Appl Microbiol Biotechnol (2013) 97:3333–3341
DOI 10.1007/s00253-012-4544-8

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Engineering *E. coli* for caffeic acid biosynthesis from renewable sugars

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Received: 29 August 2012 / Revised: 4 October 2012 / Accepted: 23 October 2012 / Published online: 21 November 2012
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Abstract Caffeic acid is a valuable aromatic compound that possesses many important pharmacological activities. In structure, caffeic acid belongs to the hydroxycinnamic acid family and can be biosynthesized from the aromatic amino acid tyrosine. In the present paper, the caffeic acid biosynthesis pathway was reconstituted in engineered *Escherichia coli* to produce caffeic acid from simple biomass sugar glucose and xylose. Different engineering approaches were utilized to optimize the production. Specifically, two parallel biosynthesis routes leading from tyrosine to caffeic acid were studied. The copy number of the intermediate biosynthesis genes was varied to find appropriate gene doses for caffeic acid biosynthesis. Three different media, including a MOPS medium, a synthetic medium, and a rich medium, were also examined to improve the production. The highest specific caffeic acid production achieved was 38 mg/L/OD. Lastly, cultivation of engineered *E. coli* in a bioreactor resulted in a production of 106 mg/L caffeic acid after 4 days.

Keywords *Escherichia coli* · Caffeic acid · Tyrosine · Heterologous biosynthesis

Introduction

There is presently a rising interest in microbial production of valuable aromatic compounds from inexpensive simple carbon sources or renewable biomass feedstocks (Gosset 2009). Notably, several industrially important products, such as styrene, *p*-hydroxystyrene, and *p*-hydroxybenzoate, have been successfully produced in engineered bacteria (Qi et al. 2007; McKenna and Nielsen 2011; Verhoef et al. 2007). Driving the progress is a recent development of metabolic engineering on microbes' aromatic amino acid biosynthesis pathway (or shikimate pathway) in which L-phenylalanine, L-tryptophan, or L-tyrosine can be used as precursors for production of desired products. However, the reported titers and yields of these products are still low, largely due to the poor availability of the substrate aromatic amino acids. Efforts have therefore been made to increase the endogenous pool size of these amino acids. For example, a number of *Escherichia coli* strains have been successfully constructed by different engineering approaches to overproduce tyrosine with high yields (Lutke-Eversloh and Stephanopoulos 2008; Santos and Stephanopoulos 2008; Juminaga et al. 2012). Based on this accomplishment, microbial biosynthesis of several tyrosine-derived compounds, including *p*-coumaric acid, melanin, flavonoids, tyrosol, and alkaloids, has been achieved (Santos and Stephanopoulos 2008; Santos et al. 2011; Satoh et al. 2012; Nakagawa et al. 2011).

Caffeic acid (3,4-dihydroxy cinnamic acid) is an important hydroxycinnamic acid that can be derived from tyrosine. It is an intriguing compound because it possesses various pharmacological activities, including antioxidant (Maurya and Devasagayam 2010; Mori and Iwahashi 2009), antitumor (Rajendra-Prasad et al. 2011; Hudson et al. 2000; Morton et al. 2000), antiviral (Ikeda et al. 2011), antidepressive (Takeda et al. 2002), and antidiabetic (Jung et al. 2006; Cheng et al. 2003) activities. In addition, caffeic acid phenethyl ester has been well documented to have anticarcinogenic and antidiabetic activities (Chuu et al. 2012; Natarajan et al. 1996; Grunberger et al. 1988; Sud'ina et al. 1993; Celik et al. 2009). As a key precursor for lignin

Electronic supplementary material The online version of this article (doi:10.1007/s00253-012-4544-8) contains supplementary material, which is available to authorized users.

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formation, caffeic acid is widely found in plants, but despite its reported pharmaceutical values, there are relatively few studies for extracting caffeic acid from natural plant sources, mainly due to the complexity and inefficiency of the separation and purification process (Ye et al. 2010; Wang et al. 2009; Leonardis et al. 2005). Therefore, engineering a microbe to make a high level of caffeic acid is of great research interest.

Caffeic acid can be biosynthesized from tyrosine through a two-step pathway, in which tyrosine is converted to *p*-coumaric acid and then to caffeic acid by tyrosine ammonia lyase (TAL) and 4-coumarate 3-hydroxylase (Coum3H), respectively (Rosler et al. 1997; Berner et al. 2006). Alternatively, 4-coumarate:CoA ligase (4CL) can convert *p*-coumaric acid to coumaroyl-CoA which becomes caffeoyl-CoA by 4-coumarate 3-hydroxylase (Gross and Zenk 1974; Kneusel et al. 1989). Notably, it has been reported that certain CoA thioesterases can hydrolyze caffeoyl-CoA to make caffeic acid (Ramirez-AhumadaMdel et al. 2006). *E. coli* native CoA thioesterases such as hydroxyphenylacetyl-CoA thioesterase have also been found to have promiscuous activity against a variety of substrates and thus could act on caffeoyl-CoA to generate caffeic acid (Song et al. 2006; Zhuang et al. 2008). Therefore, another potential pathway for caffeic acid biosynthesis is to convert tyrosine to coumaric acid, coumaroyl-CoA, caffeoyl-CoA, and finally, caffeic acid by four enzymatic reactions (Fig. 1).

Very recently, Lin et al. reported the production of caffeic acid in *E. coli* by establishing a dual biosynthesis pathway that used both coumaric acid and L-DOPA as intermediates (Lin and Yan 2012). Choi et al. biosynthesized caffeic acid in an effort to make phenylpropanoid products in *E. coli* (Choi et al. 2011). In addition, *Streptomyces fradiae* has also been utilized as a heterologous host for caffeic acid biosynthesis (Berner et al. 2006). However, the reported caffeic acid titers in these studies remain low, as the highest titer achieved so far was 50 mg/L in *E. coli* (Lin and Yan 2012). In the presented paper, two parallel biosynthesis routes leading from tyrosine to caffeic acid were reconstituted in an *E. coli* tyrosine over-producer, and the effects of different production media and biosynthetic gene copy number were investigated to improve the production. These different engineering approaches resulted in the highest reported titers of caffeic acid from glucose and the first report of caffeic acid production from xylose.

Materials and methods

Caffeic acid tolerance assay

In order to study the toxicity of caffeic acid on *E. coli*, a previously constructed *E. coli* strain rpoA14(DE3) was grown in LB medium supplemented with 0, 10, 100, and 1,000 mg/L caffeic acid standard. Twenty microliters of overnight LB culture of rpoA14(DE3) was inoculated into 1 mL of such prepared medium. After 24 h of growth at 250 rpm at 37 °C, optical density (OD) at 600 nm was measured for individual cultures.

Bacterial cultivation conditions

All strains used in this study were cultivated at 250 rpm at 37 °C. For caffeic acid production in test tube, 40 μL of overnight LB culture was inoculated into 2 mL MOPS, synthetic or rich medium and grown for 3 days. To avoid undesired formation of caffeic acid-derived *o*-quinone and poly-aromatics induced by light, the test tubes were wrapped with aluminum foil. The ingredient of the media used in this work is as follows: 1 L MOPS medium contained 1× MOPS minimal medium salt (Teknova), 2.84 g Na_2HPO_4, and 5 g glucose or xylose; 1 L synthetic medium contained 2 g NH_4Cl, 5 g $(NH_4)_2SO_4$, 2.993 g KH_2PO_4, 7.315 g K_2HPO_4, 8.372 g MOPS, 0.5 g NaCl, 2.4 g $MgSO_4$, and 5 g glucose or xylose; and 1 L rich medium contained 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 5 g glucose or xylose. Trace elements containing 0.4 mg/L Na_2EDTA, 0.03 mg/L H_3BO_3, 1 mg/L thiamine, 0.94 mg/L $ZnCl_2$, 0.5 mg/L $CoCl_2$, 0.38 mg/L $CuCl_2$, 1.6 mg/L $MnCl_2$, 3.77 mg/L $CaCl_2$, and 3.6 mg/L $FeCl_2$ were also supplemented to the synthetic and rich media. For cell cultivation, 0.1 mM IPTG and appropriate antibiotics were added at inoculation. The working concentrations of antibiotics were: 100 mg/L ampicillin, 50 mg/L kanamycin, 50 mg/L streptomycin, and 34 mg/L chloramphenicol.

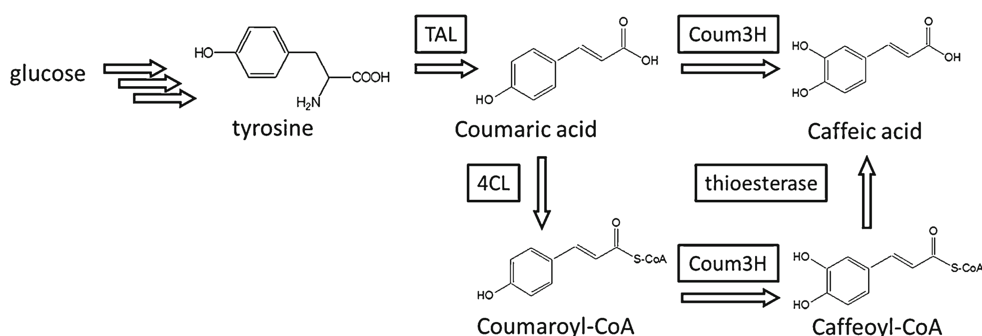

Fig. 1 Biosynthesis pathway of caffeic acid. Enzymes responsible for the reactions in the pathway are *boxed*

Construction of strains and plasmids

Strain rpoA14(DE3) (*E. coli* K12 ΔpheA ΔtyrR lacZ::$P_{LtetO\text{-}1}$-$tyrA^{fbr}aroG^{fbr}$ tyrR::$P_{LtetO\text{-}1}$-$tyrA^{fbr}aroG^{fbr}$ hisH (L82R) carrying pHACM-rpoA14) used in this study was previously constructed for tyrosine overproduction (Santos et al. 2012). Plasmid pTrc-$RgTAL^{syn}$, pTrc-$RgTAL^{syn}$–$Pc4CL^{syn}$ and pCDF-trc-$RgTAL^{syn}$-$Pc4CL^{syn}$ were constructed in our previous study (Santos et al. 2011). Plasmid pTrc-$RgTAL^{syn}$ (pCA1) contained a codon-optimized (for *E. coli*) *Rhodotorula glutinis* tyrosine ammonia lyase ($RgTAL^{syn}$; Electronic supplementary material (ESM) 1). Plasmid pTrc-$RgTAL^{syn}$-$Pc4CL^{syn}$ (pCA2) contained $RgTAL^{syn}$ and a codon-optimized *Petroselinum crispus* 4-coumarate:CoA ligase ($Pc4CL^{syn}$; ESM 2) pCA1 and pCA2 were both derived from plasmid pTrcHis2B (Invitrogen) with a low plasmid copy number (15–25 copies per cell). pCDF-trc-$RgTAL^{syn}$ (pCA3) and pCDF-trc-$RgTAL^{syn}$-$Pc4CL^{syn}$ (pCA4) were derived from pCDFDuet-1 (Novagen) and contained a relatively higher copy number than pCA1 and pCA2 (20–40 copies per cell). pCA3 was constructed by removal of $Pc4CL^{syn}$ gene from pCA4 using restriction enzyme *Sal*I digestion followed by a self-ligation. All four plasmids contain a trc promoter for gene expression. The *sam5* gene encoding a 4-coumarate 3-hydroxylase (Coum3H) from *Saccharothrix espanaensis* (Genbank accession number, DQ357071.1) was codon-optimized and synthesized by Genewiz (South Plainfield, NJ, USA; ESM 3). The Coum3H gene flanked by an *Nde*I and *Xho*I restriction sites was then subcloned into plasmid pRSFDuet-1 by *Nde*I and *Xho*I digestion followed by ligation of appropriate fragments. The resulting plasmid was named pCA5. The constructed plasmids were transformed into *E. coli* rpoA14 (DE3) with different combinations to generate strains RPC1, RPC2, RPC3, and RPC4. The description of strains and plasmids used in this study is summarized in Table 1. All enzymes used for cloning were purchased from New England Biolabs.

Analytical methods

For caffeic acid and coumaric acid quantification, 0.75 mL culture samples were extracted with ethyl acetate, air dried, and resuspended in 0.15 mL methanol containing 20 mg/L protocatechuic acid internal standard. Extract samples were then analyzed by an Applied Biosystems API2000 LC/MS/MS system equipped with a Waters symmetry 5 μm C8 column. Caffeic acid and coumaric acid were eluted using a linear gradient of 95 % acetonitrile and 5 % water (0 min) to 35 % acetonitrile and 65 % water (10 min) at a flow rate of 0.3 mL/min. For the quantification of L-tyrosine, cell-free culture supernatants were filtered through 0.2 μm pore-size polytetrafluoroethylene membrane syringe filters (VWR International) and 100 μL such-treated culture supernatant was mixed with 10 μL 1 g/L dopamine internal standard. Prepared samples were then analyzed by an Applied Biosystems API2000 LC/MS/MS system equipped with a Waters Spherisorb 5 μm C18 column. Tyrosine was eluted using an isocratic elution with 20 mM ammonium acetate (pH adjusted to 4 by formic acid) at a flow rate of 0.3 mL/min.

Bioreactor production

Twenty milliliters overnight LB culture of *E. coli* rpoA14 (DE3) harboring pCA1 and pCA2 (RPC1) was inoculated into a 2–L BioFlo110 modular fermentor system (New Brunswick Scientific) containing 1 L synthetic medium supplemented with appropriate antibiotics and 5 g/L glucose. The whole bioreactor vessel was wrapped with aluminum foil to avoid exposure of culture to light. Culture conditions were maintained as follows: air flow rate, 1 L/min; pH value, 7.6; temperature, 37 °C; and agitation, 350 rpm. Experimental samples were withdrawn every 24 h for OD_{600} measurements and product quantification.

Results

Caffeic acid tolerance of *E. coli*

In order to study the effect of caffeic acid on *E. coli* growth, strain rpoA14(DE3) was grown in LB medium supplemented with caffeic acid at final concentrations of 0, 10, 100, and 1,000 mg/L. After growth at 37 °C for 24 h, the cultures' cell densities (OD_{600}) were measured for comparison. As shown in Fig. 2, cell growth was slightly inhibited as caffeic acid concentration increased, though the presence of 1,000 mg/L caffeic acid only caused a 20 % final cell density drop, compared to the control culture without caffeic acid. The result of this assay shows that *E. coli* can tolerate high titer caffeic acid and thus has potential as a heterologous host for caffeic acid overproduction.

Caffeic acid production on glucose

In order to make caffeic acid in *E. coli*, a previously constructed tyrosine overproducer rpoA14(DE3) was transformed with plasmids pCA1 and pCA5, resulting in *E. coli* RPC1 carrying TAL and Coum3H genes (Fig. 1). Glucose was fed to the RPC1 culture at a final concentration of 5 g/L. After a 3-day cultivation at 37 °C, the engineered *E. coli* strain produced the expected caffeic acid product, as confirmed by LC/MS/MS analysis (ESM 4, Fig. S1). Therefore, a caffeic acid biosynthesis pathway was successfully reconstituted in *E. coli*, which was able to utilize inexpensive

Table 1 Plasmids and strains used in this study

Plasmid or strain	Description	Note	Source
Plasmid			
pTrcHis2B	trc promoter, pBR322ori, Amp^R		Invitrogen
pCDFDuet-1	two T7 promoters, CloDF13ori, Sp^R		Novagen
pCA1	pTrcHis2B carrying codon-optimized *R. glutinis* TAL	Relatively low copy number	Santos et al. (2011)
pCA2	pTrcHis2B carrying codon-optimized *R. glutinis* TAL and codon-optimized *P. crispus* 4CL	Relatively low copy number	Santos et al. (2011)
pCA3	pCDFDuet-1 carrying codon-optimized *R. glutinis* TAL with a trc promoter	Relatively high copy number	This study
pCA4	pCDFDuet-1 carrying codon-optimized *R. glutinis* TAL and codon-optimized *P. crispus* 4CL with trc promoters	Relatively high copy number	Santos et al. (2011)
pCA5	pRSFDuet-1 carrying codon-optimized *S. espanaensis* Coum3H	Very high copy number	This study
Strain			
rpoA14(DE3)	*E. coli* MG1655 ΔpheA ΔtyrR lacZ::$P_{LtetO-1}$–tyrA^{fbr}aroG^{fbr} tyrR::$P_{LtetO-1}$-tyrA^{fbr}aroG^{fbr} hisH(L82R) (DE3) carrying pHACM-rpoA14		Santos et al. (2011)
RPC1	rpoA14(DE3) containing pCA1 and pCA5	Low TAL copies	This study
RPC2	rpoA14(DE3) containing pCA2 and pCA5	Low TAL and 4CL copies	This study
RPC3	rpoA14(DE3) containing pCA3 and pCA5	High TAL copies	This study
RPC4	rpoA14(DE3) containing pCA4 and pCA5	High TAL and 4CL copies	This study

sugar substrate glucose to make high-value product caffeic acid.

We next set out to improve the caffeic acid production by a combination of different engineering approaches. First, a potential alternative route for making caffeic acid through coumaroyl-CoA and caffeoyl-CoA was introduced into *E. coli*. Specifically, a 4-coumarate:CoA ligase (4CL) was used to convert *p*-coumaric acid to coumaroyl-CoA, followed by Coum3H-mediated hydroxylation to make caffeoyl-CoA (Kneusel et al. 1989). De-esterification of caffeoyl-CoA to yield caffeic acid used *E. coli*'s native promiscuous thioesterase activity (Fig. 1).

Second, two sets of plasmids with relatively low and high copy numbers were constructed to vary TAL and 4CL gene doses for caffeic acid production. The transformation of these plasmids into the tyrosine overproducer rpoA14

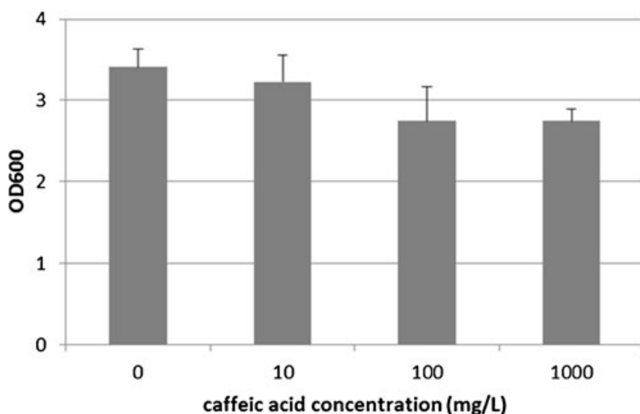

Fig. 2 *E. coli* growth inhibition by caffeic acid. *Error bars* represent the standard deviations of triplicate experiments

(DE3) yielded four *E. coli* strains, as listed in Table 1. Lastly, the constructed strains were grown in three different media, including a MOPS medium, a synthetic medium, and a rich medium, to further improve caffeic acid production on glucose.

Figure 3 shows caffeic acid, coumaric acid, and tyrosine production by different *E. coli* strains cultivated in the three media. As shown in Fig. 3a, caffeic acid was produced in *E. coli* RPC1, RPC3, and RPC4, but not in RPC2. The failure of caffeic acid biosynthesis in RPC2 was later found out by a qPCR analysis to be due to the lack of TAL and 4CL gene transcription (ESM 5 Fig. S2(a) and (b)). Similar phenomenon was observed when the same plasmid was used in our previous study, although the mechanism behind this is still unclear (Santos et al. 2011). Comparison of RPC3 and RPC4 clearly shows that addition of coumaroyl-CoA/caffeoyl-CoA biosynthesis route did not help improve the caffeic acid production on glucose. Instead, it introduced extra metabolic burden on the *E. coli* host and resulted in even lower production. Similarly, RPC1 produced more caffeic acid than RPC3 in all three media, suggesting that increasing copy number of TAL gene decreased the caffeic acid production due to the unnecessary metabolic burden. Overall, caffeic acid production in the rich medium was higher than in the other two media, which could be associated with higher cell density in rich medium as shown in Fig. 3d. In addition, glucose substrate was depleted in rich medium, but there was a big portion of un-used glucose in all *E. coli* strains grown in both the MOPS and synthetic media (data not shown). The results here indicate that engineered *E. coli* is better able to grow and thus make more caffeic acid on glucose in the rich medium.

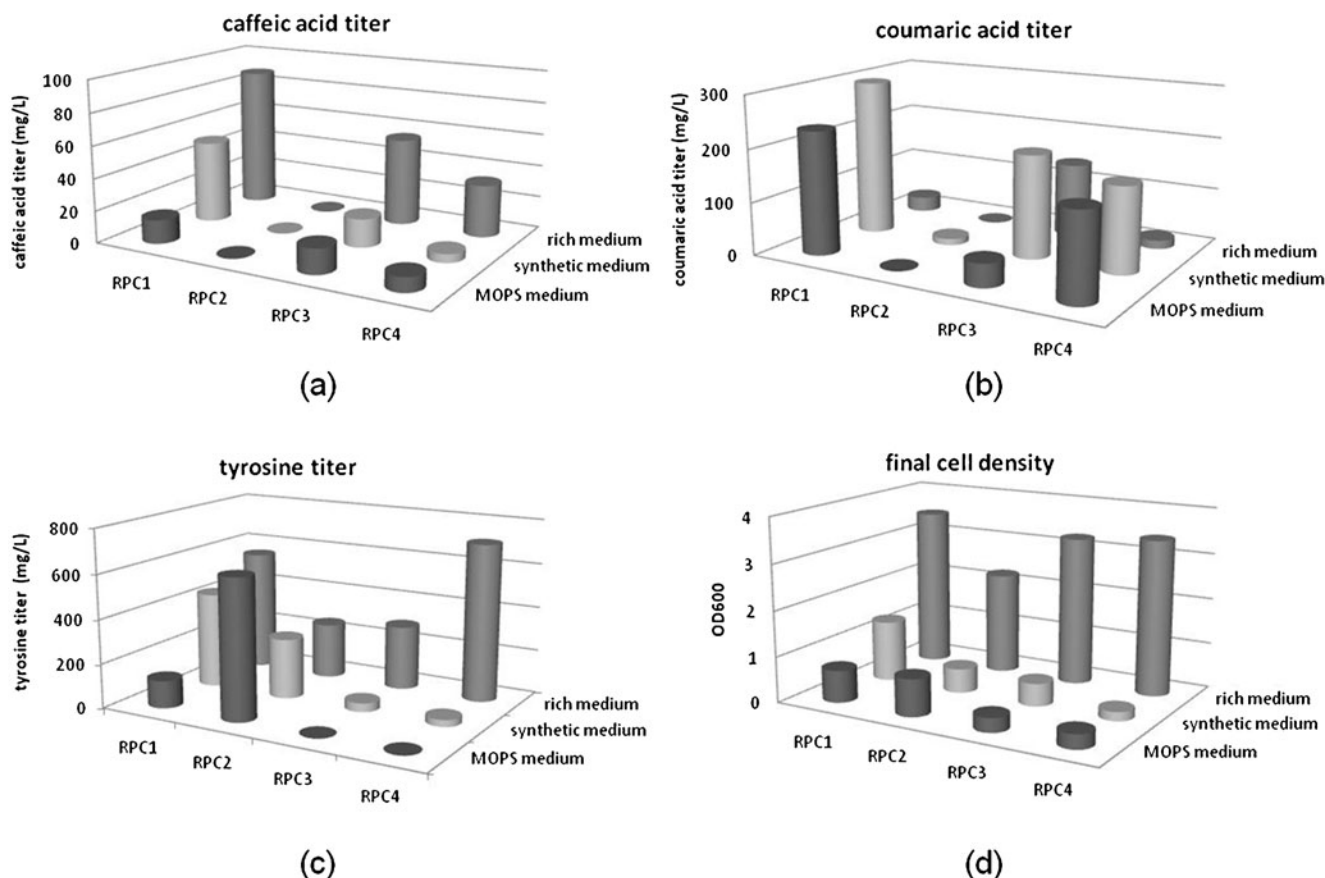

Fig. 3 Production of caffeic acid-related metabolites and cell growth of engineered *E. coli* grown on glucose. **a** Caffeic acid titer, **b** coumaric acid titer, **c** tyrosine titer, and **d** final cell density of the *E. coli* culture grown on 5 g/L glucose

Figure 3b shows that large amounts of coumaric acid was accumulated in the MOPS and synthetic media, which clearly suggests that, in these media, the production is limited by coumaric acid to caffeic acid conversion catalyzed by Coum3H. In contrast, there was significantly less coumaric acid accumulation in the rich medium, indicating that coumaric acid to caffeic acid conversion is higher in the rich medium. Notably, it was also found that a big portion of produced coumaric acid was released to the culture broth, in addition to the accumulation inside the cells. Among the four constructed strains, RPC1 produced the most coumaric acid in both the MOPS and synthetic media.

Tyrosine titers are shown in Fig. 3c. It should be noted that the rich medium contains yeast extract and thus contains around 220 mg/L tyrosine. RPC2 was found to produce a large amount of tyrosine, but it could not further use it to make coumaric acid. In the MOPS and synthetic media, RPC3 and RPC4 produced similarly low levels of tyrosine. In comparison, RPC1 made more tyrosine than RPC3 and RPC4, which translates into high caffeic acid production.

The highest caffeic acid titer of 88 mg/L was achieved in strain RPC1 grown in the rich medium. The highest specific titer, defined as caffeic acid titer divided by cell density, reached 38 mg/L/OD in RPC1 grown in the synthetic medium. These results imply that engineered *E. coli* is more efficient for making caffeic acid in the synthetic medium than in the rich medium. In addition, RPC1 was the best producer among all four strains grown on glucose.

Caffeic acid production on xylose

Xylose is an important component of renewable biomass feedstocks. Its utilization for producing industrially valuable chemicals is of current research interest. As *E. coli* natively is able to consume xylose, we studied whether xylose can be used by our engineered *E. coli* strains to make caffeic acid. Similar to production on glucose, strains containing different gene copy numbers were grown in three media supplemented with xylose to optimize caffeic acid production.

Figure 4a shows that the rich medium produced high concentrations of caffeic acid in all four strains except RPC2. Notably, RPC3 produced a slightly higher level of caffeic acid than RPC1, whereas RPC1 produce a higher level of caffeic acid than RPC3 when grown on glucose. It is therefore suggested that the effect of increasing TAL gene copy number is sugar dependent. However, mRNA

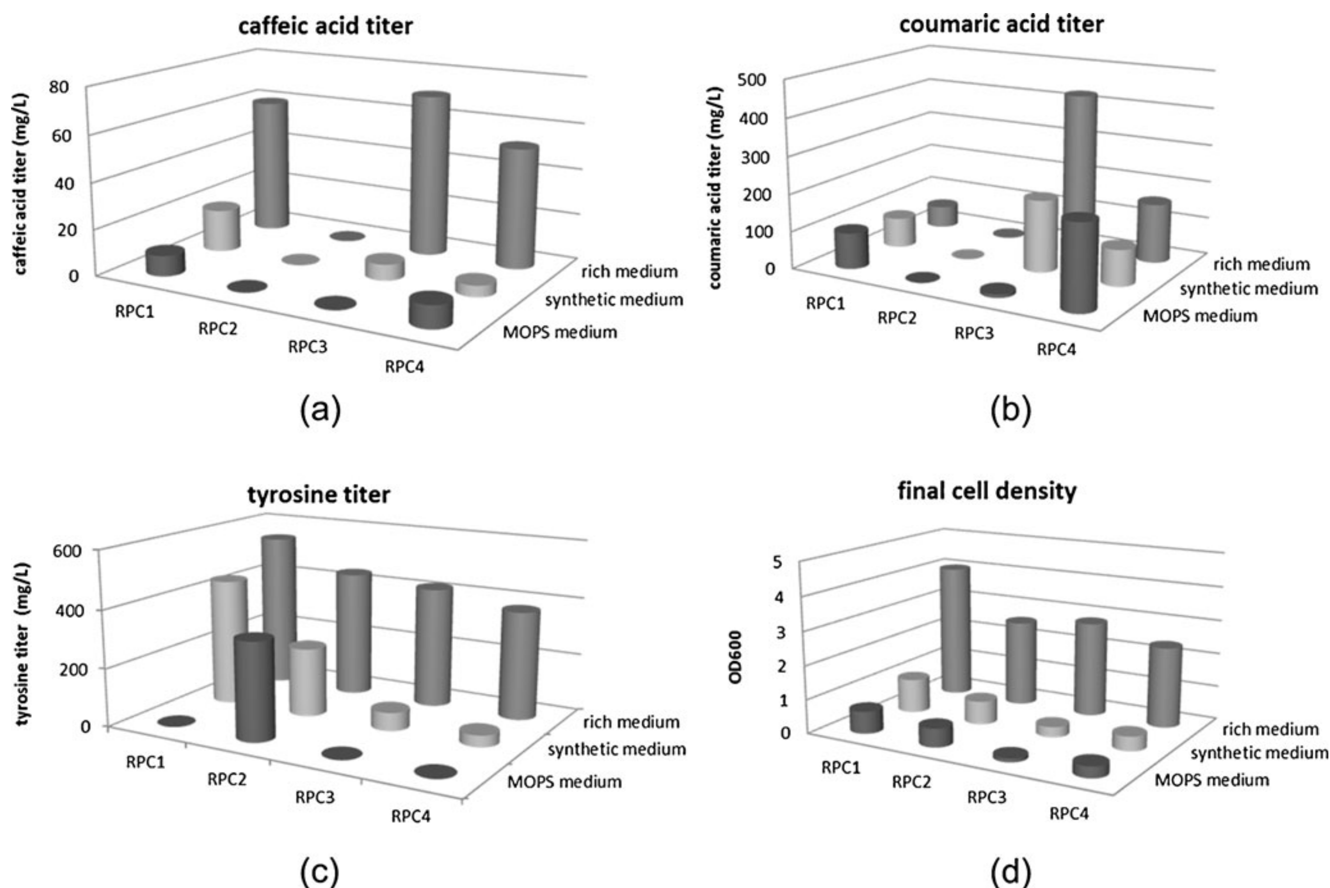

Fig. 4 Production of caffeic acid-related metabolites and cell growth of engineered *E. coli* grown on xylose. **a** Caffeic acid titer, **b** coumaric acid titer, **c** tyrosine titer, and **d** final cell density of the *E. coli* culture grown on 5 g/L xylose

quantification analysis showed that the use of different sugars did not have a significant impact on TAL gene transcription (ESM 5, Fig. S2(a)). Therefore, the improvement of caffeic acid production on xylose in RPC3 did not directly result from upregulation of TAL gene transcription. In the case of synthetic medium, RPC1 produced the most caffeic acid, whereas in the MOPS medium RPC1 and RPC4 produced similarly level of caffeic acid. Generally, caffeic acid production on xylose was lower than on glucose.

As shown in Fig. 4b, engineered *E. coli* strains, except RPC2, accumulated high titers of intermediate coumaric acid in all three media. However, coumaric acid accumulated in these media was not well converted to caffeic acid, since intermediate coumaric acid concentrations were much higher than final product caffeic acid. Figure 4c further shows that RPC1 made much more tyrosine in the synthetic medium than in the MOPS medium. Similar results were observed for RPC3 and RPC4. RPC2 was able to produce high level of tyrosine but not coumaric acid and caffeic acid. Similar to growth on glucose, cell growth on xylose in the rich medium was much better than in the other two media, as shown in Fig. 4d. The highest caffeic acid titer of 70 mg/L and the highest specific titer of 25 mg/L/OD were both achieved in strain RPC3 grown in the rich medium. Overall, the caffeic acid production on glucose was better than on xylose because glucose is a better carbon source for *E. coli* metabolism.

Bioreactor production

After establishing caffeic acid production in test tubes, we next evaluated production in a 2-L bioreactor. Glucose was chosen as the substrate as it produced a higher level of caffeic acid than xylose. *E. coli* RPC1 was the best producer on glucose, and thus it was used in this bioreactor production study. The synthetic medium was chosen as the preferred production medium (a) because it contains only inexpensive components and thus is economically attractive, and (b) because the highest specific production of 38 mg/L/OD was achieved in the synthetic medium. Although the cell density was low in test tubes, using bioreactor technique to increase cell density was expected to further boost the caffeic acid titer.

As shown in Fig. 5, production of caffeic acid was detected at day 1 and peaked at day 4. A gradual decrease

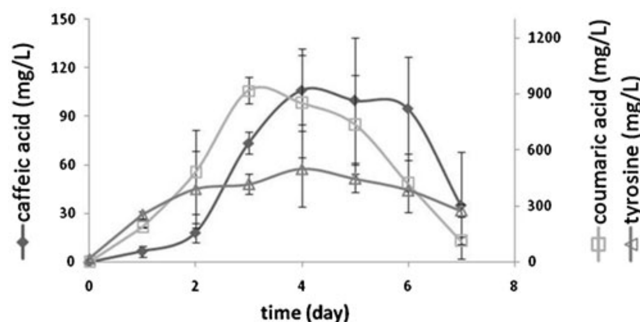

Fig. 5 Bioreactor production of caffeic acid. *Error bars* represent the standard deviations of duplicate bioreactor runs

in caffeic aid titer was observed after day 4, which should be associated with the formation of undesired *o*-quinone and its polyaromatic derivatives. The highest OD value of the bioreactor culture was 1.7, higher than the test tube culture (data not shown). The peak caffeic acid titer was 106 mg/L, in comparison with 51 mg/L achieved in a test tube in the same medium. The highest specific production of 75 mg/L/OD at day 4 was also 97 % higher than the result of the shake flask experiment. The production profiles of coumaric acid and tyrosine followed the same trend with that of caffeic acid. Notably, the cell accumulated high levels of these two compounds during the fermentation. In particular, coumaric acid titer was one order of magnitude higher than caffeic acid, highlighting the need to increase conversion of this intermediate to the final product.

Discussion

High level caffeic acid biosynthesis is an attractive research subject due to the valuable pharmacological properties of this aromatic compound. In the present study, *E. coli* was selected as a surrogate biosynthesis host due to its established advantages for heterologous natural product production (Zhang et al. 2008). A caffeic acid biosynthesis pathway was reconstituted in engineered *E. coli* and successfully converted sugar-derived tyrosine to caffeic acid by using a tyrosine ammonia lyase (TAL) and a 4-coumarate 3-hydroxylase (Coum3H). However, introduction of the alternative biosynthesis route through coumaroyl-CoA and caffeoyl-CoA did not increase production. It was therefore suggested that only the biosynthesis route involving TAL and Coum3H was active for caffeic acid production in the constructed *E. coli*. The failure of the alternative route could be due to an inactive or low activity of an *E. coli* thioesterase against caffeoyl-CoA. To further investigate the utility of this biosynthesis route, improving *E. coli* native thioestarase activity or introducing a heterologous caffeoyl-CoA thioestarase activity should be conducted.

Varying copy numbers of TAL and 4CL genes resulted in altered caffeic acid production on both glucose and xylose. Low copies of TAL gene was found to be sufficient for high titer production, whereas introduction of additional 4CL gene or increasing TAL gene copy number decreased the production in most cases, implying that a balanced biosynthetic gene copy number is crucial for final product biosynthesis. Strain RPC2 containing low copies of TAL and 4CL genes did not produce caffeic acid due to the lack of the corresponding gene transcription. A similar finding was reported in our previous study (Santos et al. 2011), yet the mechanism of this effect is not clear. Notably, although large amounts of tyrosine and coumaric acid were accumulated in all engineered strains, caffeic acid production remained relatively low. These results therefore suggest that Coum3H activity needs to be enhanced in order to improve caffeic acid biosynthesis from coumaric acid. Since the copy number of CoumH gene used in this study was high (~100 copies per cell), fine-tuning Coum3H gene copy number or utilizing Coum3H from other organisms with higher 4-coumarate 3-hydroxylase activity should be pursued to increase the coumaric acid utilization in future studies.

The rich medium was found to make the highest titer of caffeic acid because its rich nutrient content helped improve cell growth. However, since the carbon source in the rich medium was mainly consumed for the cell growth, instead of caffeic acid production, specific production level in the rich medium was lower than the synthetic medium which contained only defined salts and glucose. Among the three media, the MOPS medium showed the lowest caffeic acid production. This can be explained by the fact that it was not supplemented with trace elements and thus lacked needed metal cofactors for heterologous enzyme activity. For example, 4-coumarate 3-hydroxylase has been reported to require copper to carry out the enzymatic reaction (Nambudiri and Bhat 1972), but the MOPS medium did not contain sufficient copper to support the enzyme activity.

Caffeic acid production was further improved by cultivating RPC1 cell in a bioreactor containing the synthetic medium and glucose. Better pH control and oxygen supply were considered to help the cell growth and finally improve the caffeic acid titer to 106 mg/L, which is higher than any previous studies. However, undesired formation of *o*-quinone and its derivative led to a decrease in caffeic acid titer after a 4-day fermentation. For future studies, oxidation of caffeic acid and formation of polyaromatics need to be reduced in order to prevent the loss of caffeic acid product. In addition, metabolic engineering of the above identified pathway steps could further improve the caffeic acid heterologous production in *E. coli*.

Acknowledgments This study was supported by ARPA-E (DE-AR0000059) provided by US Department of Energy and the Singapore MIT Alliance.

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