanding therapeutic effects. Therefore, there is a great interest in developing new strategies to produce this high-value compound in a cheaper and environmentally friendly way. Curcumin heterologous production in *Escherichia coli* using artificial biosynthetic pathways was previously demonstrated using synthetic biology approaches. However, the culturing conditions to produce this compound were not optimized and so far only a two-step fermentation process involving the exchange of culture medium allowed high concentrations of curcumin to be obtained, which limits its production at an industrial scale. In this study, the culturing conditions to produce curcumin were evaluated and optimized. In addition, it was concluded that *E. coli* BL21 allows higher concentrations of curcumin to be produced than *E. coli* K-12 strains. Different isopropyl β-D-thiogalactopyranoside concentrations, time of protein expression induction and substrate type and concentration were also evaluated. The highest curcumin production obtained was 959.3 μM (95.93% of per cent yield), which was 3.1-fold higher than the highest concentration previously reported. This concentration was obtained using a two-stage fermentation with lysogeny broth (LB) and M9. Moreover, terrific broth was also demonstrated to be a very interesting alternative medium to produce curcumin because it also led to high concentrations (817.7 μM). The use of this single fermentation medium represents an advantage at industrial scale and, although the final production is lower than that obtained with the LB–M9 combination, it leads to a significantly higher production of curcumin in the first 24 h of fermentation. This study allowed obtaining the highest concentrations of curcumin reported so far in a heterologous organism and is of interest for all of those working with the heterologous production of curcuminoids, other complex polyphenolic compounds or plant secondary metabolites.

1. Introduction

Curcumin is a polyphenol found in the plant *Curcuma longa* and is well known for its several therapeutic benefits. It exhibits excellent anti-cancer potential [1,2] and has also been shown to have anti-inflammatory [3], antidiabetic [4] and anti-Alzheimer's [5] properties, among others. Despite its innumerous therapeutic applications curcumin has extremely low aqueous solubility, chemical stability and poor bioavailability, which has limited its clinical use [1,2]. Curcumin is mainly obtained using costly, energy-intensive and environmentally unfriendly extraction processes [6]. The yields obtained are low because it accumulates in low amounts over long growth periods in plants. In addition, its chemical synthesis is complex [7]. All these reasons make its heterologous biosynthetic production very interesting [6].

Recently, curcumin and other curcuminoids were produced in *Escherichia coli* using combinatorial biosynthesis [6,8–13]. Curcumin can be produced by feeding amino acids or ferulic acid (figure 1). The short pathway from ferulic acid uses two or three enzymes: 4-coumarate-CoA ligase (4CL) from different plants, diketide-CoA synthase (DCS) and curcumin synthase (CURS1) from *C. longa* [8] or curcuminoid synthase (CUS) from *Oryza sativa* [10]. CUS catalyses both steps that are catalysed separately by DCS and CURS1.

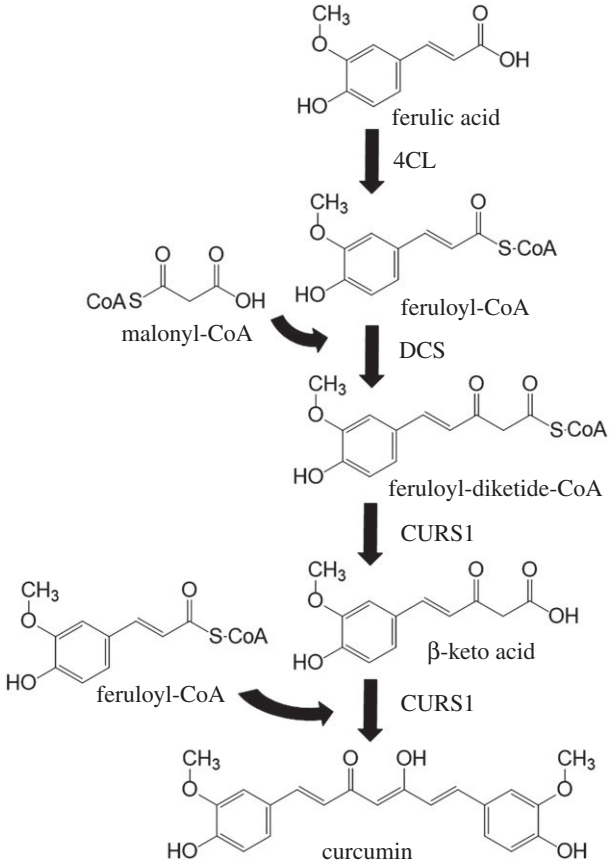

Figure 1. Curcumin biosynthetic pathway in *E. coli* using ferulic acid as substrate. 4CL, 4-coumarate-CoA ligase; DCS, diketide-CoA synthase; CURS, curcumin synthase.

In general, curcumin and other curcuminoids have been produced by heterologous hosts using two separate cultivation steps [8,10,12]. Usually the strains are first grown in lysogeny broth (LB) to produce large amounts of biomass and reach a suitable protein production level. After reaching the exponential phase, the cells are harvested and transferred to M9 modified minimal salt medium, where the substrates (amino acids or ferulic acid) are added and the curcuminoids are produced. There are also some reports of other curcuminoids produced in LB supplemented with glucose after protein expression [11,14]. Although the two-step fermentation strategy is feasible at the laboratory scale, the separation of biomass is much more difficult, laborious and expensive in large-scale fermentations. Therefore, it is very important to optimize the fermentation conditions, including media and operating parameters. In this study, we describe the production of curcumin from ferulic acid in different *E. coli* strains carrying a biosynthetic pathway previously described by our group [8]. Several fermentation parameters were studied and it was possible to obtain for the first time very high concentrations of curcumin using a single medium. The curcumin concentrations obtained in this study are the highest reported so far.

2. Material and methods

2.1. Bacterial strains and plasmids

Escherichia coli NZY5α competent cells were purchased from NZYTech (Lisbon, Portugal) and were used for molecular cloning and vector propagation. *Escherichia coli* K-12 MG1655 (DE3), *E. coli* K-12 JM109 (DE3) and *E. coli* BL21 (DE3) were tested as hosts for the expression of the curcumin biosynthetic pathway. Table 1 summarizes the characteristics of all strains and plasmids used. The construction of pCDFDuet_DCS and pRSFDuet_CURS1 was previously described by our group [8]. The DNA sequences of the codon-optimized genes are provided in the electronic supplementary material, table S1. pAC-4CL1 plasmid was provided by Claudia Schmidt-Dannert [16] through Addgene (Cambridge, MA, USA; plasmid 35947). The selected hosts were transformed with the three plasmids using electroporation.

2.2. Curcumin production

For the production of curcumin, different strains, culture media, isopropyl β-D-thiogalactopyranoside (IPTG) concentrations and times of induction were tested (electronic supplementary material, figure S1).

2.2.1. Culture media

LB, agar and super optimal broth with catabolite repression (SOC) were purchased from NZYTech and were used to prepare pre-inoculums and in the transformations. LB was also used as the production medium. In addition to LB, M9 modified minimal salt medium, MOPS (morpholinepropanesulfonic acid) minimal medium and TB (terrific broth) were used:

— M9 modified minimal salt medium contained (per litre): 40 g glucose (Acros, Geel, Belgium), 6 g Na_2HPO_4 (Scharlau, Sentmenat, Spain), 3 g KH_2PO_4 (Riel-deHaën, Seelze, Germany), 1 g NH_4Cl, 0.5 g NaCl, 15 mg $CaCl_2$ (Panreac, Barcelona, Spain), 110 mg $MgSO_4$ (Riel-deHaën), 340 mg thiamine (Fisher Scientific, Loughborough, UK) and 5 g $CaCO_3$ (Panreac) (to control the pH). Trace elements (54 mg $FeCl_3$, 4 mg $ZnCl_2$, 4 mg $CoCl_2$, 2 mg $CuCl_2$ (Riedel-deHaën), 4 mg $NaMoO_4$ and 1 mg H_2BO_3 (Merck, Kenilworth, NJ, USA)) and vitamins (0.84 mg riboflavin, 10.8 mg pantothenic acid (Sigma-Aldrich, Steinheim, Germany), 2.8 mg pyridoxine, 0.084 mg folic acid, 0.12 mg biotin (Merck) and 12.2 mg nicotinic acid (Riedel-deHaën)) were added to the medium.
— MOPS minimal medium contained (per litre): 40 g glucose, 10 ml of 0.132 M K_2HPO_4 and 100 ml of 10× MOPS mixture. The 10× MOPS mixture contained (per litre): 83.72 g MOPS (Fisher Scientific), 7.17 g tricine (ChemCruz, Dallas, TX, USA), 0.028 g $FeSO_4$ (Sigma-Aldrich), 50 ml of 1.9 M NH_4Cl, 10 ml of 0.276 M K_2SO_4 (Panreac), 0.25 ml of 0.02 M $CaCl_2{\cdot}2H_2O$, 4.2 ml of 1.25 M $MgCl_2$ (VWR, Radnor, PA, USA), 100 ml of 5 M NaCl, 0.2 ml of micronutrient solution (containing per 50 ml: 9 mg $(NH_4)_6Mo7O_{24}.4H_2O$ (Fluka, Buchs, Switzerland), 62 mg H_3BO_3 (Merck), 18 mg $CoCl_2$, 6 mg $CuSO_4$ (Sigma-Aldrich), 40 mg $MnCl_2$ (Merck) and 7 mg $ZnSO_4$ (Sigma-Aldrich)). Final pH was adjusted to 7.2 with NaOH.
— TB medium contained (per litre): 12 g tryptone (Oxoid, Basingstoke, UK), 24 g yeast extract (Oxoid), 4 ml of a 10% (v/v) glycerol solution (HiMedia, Mumbai, India), 9.4 g K_2HPO_4 (Panreac) and 2.2 g KH_2PO_4. In some experiments, 0.4–4% glycerol final concentrations were used (instead of a 0.04% glycerol final concentration) or 40 g l^{-1} glucose was supplemented to TB.

Spectinomycin (100 µg ml^{-1}) (Panreac), chloramphenicol (30 µg ml^{-1}), and kanamycin (50 µg ml^{-1}) (NZYTech) were also added to all media. IPTG (NZYTech) was added at a final concentration of 1 mM unless otherwise stated. Ferulic acid

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

strains	relevant genotype	source
E. coli NZY5α	*fhuA2Δ(argF⁻lacZ)*U169 *phoA glnV44* Φ80 *Δ(lacZ)*M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NZYTech (MB00401)
E. coli K-12 MG1655(DE3)	*F⁻ λ⁻ ilvG⁻ rfb⁻*50 *rph⁻*1 λ(DE3)	[15]
E. coli K-12 JM109(DE3)	*endA1 recA1 gyrA96 thi hsdR17* (r_k^-, m_k^+) *relA1 sup*E44 *λ⁻ Δ(lac-pro*AB) [F′ *tra*D36 *proAB lacI*^{q} *Δ(lacZ)*M15] *λ(DE3)*	Promega (P9801)
E. coli BL21(DE3)	*fhuA2 [lon] ompT gal λ(DE3) [dcm] ΔhsdS*$_B$	NEB (C2527)
plasmids	**construct**	**source**
pCDFDuet_DCS	CloDF13 *ori*, *lacI*, double T7*lac*, Strep^R; pCDFDuet-1 carrying codon-optimized DCS from *Curcuma longa*	[8]
pRSFDuet_CURS1	RSF *ori*, *lacI*, double T7*lac*, Kan^R; pRSFDuet-1 carrying codon-optimized CURS1 from *C. longa*	
pAC-4CL1	P15A *ori*, P*lac*, Cm^R, pACYC184-derived plasmid carrying 4CL1 from *Arabidopsis thaliana*	Addgene (35947)

(Acros) was added to the production medium at a final concentration of 2 mM.

2.2.2. Production conditions

Firstly, curcumin was produced in the three strains (§2.1) using the combination of LB (for biomass and protein production) and M9 (for curcumin production) previously tested [8,10]. Cultures were grown at 37°C in 50 ml LB in 250 ml flasks to an optical density at 600 nm (OD_{600}) of 0.4 (for *E. coli* K-12 MG1655(DE3)) or 0.6–0.7 (for *E. coli* K-12 JM109(DE3) and *E. coli* BL21(DE3)). The protein expression was induced with IPTG (1 mM) and 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 50 ml M9 medium in 250 ml flasks. Ferulic acid (2 mM) and IPTG (1 mM) were added at time 0 of induction in M9 medium. Both media contained IPTG at a final concentration of 1 mM.

In the case of *E. coli* BL21(DE3), the optimal IPTG concentration in LB (0.1 mM, 0.5 mM, 1 mM or 1.5 mM), need for IPTG in M9 medium (0 mM) and optimal OD_{600} (0.4–0.9) for induction of protein expression were also evaluated. Afterwards, other culture media (§2.2.1) and other combinations of media were also tested. LB, MOPS and TB were tested for the simultaneous biomass/protein and curcumin production. In these cases, the temperature was also decreased from 37°C to 26°C and the substrate was added after 5 h of protein expression. In parallel, LB or TB were also combined with MOPS. The addition of the same, but fresh, media 5 h after protein expression/before adding the substrate was also tested in the case of LB and TB. Based on the results obtained with TB, different types and concentrations of carbon sources at different phases were evaluated (§2.2.1).

All experiments were conducted in triplicate. Supernatant samples (1.5 ml) were collected for the analysis of substrate, while for the analysis of curcumin 500 µl of culture broth with cells (whole broth) was collected.

2.3. Curcumin extraction

For subsequent curcumin analysis, 500 µl of whole broth was adjusted to pH 3.0 with 6 M HCl (Fisher Scientific). Then, curcumin, which is produced intracellularly, was extracted from the cells with an equal volume of ethyl acetate (Fisher Scientific). The extracts were concentrated by solvent evaporation in a fume hood, suspended with 200 µl of acetonitrile and subjected to product analysis by ultra-high-performance liquid chromatography (UHPLC).

2.4. Ultra-high-performance liquid chromatography analysis

UHPLC analysis was used to quantify ferulic acid and curcumin using the Shimadzu Nexera-X2 (Shimadzu Corporation, Kyoto, Japan) (CBM-20A system controller, LC-30AD pump unit, DGU-20A 5R degasser unit, SPD-M20A detector unit, SIL-30AC autosampler unit, CTO-20AC column oven) system and a Grace Alltech Platinum EPS C18 column (3 µm, 150 mm × 4.6 mm) (Grace, Columbia, MD, USA). Mobile phase A was composed of water with 0.1% (v/v) of trifluoroacetic acid (Fluka). Mobile phase B was composed of acetonitrile (Fisher Scientific). For ferulic acid quantification, the following gradient was used at a flow rate of 1 ml min^{-1}: 10–20% acetonitrile (mobile phase B) for 16 min. Quantification was based on the peak areas at 310 nm for ferulic acid and the retention time was 9.7 min. For curcumin quantification, a gradient of 40–43% acetonitrile (mobile phase B) for 15 min and 43% acetonitrile for an additional 5 min was used. Curcumin was detected at 425 nm of absorbance and the retention time was 17.6 min. Curcumin used to prepare standards was purchased from Fisher Scientific.

3. Results and discussion

3.1. Selection of the best host to produce curcumin

The selection of the heterologous host for the production of curcumin is important because, depending on the strain, the concentrations reached can be very different. Indeed, the productions previously obtained in different strains were significantly different. For example, the highest curcumin concentration obtained in our previous work [8] was 187.9 µM using *E. coli* K-12 MG1655(DE3), while Katsuyama *et al.* [10] were able to obtain ≈306.7 µM using *E. coli* BLR(DE3). The fermentation conditions used in both studies were the same but the enzymes used were different (and/or from different organisms). Therefore, it was not possible to conclude whether the difference obtained was due to the strain used or, for example, due to the possibly higher catalytic efficiency of CUS compared with the combination of DCS and CURS1. Thus, the production of curcumin using the same fermentation conditions and biosynthetic pathway was tested in this study, varying only the *E. coli* strains. The productions obtained in three *E. coli* strains available in our laboratory were compared (figure 2). As it is possible to observe, the production in *E. coli* BL21 was very high (483.1 µM), 2.72-fold higher than that produced with *E. coli* K-12 MG1655 (177.9 µM). *E. coli* K-12 JM109, a strain widely used for the

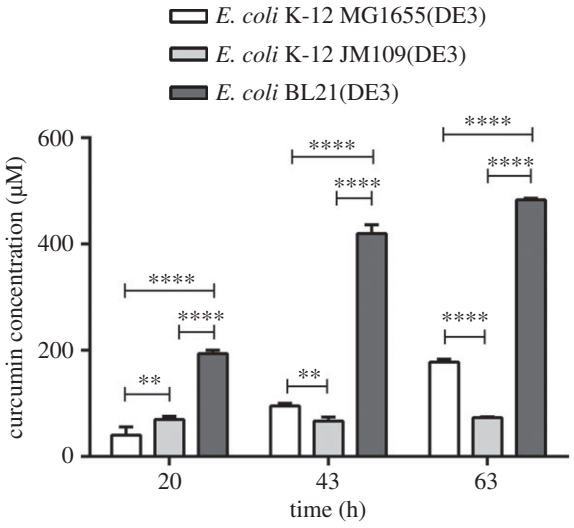

Figure 2. Curcumin production in *E. coli* K-12 MG1655 (DE3), *E. coli* K-12 JM109(DE3) and *E. coli* BL21(DE3). LB (lysogeny broth) was used for cell growth and protein expression and M9 medium for the curcumin production phase. The optical density (600 nm) at the moment of induction was 0.4 for *E. coli* K-12 MG1655 and 0.6 for *E. coli* K-12 JM109 and *E. coli* BL21. Error bars are standard deviations from triplicate experiments. Two-way ANOVA was used to determine statistically significant differences and is denoted as follows: **** indicates p-value < 0.0001 and ** indicates p-value < 0.01. See the electronic supplementary material, table S2, for more detailed information regarding statistical significance.

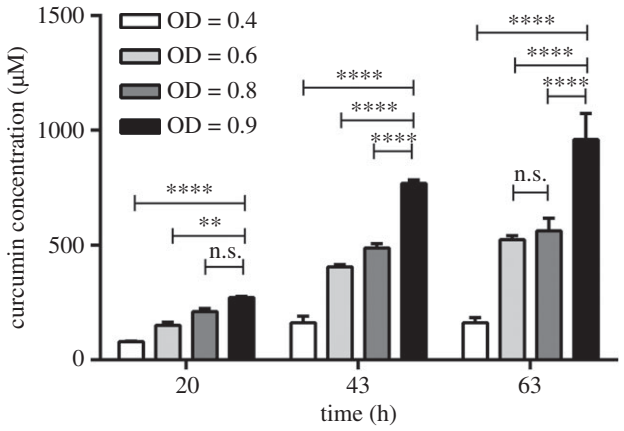

Figure 3. Effect of optical density at 600 nm (OD_{600}) at the time of induction of protein expression in curcumin production in *E. coli* BL21(DE3). LB (lysogeny broth) was used for cell growth and protein expression and M9 medium for the curcumin production phase. Error bars are standard deviations from triplicate experiments. Two-way ANOVA was used to determine statistically significant differences and is denoted as follows: **** indicates p-value < 0.0001, **indicates p-value < 0.01 and n.s. indicates no significant difference ($p > 0.05$). See the electronic supplementary material, table S3, for more detailed information regarding statistical significance.

production of various recombinant proteins due to the deletion of *recA* and *endA* that have a positive effect on plasmid stability [15], was also tested but the curcumin production was low (73.2 μM). *E. coli* BLR is a BL21-related strain, and they are both derived from B strains. B strains are excellent for protein expression because they are deficient in Lon and OmpT proteases, which degrade many heterologous proteins [17–19]. In addition, the parental strain (B834) that gave rise to BL21 has a mutation in the $hsdS_B$ gene which prevents plasmid degradation [19,20]. Besides these genetic differences, *E. coli* B strains have a more efficient central carbon metabolism, thus they tolerate higher glucose concentrations, produce less acetate and grow to higher OD than *E. coli* K-12 [15,21–24], which can be very advantageous when expressing biosynthetic pathways. Acetate, which is a growth inhibitor, accumulates less in *E. coli* BL21 because this strain presents a higher expression of acetyl-CoA synthetase during the glucose exponential phase [25]. This enzyme converts acetate to acetyl-CoA, which in turn is converted to malonyl-CoA, whose availability in the cell is very important for curcumin production [6].

The comparison of these production results also suggests that our pathway, which consisted of 4CL1 from *Arabidopsis thaliana* and DCS and CURS1 from *C. longa*, is more efficient than the pathway described by Katsuyama *et al.* [10], which consisted of 4CL from *Lithospermum erythrorhizon*, CUS and overexpression of acetyl-CoA carboxylase from *E. coli*.

3.2. Optimization of induction parameters (OD and IPTG) in *Escherichia coli* BL21(DE3) when using the combined LB and M9 medium to produce curcumin

The OD_{600} at the time of induction has proved to highly influence curcumin production when *E. coli* K-12 MG1655(DE3) was used as host [8]. Therefore, the optimal OD_{600} to induce protein expression and produce curcumin was also evaluated for *E. coli* BL21. The curcumin production obtained at different OD_{600} values for the induction of protein expression can be observed in figure 3. The addition of IPTG at an OD_{600} of 0.9 yielded the highest production titre (959.3 μM), thus suggesting that induction should be performed at high OD_{600} values. An early induction can probably impose a metabolic burden on the host strain associated with protein overexpression. When the cells are induced after the exponential phase there is a higher cell density for product formation. However, after the exponential phase the metabolic state of the cells may not be favourable for protein expression because they may be under stressful conditions and trigger a response that increases protease levels, which can reduce the yield of heterologous proteins [26]. Protein induction using *E. coli* BL21 could be performed at a later stage than when *E. coli* K-12 MG1655(DE3) was used ($OD_{600} = 0.4$), probably because *E. coli* BL21 lacks some proteases and is less sensitive to growth conditions, usually growing to a higher OD as stated before [15].

The optimal IPTG concentration for protein induction and consequently substrate conversion was also investigated. In previous studies, IPTG was added to a final concentration of 1 mM in LB and then in M9 minimal medium. In figure 4, it is possible to compare the curcumin production for different IPTG concentrations tested (0.1, 0.5, 1.0 and 1.5 mM). These concentrations were tested in both LB and M9 medium. In addition, in one of the tests 1 mM of IPTG was added to LB and no IPTG was added to the M9 medium. The study of the best IPTG concentration is important because protein expression does not respond predictably to IPTG concentration. IPTG is actively transported across the cell membrane by permeases or permease-independent pathways [27] and, therefore, the IPTG that enters each cell is highly variable. As can be seen in figure 4, the highest curcumin production (822.6 μM) was obtained when 0.1 mM of IPTG was added and the increase in the production is statistically

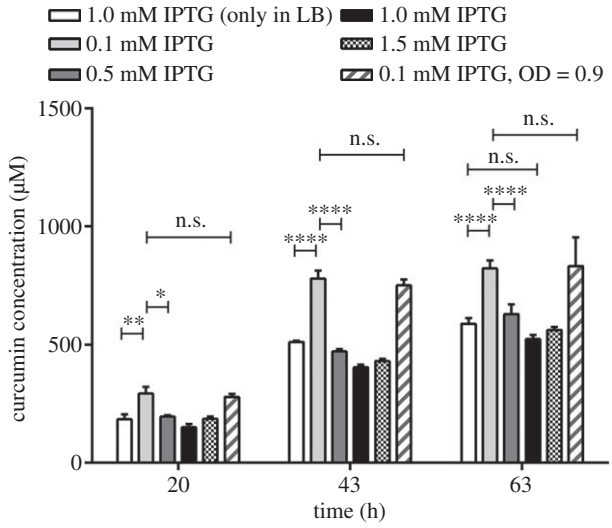

Figure 4. Effect of IPTG (isopropyl β-D-1-thiogalactopyranoside) concentration in curcumin production by *E. coli* BL21(DE3). IPTG was added to LB (lysogeny broth) for protein expression: 0.1, 0.5, 1.0 or 1.5 mM (final concentration) and the same correspondent concentrations were added to the M9 medium. In one of the assays IPTG (1.0 mM) was only added to LB medium. Protein expression was induced when cells reached an optical density (OD_{600nm}) of 0.6 unless otherwise stated in the legend. Error bars are standard deviations from triplicate experiments. Two-way ANOVA was used to determine statistically significant differences and is denoted as follows: **** indicates p-value < 0.0001, ** indicates p-value < 0.01, * indicates $0.01 < p\text{-value} \leq 0.05$ and n.s. indicates no significant difference ($p > 0.05$). See the electronic supplementary material, table S4, for more detailed information regarding statistical significance.

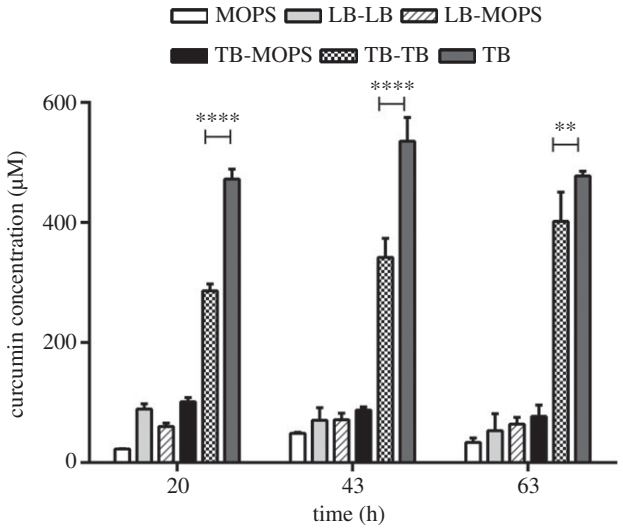

Figure 5. Curcumin production over time in different culture media in *E. coli* BL21(DE3). Curcumin was produced in MOPS (morpholinepropanesulfonic acid) medium, in LB (lysogeny broth) and TB (terrific broth). In some cases, different media were used for the two phases (LB-MOPS, TB-MOPS) or the same medium was used but before the substrate was added the cells were centrifuged and resuspended in fresh medium (LB–LB, TB–TB). Protein expression was induced with 1.0 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) when cells reached an optical density (OD_{600nm}) of 0.6. Error bars are standard deviations from triplicate experiments. Two-way ANOVA was used to determine statistically significant differences and is denoted as follows: **** indicates p-value < 0.0001 and ** indicates p-value < 0.01. See the electronic supplementary material, table S5, for more detailed information regarding statistical significance.

significant ($p < 0.0001$). Higher IPTG concentrations did not lead to titre enhancement, suggesting that metabolic burden effects may be affecting productivity [28–30]. High expression of heterologous proteins can have a negative and unpredictable effect on the cell growth while expression levels that are too low might reduce the encounter frequency between the enzymes and the substrates [28–31]. Owing to this unpredictability it is important to optimize the IPTG concentration. The need to add IPTG in the phase of production (in M9 medium) was also evaluated (figure 4, white bar). By comparing the result obtained (589.8 μM) with that where 1 mM of IPTG was added in both steps (LB and M9 medium) (524.8 μM), it is possible to conclude that the addition of IPTG in the production phase may not be needed. This possibly occurred because in this case the metabolic burden is reduced.

Finally, we evaluated whether the curcumin concentration could be improved by inducing the cells at an OD of 0.9, which proved to be favourable (figure 3), and by adding only 0.1 mM of IPTG. As can be observed in figure 4, the combination of both optimal parameters did not significantly increase the production (831.9 μM). The initial conditions tested, OD of 0.9 and IPTG of 1 mM (figure 3), proved to be the best conditions to produce curcumin in this culture medium.

3.3. Optimization of culture media to produce curcumin using *Escherichia coli* BL21(DE3)

In this study, different cultivation media for the entire production bioprocess were evaluated in order to avoid medium exchange in the middle of the process. This would make the curcumin production easier, more attractive and also more economically viable for an industrial scale-up. LB and M9 medium were previously tested in a one-step cultivation strategy using *E. coli* K-12 MG1655(DE3) but it was concluded that the productions were very low (data not shown). Therefore, other media such as TB and MOPS were tested because they were previously used to successfully produce plant secondary metabolites [32,33]. In addition, different media combinations (LB-MOPS and TB-MOPS) and exchange to the same, but fresh, medium (LB–LB and TB–TB) were also tested with the aim of finding a combination that allowed higher titres to be obtained. The productions obtained can be observed in figure 5. TB medium, from all the new media tested, proved to be the best one to produce curcumin (535.8 μM at 43 h). In addition, in this medium it is possible to obtain higher concentrations of curcumin in the first 24 h than when using the combination LB–M9, which represents an advantage for industrial-scale production. The other combinations tested led to low curcumin productions. The use of fresh TB medium in the production phase also allowed significantly higher concentrations of curcumin to be obtained but the high content of the carbon source probably had an inhibitory effect when compared with the case where the same TB was used in both phases. TB is a phosphate-buffered rich medium and has 20% more tryptone and 380% more yeast extract than LB. In addition, TB also has glycerol as an extra carbon source. This high nutritive content and the presence of potassium phosphates that prevent a drop in pH of the medium during bacterial growth allow *E. coli* to maintain

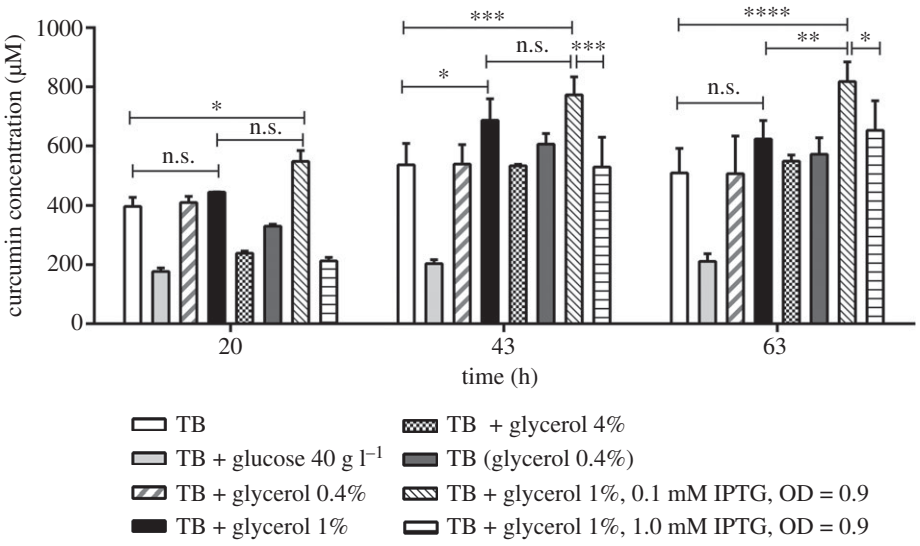

Figure 6. Effect of different carbon source concentrations in TB (terrific broth) in curcumin production by *E. coli* BL21(DE3). All experiments contained 0.04% (v/v) glycerol at the beginning of the fermentation except TB (glycerol 0.4%), which contained 0.4% glycerol. In the other experiments, glycerol or glucose were supplemented when the susbtrate was added (5 h after protein expression induction). Protein expression was induced with 1.0 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) when cells reached an optical density (OD_{600nm}) of 0.6, unless otherwise stated in the legend. Error bars are standard deviations from triplicate experiments. Two-way ANOVA was used to determine statistically significant differences and is denoted as follows: **** indicates p-value $<$ 0.0001, *** indicates p-value $<$ 0.001, ** indicates p-value $<$ 0.01, * indicates 0.01 $<$ p-value $\leq$ 0.05 and n.s. indicates no significant difference ($p >$ 0.05). See the electronic supplementary material, table S6, for more detailed information regarding statistical significance.

an extended growth phase and therefore to obtain greater yields of recombinant protein and plasmid DNA [34]. M9 modified minimal medium was also buffered (by the addition of $CaCO_3$), which was essential to obtain the high concentrations reported (figures 2–4) in this medium [35,36].

3.4. Optimization of carbon source concentration in terrific broth medium

TB was demonstrated to be an optimal medium for both stages of curcumin production (figure 5). Therefore, the effect of induction time in TB medium was evaluated and, although at an OD_{600} of 0.8–0.9 the production was higher than at an OD_{600} of 0.4 and 0.6 (data not shown), the differences were not statistically significant ($p > 0.05$). In parallel, different concentrations of glycerol were tested with the goal of improving the production (figure 6). TB contained 0.04% (v/v) glycerol at the beginning of the fermentation except in one case where a 0.4% concentration was tested (figure 6, TB (glycerol 0.4%)). This concentration was tested as it is the concentration present in most TB formulations [34]. In this study, glycerol or glucose were also supplemented when the substrate was added (5 h after induction with IPTG). As can be seen in figure 6, the supplementation of glucose had a negative/inhibitory effect on curcumin production. The presence of glucose, a preferred carbon source, represses the synthesis and activity of key proteins for transport and metabolism of glycerol [37]. In addition, the supplementation of glucose is known to enhance acetic acid production, which reduces the pH of the medium. This acidic shift in a first phase inhibits protein production and then retards *E. coli* growth [38,39]. Glucose accumulation can also promote the growth of plasmid-free cells [40].

Regarding the different concentrations of glycerol tested, the best result was obtained when 0.04% glycerol was added at the beginning of the fermentation and then 1% of glycerol was added with the substrate (686.7 μM at 43 h). This experiment was repeated but in the conditions previously optimized for the LB and M9 combination—the protein expression was induced at an OD of 0.9 and 0.1 mM or 1.0 mM IPTG was tested. At an OD of 0.9 and 0.1 mM IPTG, it was possible to improve curcumin production (817.7 μM at 63 h) by 31% at 63 h. This production is very high and almost equivalent to the highest one obtained using the LB and M9 combination (959.3 μM), which clearly demonstrates that the TB medium should be considered at industrial scale for the production of curcumin because it allows the operational process to be simplified and reduces the operational costs related to the exchange of the culture medium. In addition, this medium allows a higher production of curcumin to be obtained in the first 24 h of fermentation than the combination of LB–M9. To further decrease the costs of the fermentation medium (TB), alternative low-cost substrates could be considered. For instance, corn steep liquor could be tested as a nitrogen source because it is an inexpensive residue compared with the commonly used sources, namely yeast extract and tryptone. In addition, crude glycerol arising from biodiesel production could be evaluated towards the development of a more sustainable process.

4. Conclusion

The optimization of curcumin fermentation conditions is essential to increase the production and yields of this important therapeutic agent. So far, curcumin has only been produced with high yields in a two-step fermentation that involves the exchange of fermentation medium, which is not ideal at an industrial scale. Herein we study different parameters including different *E. coli* strains, IPTG concentrations, time of protein expression induction and substrate type and concentration. *Escherichia coli* BL21(DE3) enabled

more curcumin to be produced than the other strains tested. In the end, we were able not only to increase curcumin production from ferulic acid by 3.1-fold, but also to produce it using a single fermentation medium without any significant decrease in the production or yield. TB proved to be an optimum culture medium to produce curcumin. The productions obtained in this study, 817.7 µM (301 mg l^{-1}) in TB and 959.3 µM (353 mg l^{-1}) in the combined LB and M9 medium, are the highest reported so far, as well as the per cent yields of 81.8–95.9%. In the future, the potential of industry by-products and residues could be tested in curcumin production aiming at the development of a sustainable bioprocess based on the circular bioeconomy concept. In addition, factorial experiment designs can be used to optimize other relevant variables (e.g. duration of protein expression before substrate addition) before starting the bioprocess scale-up. In bioreactor production, other parameters will have to be taken into account, such as aeration and agitation rates that may influence cellular growth and, consequently, curcumin production. Additionally, the availability of the natural precursor malonyl-CoA can be studied and further improved by different metabolic engineering strategies.

Data accessibility. The datasets for this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. M.R.C. performed the experiments and analysed the results, J.L.R. assisted in study conception and design, and wrote the manuscript, L.R.R. assisted in study conception and design, coordinated the study and provided feedback and suggestions on the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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References

1. Zaman MS *et al.* 2016 Curcumin nanoformulation for cervical cancer treatment. *Sci. Rep.* **6**, 20051. (doi:10.1038/srep20051)
2. Nagahama K, Utsumi T, Kumano T, Maekawa S, Oyama N, Kawakami J. 2016 Discovery of a new function of curcumin which enhances its anticancer therapeutic potency. *Sci. Rep.* **6**, 1–14. (doi:10.1038/srep30962)
3. Kunnumakkara AB, Bordoloi D, Padmavathi G, Monisha J, Roy NK, Prasad S, Aggarwal BB. 2016 Curcumin, the golden nutraceutical: multitargeting for multiple chronic diseases. *Br. J. Pharmacol.* **174**, 325–1348. (doi:10.1111/bph.13621)
4. Weisberg S, Leibel R, Tortoriello D. 2016 Proteasome inhibitors, including curcumin, improve pancreatic β-cell function and insulin sensitivity in diabetic mice. *Nutr. Diabetes* **6**, e205. (doi:10.1038/nutd.2016.13)
5. Shen L, Liu C-C, An C-Y, Ji H-F. 2016 How does curcumin work with poor bioavailability? Clues from experimental and theoretical studies. *Sci. Rep.* **6**, 1–10. (doi:10.1038/srep20872)
6. Rodrigues JL, Prather KL, Kluskens L, Rodrigues L. 2015 Heterologous production of curcuminoids. *Microbiol. Mol. Biol. Rev.* **79**, 39–60. (doi:10.1128/MMBR.00031-14)
7. Rao EV, Sudheer P. 2011 Revisiting curcumin chemistry part I: a new strategy for the synthesis of curcuminoids. *Indian J. Pharm. Sci.* **73**, 262–270. (doi:10.4103/0250-474X.93508)
8. Rodrigues J, Araújo R, Prather K, Kluskens L, Rodrigues L. 2015 Production of curcuminoids from tyrosine by a metabolically engineered *Escherichia coli* using caffeic acid as an intermediate. *Biotechnol. J.* **10**, 599–609. (doi:10.1002/biot.201400637)
9. Katsuyama Y, Hirose Y, Funa N, Ohnishi Y, Horinouchi S. 2010 Precursor-directed biosynthesis of curcumin analogs in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **74**, 641–645. (doi:10.1271/bbb.90866)
10. Katsuyama Y, Matsuzawa M, Funa N, Horinouchi S. 2008 Production of curcuminoids by *Escherichia coli* carrying an artificial biosynthesis pathway. *Microbiology* **154**, 2620–2628. (doi:10.1099/mic.0.2008/018721-0)
11. Wang S, Zhang S, Zhou T, Zeng J, Zhan J. 2013 Design and application of an *in vivo* reporter assay for phenylalanine ammonia-lyase. *Appl. Microbiol. Biotechnol.* **97**, 7877–7885. (doi:10.1007/s00253-013-5122-4)
12. Rodrigues JL, Couto MR, Araújo RG, Prather KL, Kluskens L, Rodrigues LR. 2017 Hydroxycinnamic acids and curcumin production in engineered *Escherichia coli* using heat shock promoters. *Biochem. Eng. J.* **125**, 41–49. (doi:10.1016/j.bej.2017.05.015)
13. Kim EJ, Cha MN, Kim B-G, Ahn J-H. 2017 Production of curcuminoids in engineered *Escherichia coli*. *J. Microbiol. Biotechnol.* **27**, 975–982. (doi:10.4014/jmb.1701.01030)
14. Wang S, Zhang S, Xiao A, Rasmussen M, Skidmore C, Zhan J. 2015 Metabolic engineering of *Escherichia coli* for the biosynthesis of various phenylpropanoid derivatives. *Metab. Eng.* **29**, 153–159. (doi:10.1016/j.ymben.2015.03.011)
15. Phue JN, Lee SJ, Trinh L, Shiloach J. 2008 Modified *Escherichia coli* B (BL21), a superior producer of plasmid DNA compared with *Escherichia coli* K (DH5α). *Biotechnol. Bioeng.* **101**, 831–836. (doi:10.1002/bit.21973)
16. Watts KT, Lee PC, Schmidt-Dannert C. 2006 Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. *BMC Biotechnol.* **6**, 1–12. (doi:10.1186/1472-6750-6-22)
17. Gottesman S. 1996 Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.* **30**, 465–506. (doi:10.1146/annurev.genet.30.1.465)
18. Grodberg J, Dunn JJ. 1988 ompT encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **170**, 1245–1253. (doi:10.1128/jb.170.3.1245-1253.1988)
19. Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF. 2009 Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21 (DE3) and comparison of the *E. coli* B and K-12 genomes. *J. Mol. Biol.* **394**, 653–680. (doi:10.1016/j.jmb.2009.09.021)
20. Rosano GL, Ceccarelli EA. 2014 Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front. Microbiol.* **7**, 1–17. (doi:10.3389/fmicb.2014.00172)
21. Negrete A, Ng W-I, Shiloach J. 2010 Glucose uptake regulation in *E. coli* by the small RNA SgrS: comparative analysis of *E. coli* K-12 (JM109 and MG1655) and *E. coli* B (BL21). *Microb. Cell Fact.* **9**, 1–9. (doi:10.1186/1475-2859-9-75)
22. Shiloach J, Kaufman J, Guillard A, Fass R. 1996 Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by *Escherichia coli* BL21 (λDE3) and *Escherichia coli* JM109. *Biotechnol. Bioeng.* **49**, 421–428. (doi:10.1002/(SICI)1097-0290(19960220)49:4<421::AID-BIT9>3.0.CO;2-R)
23. Shiloach J, Reshamwala S, Noronha SB, Negrete A. 2010 Analyzing metabolic variations in different bacterial strains, historical perspectives and current trends–example *E. coli*. *Curr. Opin. Biotechnol.* **21**, 21–26. (doi:10.1016/j.copbio.2010.01.001)

24. Yoon SH *et al.* 2012 Comparative multi-omics systems analysis of *Escherichia coli* strains B and K-12. *Genome Biol.* **13**, R37. (doi:10.1186/gb-2012-13-5-r37)
25. Castaño-Cerezo S, Bernal V, Röhrig T, Termeer S, Cánovas M. 2015 Regulation of acetate metabolism in *Escherichia coli* BL21 by protein N^{ϵ}-lysine acetylation. *Appl. Microbiol. Biotechnol.* **99**, 3533–3545. (doi:10.1007/s00253-014-6280-8)
26. Carneiro S, Ferreira EC, Rocha I. 2013 Metabolic responses to recombinant bioprocesses in *Escherichia coli*. *J. Biotechnol.* **164**, 396–408. (doi:10.1016/j.jbiotec.2012.08.026)
27. Fernández-Castané A, Vine CE, Caminal G, López-Santín J. 2012 Evidencing the role of lactose permease in IPTG uptake by *Escherichia coli* in fed-batch high cell density cultures. *J. Biotechnol.* **157**, 391–398. (doi:10.1016/j.jbiotec.2011.12.007)
28. Moon TS, Yoon S-H, Lanza AM, Roy-Mayhew JD, Prather KLJ. 2009 Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* **75**, 589–595. (doi:10.1128/AEM.00973-08)
29. Moon TS, Dueber JE, Shiue E, Prather KLJ. 2010 Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. *Metab. Eng.* **12**, 298–305. (doi:10.1016/j.ymben.2010.01.003)
30. Jones KL, Kim S-W, Keasling J. 2000 Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metab. Eng.* **2**, 328–338. (doi:10.1006/mben.2000.0161)
31. Donovan RS, Robinson CW, Glick B. 1996 Review: optimizing inducer and culture conditions for expression of foreign proteins under the control of thelac promoter. *J. Ind. Microbiol.* **16**, 145–154. (doi:10.1007/BF01569997)
32. Wu J, Du G, Zhou J, Chen J. 2013 Metabolic engineering of *Escherichia coli* for (2S)-pinocembrin production from glucose by a modular metabolic strategy. *Metab. Eng.* **16**, 48–55. (doi:10.1016/j.ymben.2012.11.009)
33. Leonard E, Yan Y, Fowler ZL, Li Z, Lim C-G, Lim K-H, Koffas MA. 2008 Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. *Mol. Pharm.* **5**, 257–265. (doi:10.1021/mp7001472)
34. Tartof K, Hobbs C. 1987 Improved media for growing plasmid and cosmid clones. *Focus* **9**, 12.
35. Niu D, Tian K, Prior BA, Wang M, Wang Z, Lu F, Singh S. 2014 Highly efficient L-lactate production using engineered *Escherichia coli* with dissimilar temperature optima for L-lactate formation and cell growth. *Microb. Cell Fact.* **13**, 1–11. (doi:10.1186/1475-2859-13-78)
36. Mazumdar S, Lee J, Oh M-K. 2013 Microbial production of 2, 3 butanediol from seaweed hydrolysate using metabolically engineered *Escherichia coli*. *Bioresour. Technol.* **136**, 329–336. (doi:10.1016/j.biortech.2013.03.013)
37. Deutscher J, Francke C, Postma PW. 2006 How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev.* **70**, 939–1031. (doi:10.1128/MMBR.00024-06)
38. De Mey M, De Maeseneire S, Soetaert W, Vandamme E. 2007 Minimizing acetate formation in *E. coli* fermentations. *J. Ind. Microbiol. Biotechnol.* **34**, 689–700. (doi:10.1007/s10295-007-0244-2)
39. Romano D, Molla G, Pollegioni L, Marinelli F. 2009 Optimization of human D-amino acid oxidase expression in *Escherichia coli*. *Protein Expr. Purif.* **68**, 72–78. (doi:10.1016/j.pep.2009.05.013)
40. Neubauer P, Lin H, Mathiszik B. 2003 Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli*. *Biotechnol. Bioeng.* **83**, 53–64. (doi:10.1002/bit.10645)