Electronic Journal of Biotechnology 38 (2019) 19–26

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Review

Metabolic engineering strategies for caffeic acid production in *Escherichia coli*

Georgina Hernández-Chávez, Alfredo Martinez, Guillermo Gosset *

Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Morelos 62250, Mexico

ARTICLE INFO

Article history:
Received 23 July 2018
Accepted 20 December 2018
Available online 29 December 2018

Keywords:
Aromatics
Biosynthesis of caffeic acid
Caffeic acid
Engineered microorganisms
Escherichia coli
Hydroxycinnamic acids
Industrial biotechnology
Metabolic engineering
Natural products
p-Coumaric acid
Phenylpropanoid pathway

ABSTRACT

Caffeic acid (CA; 3,4-dihydroxycinnamic acid) is an aromatic compound obtained by the phenylpropanoid pathway. This natural product has antioxidant, antitumor, antiviral, and anti-inflammatory activities. It is also a precursor of CA phenethyl ester (CAPE), a compound with potential as an antidiabetic and liver-protective agent. CA can be found at low concentrations in plant tissues, and hence, its purification is difficult and expensive. Knowledge regarding the pathways, enzymes, and genes involved in CA biosynthesis has paved the way for enabling the design and construction of microbial strains with the capacity of synthesizing this metabolite. In this review, metabolic engineering strategies for the generation of *Escherichia coli* strains for the biotechnological production of CA are presented and discussed.

How to cite: Hernández-Chávez G, Martinez A, Gosset G. Metabolic engineering strategies for caffeic acid production in *Escherichia coli*. Electron J Biotechnol 2019;38. https://doi.org/10.1016/j.ejbt.2018.12.004.

Contents

1. Introduction

Hydroxycinnamic acids (mainly *p*-coumaric, sinapic, ferulic, and caffeic acids [CA]) are a group of compounds within the phenolic acids characterized by their important antioxidant properties (Fig. 1A) [1]. CA (3-(3,4-dihydroxyphenyl)-2-propenoic acid) is present in a wide variety of plants and fruits (Fig. 1B). Importantly, abundance of CA has been reported in citrus; berries; peel of vegetables such as potatoes; bran cereals; seeds such as sunflower seed; or herbs such as sage, oregano, and spearmint [2,3]. CA is related to quinic acid in chlorogenic acid (5-O-caffeoylquinic acid), present in coffee [4], and its CA phenethyl ester (CAPE) is present in abundance in honeybee propolis [5] (Fig. 1C). These esters have shown important anticancer, antioxidant, and antiviral activities [6].

* Corresponding author.
E-mail address: gosset@ibt.unam.mx (G. Gosset).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.12.004

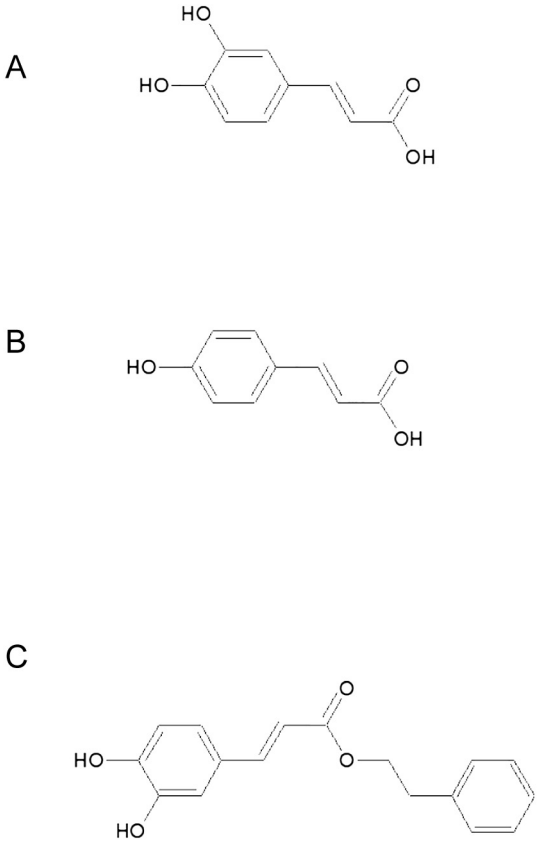

Fig. 1. Chemical structures of caffeic acid (A), *p*-coumaric acid (B), and caffeic acid phenethyl ester (CAPE) (C).

Hydroxyl groups in CA and its phenyl ethyl ester analog, in association with the carbonyl moiety, contribute importantly to the antioxidant activity [7]. Their relative positions in para- and ortho- within the aromatic ring add to the stabilization of the resonance energy in the phenolic acid [8]. This effect is more remarkable in CA and its phenyl ethyl ester analog, with two hydroxyl substitutions, compared to their mono hydroxyl analog ferulic acid [8]. Additional to the hydroxy substituents, the unsaturated side chain contributes to structure stabilization by electron delocalization [9]. The overall structure also makes it an appropriate chelating molecule, which can coordinate oxidizing metals [10].

Antioxidant properties of CA allow diverse applications in food, cosmetic, and medical industries, either as an additive to prevent the oxidation of components in cosmetic formulations or as a key ingredient in some other products, where it is present as either a free acid or a phenyl ethyl ester [11]. Oxidation of components in commercial products leads to changes in appearance, color, or homogeneity and can be an important asset for their commercialization [12,13]. Because of the limited solubility of CA in water, products derived from its basic structure have been produced [14]. Purification of CA from plants is mainly performed by solvent extraction and column chromatography, which yields very low amounts [15] mainly because of the extremely low accumulation in plants and poor method yields. Caffeic acid can also be obtained through chlorogenic acid deesterification, generating quinic acid as a side product [11].

CA is an intermediate in plant phenylpropanoid metabolism [16]. In plants, the synthesis of CA starts with a deamination reaction of the amino acid L-phenylalanine catalyzed by the enzyme phenylalanine ammonia lyase (PAL) to yield cinnamic acid [17]. This compound is hydroxylated at the fourth position of the benzyl ring to generate *p*-coumaric acid in a reaction catalyzed by cinnamate 4-hydroxylase (C4H) and hydroxylated again at the third position by the enzyme *p*-coumarate 3-hydroxylase (C3H) to generate CA. Both C3H and C4H are cytochrome P450-dependent monooxygenases [18].

2. Microbial production of caffeic acid

The aromatic compounds are a diverse class of chemicals. These compounds are generated in nature mostly by plants and as a result of human industrial activity. The carbon from these compounds is recycled mainly by microorganisms through diverse degradation strategies. One of the strategies involves a hydroxylation step that increases solubility and activates the compound for subsequent degradation steps [19]. Some of these enzymes can employ *p*-coumaric acid as the substrate and yield CA as the product (Fig. 2). The hydroxylation of *p*-coumaric acid is considered a rare occurrence in microorganisms [20]. However, the conversion of *p*-coumaric acid to CA has been reported for several microbial species including the filamentous fungi *Gliocladium deliquescens* and the bacterium *Streptomyces griseus* [21]. An enzyme displaying *p*-coumaric acid hydroxylation activity was purified from *Streptomyces nigrifaciens*. This enzyme was shown to hydroxylate several monophenols at an optimal temperature of 40°C [20]. Although this report was not specifically focused on CA production, the enzyme from *S. nigrifaciens* can be used in a conversion process for generating CA in vitro or in vivo.

The white rot fungus *Pycnoporus cinnabarinus* is a natural CA producer. This organism was employed to develop a production process for the transformation of *p*-coumaric acid to CA. The toxicity of *p*-coumaric acid was avoided by feeding this substrate and maintaining a concentration in the culture medium lower than 500 mg/L. In a complex culture medium with yeast extract and glucose, 257 mg/L of CA was produced with a 21% molar yield from *p*-coumaric acid [22]. Another example of a biotechnological process for CA production was based on the actinomycete *Streptomyces caeruleus*. The best culture conditions were observed when this organism was pregrown in nutrient broth, which resulted in the synthesis of 150 mg/L of CA from 5 mM *p*-coumaric acid in 96 h with a 16.6% molar yield [23].

3. Production of caffeic acid with engineered microorganisms

The examples described above illustrate the feasibility of generating biotechnological processes to produce CA by employing microorganisms, as they are found in nature. With the application of metabolic engineering strategies, it is now possible to enhance the production capacity of some microorganisms to synthesize molecules that are not part of their metabolic repertory. Furthermore, these techniques enable the transfer of genetic information between species, thus allowing the heterologous expression of genes encoding the key enzymes for CA production to microorganisms that cannot produce this compound. In addition, the molecular tools applied for genetic engineering can also be employed to modify protein functions, thus enabling the engineering of enzymes to enhance their catalytic activity. The biosynthesis of CA by recombinant strains can proceed from the conversion of the direct precursor *p*-coumaric acid, from L-tyrosine, or from simple carbon sources such as glucose. Each of these approaches has specific advantages and technical challenges, which are discussed in the following examples.

3.1. Production of caffeic acid from p-coumaric acid

The generation of an engineered *Escherichia coli* strain with the capacity of converting *p*-coumaric acid to CA requires the expression of genes encoding an enzyme with hydroxylating activity toward the substrate. The cytochrome P450 monooxygenase CYP199A2 from *Rhodopseudomonas palustris* displays oxidation activity for aromatic carboxylic acids [24]. To express this enzyme in *E. coli*, the *CYP199A2* gene was cloned into the vector pET21a under the transcriptional

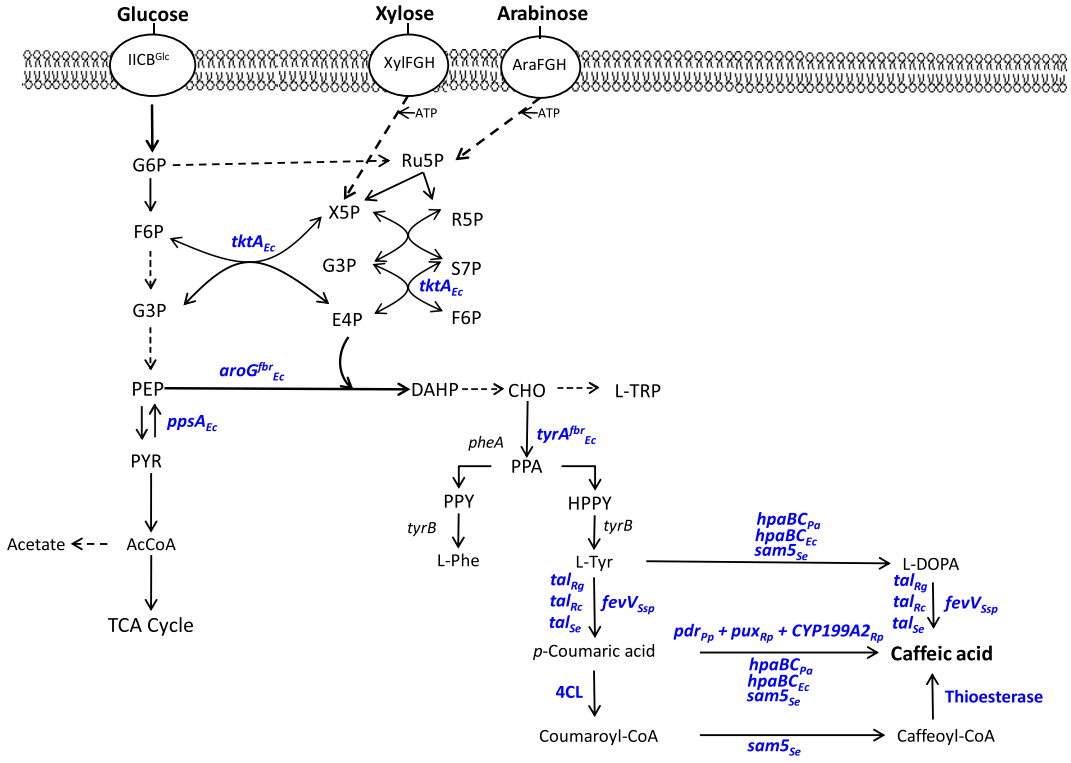

Fig. 2. Central metabolism, biosynthetic pathways, and transport pathways of aromatic compounds in engineered *E. coli*. Dashed arrows indicate multiple enzyme reactions. EI, PTS enzyme I; HPr, PTS phosphohistidine carrier protein; EIIA, PTS glucose-specific enzyme II; PTS IICBGlc, integral membrane glucose permease; GalP, galactose permease; XylFGH, xylose transport proteins, AraFGH, arabinose transport proteins; DAHPS, DAHP synthase; *aroG*^{fbr}, gene encoding a feedback inhibition-resistant mutant version of DAHPS; TktA, transketolase; TyrB, tyrosine aminotransferase gene; PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamate 4-hydroxylase; AaeXAB, efflux pump from *E. coli*; SprABC, efflux pump from *P. putida*; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; X5P, xylulose-5-phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; TCA, tricarboxylic acid.

control of the T7 promoter and induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG). To provide the redox partners for CYP199A2, the genes encoding putidaredoxin reductase (Pdr) from *Pseudomonas putida* and palustrisredoxin (Pux) from *R. palustris* were coexpressed [24] (Fig. 2). A recombinant *E. coli* strain expressing these three genes converted 1 mM *p*-coumaric acid to CA in 240 min. In an effort to improve this production system, protein engineering was employed to generate CYP199A2 mutants. The crystal structure of CYP199A2 is known; on the basis of this information, the amino acid residue L-phenylalanine at position 185 (F185) was identified as important in substrate binding [25]. Employing site-directed mutagenesis, F185 was replaced by nine different amino acid residues. It was determined that the mutant F185L, having L-phenylalanine replaced by L-leucine, displayed a 5.5-fold higher rate of *p*-coumaric hydroxylation, than the wild-type CYP199A2. In shake flask cultures of *E. coli* expressing the F185L mutant with either glucose or glycerol as energy sources, 20 mM *p*-coumaric acid was converted to 15 mM CA (2.8 g/L) in 24 h. In contrast, cells expressing the wild-type CYP199A2 produced 4.3 mM CA under the same conditions [26]. This work illustrates the value of applying protein engineering approaches to improve enzyme catalytic activity, which results in a 3.5-fold increase in CA titer (Table 1). Several microorganisms have the natural ability to degrade aromatic compounds. It has been observed that some of them have in their chromosome genes encoding the two subunits of the enzyme 4-hydroxyphenlacetate 3-hydroxylase (4HPA3H). This protein is a two-component flavin-dependent monooxygenase that participates in the catabolism of organic compounds [27]. In another study, the catalytic activity toward cinnamic acid derivatives was investigated with the enzyme 4HPA3H from *Pseudomonas aeruginosa* PAO1 expressed in *E. coli*. It was determined that this enzyme can employ 4-hydroxyphenlacetate, *p*-coumaric acid, tyrosol, 3-(4-hydroxyphenyl)propanoic acid, ferulic acid, and coniferaldehyde as substrates. The recombinant *E. coli* strain expressing 4HPA3H from *P. aeruginosa* PAO1 was employed to develop a process to produce CA. It was determined in shake flask experiments that *p*-coumaric acid at concentrations of 40 and 100 mM inhibited CA production by 36% and 95%, respectively. Therefore, the production of CA was evaluated by repeated feeding of 20 mM *p*-coumaric acid. By following this scheme and using a culture medium containing glycerol as the energy source, 56.6 mM CA (10.2 g/L) was produced after repeated addition of substrate in 24 h [28]. This represents the highest CA titer produced from *p*-coumaric acid.

3.2. Production of caffeic acid from L-tyrosine

Production of CA from L-tyrosine avoids the use of *p*-coumaric acid as a substrate but requires an additional deamination step that should be expressed in the production host [29]. This scheme was evaluated in *E. coli* by employing the enzymes tyrosine ammonia lyase (TAL) from *Rhodotorula glutinis* and either C3H from *Saccharothrix espanaensis* or cytochrome P450 CYP199A2 from *R. palustris*. The genes encoding these enzymes were synthesized and codon-optimized for *E. coli*. The first part of such study consisted in determining conditions for the production of *p*-coumaric acid from L-tyrosine. This was evaluated by expressing the TAL gene from *R. glutinis* in plasmids with distinct copy numbers. The highest *p*-coumaric acid production, i.e., 2.62 mM (472 mg/L), was observed when employing the high-copy number plasmid pRSFDuet-1. Production of CA from L-tyrosine was evaluated by expressing genes encoding TAL and C3H. Each of the two genes was expressed individually in plasmids, transcribed from either the T7 or the P_{tet} promoters. Several plasmids with dissimilar copy numbers were employed, thereby enabling the identification of the best combination to express these two enzymes. The study reported the highest CA titer, i.e., 1 mM (180 mg/L), using

Table 1
Production of caffeic acid with engineered *E. coli* strains.

Promoter	Inducer	Gene(s)	Organism	Precursor compound	Carbon source	Process temperature	Caffeic acid titer	Ref.
T7	IPTG 1 mM	*CYP199A2*	*R. palustris*	*p*-Coumaric acid	Glycerol	30°C	15 mM (2800 mg/L)	[26]
T7		*pdR*	*P. putida*					
T7		*pux*	*R. palustris*					
T7	IPTG 1 mM	*hpaB hpaC*	*P. aeruginosa* PAO1	*p*-Coumaric acid	Glycerol	30°C	56.6 mM (10,200 mg/L)	[28]
*dnaK*p	48°C, 5 min	*CYP199A2*	*R. palustris*	*p*-Coumaric acid	Glucose	37°C	2 μM (0.37 mg/L)	[39]
*dnaK*p		*pdR*	*P. putida*					
*dnaK*p		*pux*	*R. palustris*					
T7	IPTG 1 mM	*tal*	*R. glutinis*	L-Tyrosine	Glucose	26°C	1.56 mM (280 mg/L)	[29]
T7		*CYP199A2*	*R. palustris*					
P_{tet}	aTC[a] 100 ng/mL	*pdR*	*P. putida*					
P_{tet}		*pux*	*R. palustris*					
$P_L$lacO1	IPTG 0.2 mM	$aroG^{fbr}$	*E. coli*	Not added	Glucose + glycerol	30°C	0.28 mM (50.2 mg/L)	[32]
$P_L$lacO1		$tyrA^{fbr}$	*E. coli*					
$P_L$lacO1		*ppsA*	*E. coli*					
$P_L$lacO1		*tktA*	*E. coli*					
$P_L$lacO1		*tal*	*R. capsulatus*					
$P_L$lacO1		*hpaB hpaC*	*E. coli* MG1655					
T7	IPTG 1 mM	$aroG^{fbr}$	*E. coli*	Not added	Glucose	26°C	0.84 mM (150 mg/L)	[30]
T7		$tyrA^{fbr}$	*E. coli*					
T7		*tal*	*S. espanaensis*					
T7		*sam5*	*S. espanaensis*					
		Δ*tyrR*	*E. coli*					
$P_{LtetO-1}$	Constitutive	$aroG^{fbr}$	*E. coli*	Not added	Glucose	37°C	0.59 mM (106 mg/L)	[34]
$P_{LtetO-1}$	Constitutive	$tyrA^{fbr}$	*E. coli*					
P_{trc}	IPTG 0.1 mM	*tal*	*S. espanaensis*					
T7	IPTG 0.1 mM	*sam5*	*S. espanaensis*					
$P_L$lacO1	IPTG 0.2 mM	$aroG^{fbr}$	*E. coli*	Not added	Glucose + glycerol	37°C	4.28 mM (766.7 mg/L)	[35]
$P_L$lacO1		$tyrA^{fbr}$	*E. coli*					
$P_L$lacO1		*ppsA*	*E. coli*					
$P_L$lacO1		*tktA*	*E. coli*					
$P_L$lacO1		*hpaB hpaC*	*E. coli* MG1655					
$P_L$lacO1		*tal*	*R. glutinis*					
		Δ*pheLA-tyrA*	*E. coli*					
T7	IPTG 0.08 mM	$aroG^{fbr}$	*E. coli*	Not added	Cellulose from kraft pulp[b]	26°C	1.3 mM (233 mg/L)	[38]
T7		$tyrA^{fbr}$	*E. coli*					
T7		*ppsA*	*E. coli*					
T7		*tktA*	*E. coli*					
T7		*fevV*	*Streptomyces* sp. WK-5344					
T7		*hpaB hpaC*	*P. aeruginosa* PAO1					
		Δ*tyrR*	*E. coli*					

[a] Anhydrotetracycline.
[b] Kraft pulp was hydrolyzed with Cellic CTec2 cellulose at 2.5 FPU/g glucan for 6 h at 45°C and then added to the culture medium.

genes encoding TAL and C3H activities. The use of the enzyme CYP199A2 from *R. palustris* was evaluated for producing CA from *p*-coumaric acid. The redox partners Pdr and Pux were expressed simultaneously with CYP199A2. Production of CA from L-tyrosine was evaluated by employing TAL from *R. glutinis* and CYP199A2 with its redox partners in different genetic configurations. From this study, it was determined that the expression of the genes individually, as opposed to operons, provides better results, leading to the production of 1.56 mM CA (280 mg/L). It was also concluded that higher CA production is obtained with CYP199A2 and its redox partners than with C3H. In the previous studies, it was determined that titers of CA were lower at 30 or 37°C, with 26°C being the best temperature for production. A general trend was found in this study, where high copy numbers of plasmids correlated with increased product titer [29].

3.3. Production of caffeic acid from glucose and glycerol

The generation of an engineered *E. coli* strain with the ability to convert a simple carbon source such as glucose to CA involves genetic modifications that increase carbon flow to the L-tyrosine biosynthetic pathway, as well as the expression of genes encoding enzymes catalyzing deamination and hydroxylation reactions. To construct such a strain, the gene *tal* encoding the enzyme TAL from the actinomycete *S. espanaensis* was obtained. Further, codon optimization of *tal* was performed by employing the GeneGPS™ program from ATUM (https://www.atum.bio/), and the resulting encoded enzyme was designated opTAL. The genes encoding TAL and opTAL from *S. espanaensis* were cloned in the plasmid pET-28a(+) (Novagen) and placed under the transcriptional control of the T7 promoter. Production of *p*-coumaric acid was determined in recombinant *E. coli* strains expressing either one of these proteins; it was found that the expression of opTAL caused a twofold to fourfold increase in the product (144 mg/L) when compared to that of TAL [30]. Culture conditions were M9 minimal medium with 15 g/L glucose and 1 mM IPTG. The gene *sam5* encoding C3H from *S. espanaensis* was expressed in a plasmid as an operon, with the genes encoding either TAL (pET-T5) or opTAL (pET-opT5). *E. coli* strains transformed with pET-T5 and pET-opT5 produced CA at titers of 42 and 14 mg/L, respectively [30]. Unexpectedly, the titer of CA did not improve in the strain expressing opTAL. This result can be explained considering that C3H activity might be limiting in such strains. To improve the current production microbial system, a strategy was followed to construct an L-tyrosine overproducer strain. The modifications included disruption of the *tyrR* gene encoding the TyrR repressor, as well as expression of the genes $aroG^{fbr}$ and $tyrA^{fbr}$ encoding feedback inhibition-resistant mutant versions of the enzymes 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase ($AroG^{fbr}$) and chorismate mutase/prephenate dehydrogenase ($TyrA^{fbr}$), respectively. The genes $aroG^{fbr}$ and $tyrA^{fbr}$ were placed under the control of the T7 promoter in a medium-copy number plasmid. When transformed with the plasmids pET-T5 and pET-opT5, the

resulting strains produced 974 and 805 mg/L of *p*-coumaric acid from glucose, respectively. When these strains with the plasmids pET-T5 and pET-opT5 also expressed the gene encoding C3H, 150 and 40 mg/L of CA were produced, respectively [30]. Although the amount of CA was higher in these strains than in the non-L-tyrosine overproducer counterparts, the improvement was not proportional to that observed for *p*-coumaric acid. It can be observed that approximately 90% of the produced *p*-coumaric acid is not converted to CA in these strains. These results clearly indicate that C3H activity is a limiting factor in CA production.

It has been determined that some *E. coli* strains have in their chromosome the genes *hpaB* and *hpaC*, which encode the two subunits of 4HPA3H. These enzymes were evaluated for generating CA from *p*-coumaric acid. This scheme would avoid the use of cytochrome P450-dependent monooxygenases, as these plant membrane proteins have been reported to display low activity when expressed in bacterial systems [31]. It was determined that 4HPA3H from *E. coli* MG1655 can employ *p*-coumaric acid as the substrate to generate CA [31]. This enzyme can also convert L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). The authors also determined that the enzyme TAL from *Rhodobacter capsulatus* (*Rc*TAL) can accept L-tyrosine and L-DOPA as substrates and generate *p*-coumaric acid and CA, respectively. On the basis of these results, a CA-producing strain was generated by expressing in *E. coli* the genes encoding *Rc*TAL and 4HPA3H from *E. coli* MG1655. This scheme would constitute a dual pathway for the de novo synthesis of CA. The CA-producing strain was further modified to increase L-tyrosine synthesis capacity by expressing feedback inhibition-resistant mutant versions of genes encoding TyrA and DAHP synthase, as well as wild-type versions of genes encoding phosphoenolpyruvate synthase (*ppsA*) and transketolase (*tktA*). A strain with such phenotype produced 50.2 mg/L of CA after 48 h of fermentation in M9 minimal salt medium with 10 g/L glycerol and 2.5 g/L glucose. It should be noted that a relatively large amount of both L-DOPA (75.3 mg/L) and L-tyrosine (25.1 mg/L) accumulated in the culture medium, which indicates that *Rc*TAl activity is a limiting factor [32].

In another report, an *E. coli* strain with the capacity of synthesizing CA from glucose was generated by employing an L-tyrosine overproducer with the following genetic modifications: Δ*pheA* Δ*tyrR* *lacZ*::$P_{LtetO-1}$–$tyrA^{fbr}$ $aroG^{fbr}$ *tyrR*::$P_{LtetO-1}$–$tyrA^{fbr}$ $aroG^{fbr}$ *hisH* (L82R) [33]. It should be noted that this strain was constructed by inserting in the chromosome two copies of the genes $tyrA^{fbr}$ and $aroG^{fbr}$ under the control of constitutive promoters. This strain was transformed with combinations of plasmids expressing the codon-optimized genes RgTAL, Pc4CL, and *sam5* encoding TAL from *R. glutinis*, 4-coumarate:CoA ligase from *Petroselinum crispus*, and C3H from *S. espanaensis*, respectively. In addition to the generation and characterization of CA-producing strains by following the schemes described above, a novel biosynthetic route was evaluated consisting on the 4CL-dependent conversion of *p*-coumaric acid to coumaryl-CoA, followed by its hydroxylation by C3H to generate caffeoyl-CoA. This compound would be de-esterified to CA by the thioesterase activity of a native *E. coli*.

A combinatorial study in shake flasks was performed by varying the expression levels of the heterologous genes using plasmids with either low or high copy numbers and growing the recombinant strains in three different culture media. It was determined that the expression of the coumaroyl-CoA/caffeoyl-CoA route in a strain that also expresses the RgTAL/C3H route does not result in increased CA production. This result can be explained by the high metabolic burden caused by the simultaneous expression of both biosynthetic routes. This result can also be explained assuming a low activity of the native *E. coli* thioesterase against caffeoyl-CoA. A potential solution to this issue would be overexpression of the native on a heterologous caffeoyl-CoA thioesterase. Additionally, a negative effect on production, related to metabolic burden, was detected in some of the strains carrying a high-copy number plasmid. These results highlight the importance of fine-tuning gene expression as a strategy to optimize strain performance. Bioreactor production of CA was evaluated with a strain expressing RgTAL and C3H in synthetic medium with glucose as the carbon source. The highest CA titer (106 mg/L) was detected at day 4; however, a decrease in the concentration of this product was observed starting at that point, with a level of 40 mg/L detected at day 7. It is assumed that CA is lost by its oxidative conversion to *o*-quinone, which can result in the formation of polyaromatic derivatives. This is an important issue that needs to be addressed to improve productivity. It should be noted that in these cultures, *p*-coumaric acid accumulated at a level tenfold higher than the CA level. This result indicates that C3H activity should be increased to improve CA yield [34].

The catalytic capacity to convert *p*-coumaric acid to CA was compared by employing 4HPA3H enzymes from *E. coli* MG1655 and *Thermus thermophilus* HB8. The genes encoding these enzymes were amplified by PCR and cloned in an expression vector. When feeding *p*-coumaric acid to *E. coli* strains expressing either of these two enzymes, it was determined that 4HPA3H from *E. coli* MG1655 was twofold more active with this substrate than the protein from *T. thermophilus*. The strain expressing 4HPA3H from *E. coli* MG1655 was employed to develop a process for the one-step conversion of *p*-coumaric acid to CA. To avoid *p*-coumaric acid toxicity, which was evident at a concentration of 5 g/L, a scheme based on substrate feeding was employed to produce 3.82 g/L of CA in 24 h [35]. At the time this result was reported, this was the highest caffeic titer produced by a recombinant strain. With the purpose of generating an *E. coli* strain for the synthesis of CA from a simple carbon source, a metabolic engineering approach was followed to increase carbon flow for the synthesis of L-tyrosine. The strategy consisted on deleting the gene *pheLA*, which encodes an enzyme directing carbon flow into the L-phenylalanine pathway, while expressing the gene $tyrA^{fbr}$ encoding a feedback inhibition-resistant mutant version of the enzyme chorismate mutase/prephenate dehydrogenase, which participates in L-tyrosine biosynthesis. In addition, the genes *ppsA*, *tktA*, and $aroG^{fbr}$ encoding transketolase, phosphoenolpyruvate synthetase, and a feedback inhibition-resistant mutant version of DAHP, respectively, were expressed. A strain with such modifications produced 718 mg/L of L-tyrosine in M9 minimal salt media containing 2.5 g/L glucose and 10 g/L glycerol. This strain was further modified to obtain a *p*-coumaric acid producer by expressing a codon-optimized version of the gene RgTAL encoding a TAL enzyme from *R. glutinis*. The recombinant *E. coli* strain produced 294 mg/L of *p*-coumaric acid under the same growth conditions employed for L-tyrosine production. To transform this strain into a CA producer, the gene encoding 4HPA3H from *E. coli* MG1655 was expressed. Three genetic configurations were tested to express simultaneously the genes RgTAL and 4HPA3H. It was determined that the construct where each gene had its own promoter yielded the best results. The strain with this configuration and grown in M9 minimal salt medium containing 2.5 g/L glucose and 10 g/L glycerol produced 766 mg/L CA, although 7 and 53 mg/L of *p*-coumaric acid and L-tyrosine were also detected in the culture medium, respectively [35].

3.4. Production of caffeic acid from lignocellulosic feedstock

The production of CA employing a lignocellulosic feedstock was evaluated using an L-tyrosine-overproducing *E. coli* strain [36]. This strain was modified by expressing the genes *hpaBC* encoding 4HPA3H from *P. aeruginosa* and *fevV* encoding ammonia lyase from *Streptomyces* sp. WK-5344. The enzyme FevV is highly specific for L-tyrosine as the substrate [37]. The generated *E. coli* strain was named YD01, and it was shown to produce CA from glucose, although the amount was not reported. This strain was evaluated for CA production from cellulose in kraft pulp. This simultaneous saccharification and fermentation (SSF) experiment was performed by supplementing various amounts of the cellulose cocktail Cellic CTec2 and 0.563 g of kraft pulp. The maximum CA concentration (233 mg/L) was produced

when employing an enzyme loading of 2.5 filter paper units/g glucan after 310 h of cultivation. This study also demonstrated that glucose limitation in the culture medium increases CA yields. In another set of experiments, separate hydrolysis and fermentation (SHF) of kraft pulp and filter paper was performed and the hydrolysate was employed as part of the culture medium. In cultures with the strain YD01, a lower level of CA and higher level of acetic acid were observed when employing kraft pulp hydrolysate. This suggests that potential inhibitors were present in kraft pulp hydrolysate. It was determined that furfural, 5-hydroxymethylfurfural, levulinic acid, syringaldehyde, and syringic acid were significantly higher in kraft pulp hydrolysate than in filter paper hydrolysate. These results indicate that SSF conditions for improving CA production with *E. coli* should aim at limiting glucose and accumulation of inhibitors [38].

3.5. Use of heat shock promoters for gene expression in strains for hydroxycinnamic acid production

The *E. coli* strains generated for CA production are based on the expression of native and heterologous genes from promoters dependent on chemical induction. This method for controlling gene expression is effective for laboratory-scale experiments. However, in large-scale commercial production, chemical induction poses problems such as high cost, toxicity, and contamination of the desired product. A recent report evaluated the use of heat shock promoters to control the expression of genes related to the synthesis of hydroxycinnamic acids and curcumin. A strain with the capacity of producing CA from L-tyrosine was generated by expressing genes encoding TAL from *R. glutinis* and C3H from *S. espanaensis* or cytochrome P450 CYP199A2 from *R. palustris* with its redox partners, Pux from *R. palustris*, and Pdr from *P. putida*. The heat shock promoter *dnaK* was employed in this part of the study, and induction conditions consisted of increasing culture temperature to 48°C for 5 min. It was determined that CA production levels were lower when using *dnaK* than when using the T7 promoter. To improve translation efficiency, new synthetic ribosome-binding sites (RBSs) with higher translation initiation rates were designed for each gene by employing RBS calculator software (https://salislab.net/software/). It was determined that such RBSs led to the production of *p*-coumaric acid to a level higher than that observed with the T7 promoter. The *ibpA* heat shock promoter was also evaluated for the expression of the genes involved in *p*-coumaric and CA production. For the expression of the redox partners Pux and Pdr, it was concluded that no significant difference was found when comparing promoters of *dnaK* or *ibpA*. Several strains were tested by employing various promoters and RBSs. It was determined that the use of optimized RBSs and the *dnaK* promoter for the expression of genes encoding CYP199A2, Pux, and Pdr resulted in the highest-level production of CA from *p*-coumaric acid (0.37 mg/L) among tested strains [39]. These results are promising, and it is clear that various aspects such as the evaluation of other heat shock promoters, RBS optimization, and heat induction protocols could lead to an efficient system for gene induction without the need to add chemicals for controlling gene expression.

4. Conclusions and outlook

The examples discussed above represent the current state of the art regarding biological technologies to produce CA. The development of recombinant *E. coli* strains and production processes has enabled the synthesis of this natural product from diverse precursor and carbon sources from the milligram to the gram level. These advances should pave the way to efficient processes that will enable the sustainable production of this useful compound. However, there remain technical challenges to overcome to improve the current production schemes. Several examples highlight a common outcome observed when extending a biosynthetic pathway in an engineered strain, where a reduction in the amount of the novel product is observed. This is illustrated as a reduction in the product titer of 60% when comparing *p*-coumaric to L-tyrosine production by *E. coli* strains differing only in the expression of the gene RgTAL [35]. This result can be explained considering that the activity of the last enzyme introduced in the pathway cannot cope with the carbon flux from the preceding enzyme. In this case, this assumption is supported by the detection of trace amounts of L-tyrosine in the culture medium, suggesting that RgTAL activity is not optimal. The search for more active TAL enzymes either through natural diversity or by protein engineering should be evaluated as a strategy for strain improvement. Both natural and engineered versions of the TAL enzyme from diverse sources have been shown to display improved catalytic properties. It remains to be determined whether the expression of these enzymes would improve current *E. coli* CA-producing strains [40,41,42].

The fine tuning of gene expression in heterologous pathways by employing static or dynamic regulation is an approach that has been shown to increase flow to the desired product in various microbial hosts [43,44,45,46]. This strategy can be combined with the use of protein scaffolds, that colocalize enzymes in a pathway, thereby improving the synthetic efficiency [47,48]. When applied to a CA-producing strain, these strategies have the potential of reducing intermediate accumulation and increasing the final product.

Another important issue detected in these studies is the toxic effects of *p*-coumaric acid and CA on *E. coli*, which cause a decrease in growth rate and productivity. A process solution to this problem involves pulse feeding to avoid the accumulation of *p*-coumaric acid to a toxic level [35]. From a strain design perspective, tolerance engineering can be employed to increase resistance to toxic chemicals [49]. This approach includes membrane transport engineering as a method to reduce the intracellular concentration of a toxic compound. In *E. coli*, the gene *aaeXAB* encodes an efflux pump for several aromatic compounds. The overexpression of this gene in *E. coli* resulted in a twofold increase in tolerance to *p*-coumaric acid [50,51]. The identification of other efflux pumps that can employ *p*-coumaric acid or CA as substrates should provide further targets for production strain optimization [52].

The overexpression of chaperones has been shown to increase tolerance to toxic compounds [49]. In the case of *Clostridium acetobutylicum* ATCC 824, the overexpression of the heat shock *groESL* operon encoding the chaperones GroES and GroEL resulted in 85% less growth inhibition by butanol [53]. In another example, the overexpression of the gene *ibpA* encoding a chaperone in an *E. coli* strain modified for isopentenol synthesis increased production of and tolerance to this product [54]. These results indicate that overexpression of chaperones in a CA-producing strain is an improvement strategy that should be evaluated.

Several studies have compared the effect of employing either glucose or glycerol as energy sources to regenerate NADH from NAD^+. A higher capacity to produce CA from either L-tyrosine or *p*-coumaric acid was detected when using glycerol than using glucose [26,28]. Further research is required to determine the basis of such results. It will also be interesting to determine the effects on CA production capacity using other carbon sources, especially those originating from lignocellulose hydrolysates.

In the studies reviewed here, several enzymes have been expressed and evaluated in *E. coli* to generate the heterologous pathways for CA production. The genes encoding these proteins originate from several species. However, in these reports, only a very small fraction of the known biological diversity has been assessed. The mining of genome and metagenome data should yield many candidate enzymes that have the potential to improve current production strains. The characterization of such novel enzymes should yield valuable data to improve the production of CA and other useful compounds.

Conflict of interest

The authors declare that they have no conflict of interest.

Financial support

This work was supported by a grant from Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica PAPIIT/UNAM IT200217.

References

[1] Gülcin I. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology 2006;217(2–3):213–20. https://doi.org/10.1016/j.tox.2005.09.011.
[2] Zheng W, Wang SW. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J Agric Food Chem 2003;51(2):502–9. https://doi.org/10.1021/jf020728u.
[3] Kivilompolo M, Obůrka V, Hyötyläinen T. Comparison of GC–MS and LC–MS methods for the analysis of antioxidant phenolic acids in herbs. Anal Bioanal Chem 2007;388(4):881–7. https://doi.org/10.1007/s00216-007-1298-8.
[4] Manach C, Scalbert A, Morand C, et al. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79(5):727–47. https://doi.org/10.1093/ajcn/79.5.727.
[5] Göçer H, Gülçin I. Caffeic acid phenethyl ester (CAPE): correlation of structure and antioxidant properties. Int J Food Sci Nutr 2011;62(8):821–5. https://doi.org/10.3109/09637486.2011.585963.
[6] Sud'ina GF, Mirzoeva OK, Pushkareva MA, et al. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. FEBS Lett 1993;329(1–2):21–4. https://doi.org/10.1016/0014-5793(93)80184-V.
[7] Andreasen MF, Landbo AK, Christensen LP, et al. Antioxidant effects of phenolic rye (*Secale cereale* L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. J Agric Food Chem 2001;49(8):4090–6. https://doi.org/10.1021/jf0101758.
[8] Chen JH, Ho CT. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. J Agric Food Chem 1997;45(7):2374–8. https://doi.org/10.1021/jf970055t.
[9] Razzaghi-Asl N, Garrido J, Khazraei H, et al. Antioxidant properties of hydroxycinnamic acids: a review of structure–activity relationships. Curr Med Chem 2013;20(36):4436–50. https://doi.org/10.2174/09298673113209990141.
[10] Magnani C, Isaac VLB, Correa MA, et al. Caffeic acid: a review of its potential use in medications and cosmetics. Anal Methods 2014;6:3203–10. https://doi.org/10.1039/C3AY41807C.
[11] Sato Y, Itagaki S, Kurokawa T, et al. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. Int J Pharm 2011;403(1–2):136–8. https://doi.org/10.1016/j.ijpharm.2010.09.035.
[12] Kaur C, Kapoor HC. Antioxidants in fruits and vegetables — the millennium's health. Int J Food Sci Technol 2001;36(7):703–25. https://doi.org/10.1111/j.1365-2621.2001.00513.x.
[13] Gülcin I. Antioxidant activity of food constituents: an overview. Arch Toxicol 2012;86(3):345–91. https://doi.org/10.1007/s00204-011-0774-2.
[14] Silva T, Oliveira C, Borges F. Caffeic acid derivatives, analogs and applications: a patent review (2009–2013). Expert Opin Ther Pat 2014;24(11):1257–70. https://doi.org/10.1517/13543776.2014.959492.
[15] Cheetham PSJ, Banister NE. Production and uses of caffeic acid and derivatives thereof. United States Patent, 2000. Number 6,066,311.
[16] Saltveit ME. Synthesis and metabolism of phenolic compounds. Fruit and vegetable phytochemicals: chemistry and human health; 2017. p. 115–24. Chapter 5, https://doi.org/10.1002/9781119158042.ch5.
[17] Zhang X, Liu CJ. Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol Plant 2015;8(1):17–27. https://doi.org/10.1016/j.molp.2014.11.001.
[18] Vogt T. Phenylpropanoid biosynthesis. Mol Plant 2010;3(1):2–20. https://doi.org/10.1093/mp/ssp106.
[19] Fuchs G, Boll M, Heider J. Microbial degradation of aromatic compounds — from one strategy to four. Nat Rev Microbiol 2011;9(11):803–16. https://doi.org/10.1038/nrmicro2652.
[20] Nambudiri AMD, Bhat JV, Subba Rao PV. Conversion of *p*-coumaric acid into caffeic acid by *Streptomyces nigrifaciens*: purification and properties of the hydroxylating enzyme. Biochem J 1972;130(2):425–33. https://doi.org/10.1042/bj1300425.
[21] Torres y Torres JL, Rosazza JPN. Microbial transformations of *p*-coumaric acid by *Bacillus megaterium* and *Curvularia lunata*. J Nat Prod 2001;64(11):1408–14. https://doi.org/10.1021/np010238g.
[22] Estrada Alvarado I, Navarro D, Record E, et al. Fungal biotransformation of *p*-coumaric acid into caffeic acid by *Pycnoporus cinnabarinus*: an alternative for producing a strong natural antioxidant. World J Microbiol Biotechnol 2003;19(2):157–60. https://doi.org/10.1023/A:1023264200256.
[23] Sachan A, Ghosh S, Sen SK. Co-production of caffeic acid and *p*-hydroxybenzoic acid from p-coumaric acid by *Streptomyces caeruleus* MTCC 6638. Appl Microbiol Biotechnol 2006;71(5):720–7. https://doi.org/10.1007/s00253-005-0197-1.
[24] Furuya T, Kino K. Regioselective oxidation of indole- and quinolinecarboxylic acids by cytochrome P450 CYP199A2. Appl Microbiol Biotechnol 2010;85(6):1861–8. https://doi.org/10.1007/s00253-009-2207-1.
[25] Bell SG, Feng X, Forward I, et al. Crystal structure of CYP199A2, a *para*-substituted benzoic acid oxidizing cytochrome P450 from *Rhodopseudomonas palustris*. J Mol Biol 2008;383(3):561–74. https://doi.org/10.1016/j.jmb.2008.08.033.
[26] Furuya T, Arai Y, Kino K. Biotechnological production of caffeic acid by bacterial cytochrome P450 CYP199A2. Appl Environ Microbiol 2012;78(17):6087–94. https://doi.org/10.1128/AEM.01103-12.
[27] Galán B, Díaz E, Prieto MA, et al. Functional analysis of the small component of the 4-hydroxyphenylacetate 3-monooxygenase of *Escherichia coli* W: a prototype of a new flavin: NAD(P) H reductase subfamily. J Bacteriol 2000;182(3):627–36. https://doi.org/10.1128/JB.182.3.627-636.2000.
[28] Furuya T, Kino K. Catalytic activity of the two-component flavin-dependent monooxygenase from *Pseudomonas aeruginosa* toward cinnamic acid derivatives. Appl Microbiol Biotechnol 2014;98(3):1145–54. https://doi.org/10.1007/s00253-013-4958-y.
[29] Rodrigues JL, Araújo RG, Prather KL, et al. Heterologous production of caffeic acid from tyrosine in *Escherichia coli*. J Enzyme Microb Technol 2015;71:36–44. https://doi.org/10.1016/j.enzmictec.2015.01.001.
[30] Kang SY, Choi O, Lee JK, et al. Artificial biosynthesis of phenylpropanoic acids in a tyrosine overproducing *Escherichia coli* strain. Microb Cell Fact 2012;11(1):153. https://doi.org/10.1186/1475-2859-11-153.
[31] Kim YH, Kwon T, Yang HJ, et al. Gene engineering, purification, crystallization and preliminary X-ray diffraction of cytochrome P450 *p*-coumarate-3-hydroxylase (C3H), the *Arabidopsis* membrane protein. Protein Expr Purif 2011;79(1):149–55. https://doi.org/10.1016/j.pep.2011.04.013.
[32] Lin Y, Yan Y. Biosynthesis of caffeic acid in *Escherichia coli* using its endogenous hydroxylase complex. Microb Cell Fact 2012;11(1):42. https://doi.org/10.1186/1475-2859-11-42.
[33] Santos CNS, Xiao W, Stephanopoulos G. Rational, combinatorial, and genomic approaches for engineering L-tyrosine production in *Escherichia coli*. PNAS 2012;109(34):13538–43. https://doi.org/10.1073/pnas.1206346109.
[34] Zhang H, Stephanopoulos G. Engineering *Escherichia coli* for caffeic acid biosynthesis from renewable sugars. Appl Microbiol Biotechnol 2013;97(8):3333–41. https://doi.org/10.1007/s00253-012-4544-8.
[35] Huang Q, Lin Y, Yan Y. Caffeic acid production enhancement by engineering a phenylalanine over-producing *Escherichia coli* strain. Biotechnol Bioeng 2013;110(12):3188–96. https://doi.org/10.1002/bit.24988.
[36] Nakagawa A, Minami H, Kim J, et al. A bacterial platform for fermentative production of plant alkaloids. Nat Commun 2011;2(326). https://doi.org/10.1038/ncomms1327.
[37] Kawaguchi H, Uematsu K, Ogino C, et al. Simultaneous saccharification and fermentation of kraft pulp by recombinant *Escherichia coli* for phenyllactic acid production. Biochem Eng J 2014;88:188–94. https://doi.org/10.1016/j.bej.2014.04.014.
[38] Kawaguchi, Katsuyama Y, Danyao D, et al. Caffeic acid production by simultaneous saccharification and fermentation of kraft pulp using recombinant *Escherichia coli*. Appl Microbiol Biotechnol 2017;101(13):5279–90. https://doi.org/10.1007/s00253-017-8270-0.
[39] Rodrigues JL, Couto MR, Araújo RG, et al. Hydroxycinnamic acids and curcumin production in engineered *Escherichia coli* using heat shock promoters. Biochem Eng J 2017;125:41–9. https://doi.org/10.1016/j.bej.2017.05.015.
[40] Zhou S, Liu P, Chen J, et al. Characterization of mutants of a tyrosine ammonia-lyase from *Rhodotorula glutinis*. Appl Microbiol Biotechnol 2016;100(24):10443–52. https://doi.org/10.1007/s00253-016-7672-8.
[41] Weise NJ, Ahmed ST, Parmeggiani F, et al. Zymophore identification enables the discovery of novel phenylalanine ammonia lyase enzymes. Sci Rep 2017;7(1):13691. https://doi.org/10.1038/s41598-017-13990-0.
[42] Jendresen CB, Stahlhut SG, Li M, et al. Highly active and specific tyrosine ammonia-lyases from diverse origins enable enhanced production of aromatic compounds in bacteria and *Saccharomyces cerevisiae*. Appl Environ Microbiol 2015;81(13):4458–76. https://doi.org/10.1128/AEM.00405-15.
[43] Xu P, Rizzoni EA, Sul SY, et al. Improving metabolic pathway efficiency by statistical model-based multivariate regulatory metabolic engineering. ACS Synth Biol 2017;6(1):148–58. https://doi.org/10.1021/acssynbio.6b00187.
[44] Hwang HJ, Lee SY, Lee PC. Engineering and application of synthetic *nar* promoter for fine-tuning the expression of metabolic pathway genes in *Escherichia coli*. Biotechnol Biofuels 2018;11(1):103. https://doi.org/10.1186/s13068-018-1104-1.
[45] Quax TE, Claassens NJ, Söll D, et al. Codon bias as a means to fine-tune gene expression. Mol Cell 2015;59(2):149–61. https://doi.org/10.1016/j.molcel.2015.05.035.
[46] Xu P, Li L, Zhang F, et al. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. PNAS 2014;111(31):11299–304. https://doi.org/10.1073/pnas.1406401111.
[47] Moon TS, Dueber JE, Shiue E, et al. Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. Metab Eng 2010;12(3):298–305. https://doi.org/10.1016/j.ymben.2010.01.003.
[48] Wang Y, Yu O. Synthetic scaffolds increased resveratrol biosynthesis in engineered yeast cells. J Biotechnol 2012;157(1):258–60. https://doi.org/10.1016/j.jbiotec.2011.11.003.
[49] Mukhopadhyay A. Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. Trends Microbiol 2015;23(8):498–508. https://doi.org/10.1016/j.tim.2015.04.008.
[50] Van Dyk TK, Templeton LJ, Cantera KA, et al. Characterization of the *Escherichia coli* AaeAB efflux pump: a metabolic relief valve? J Bacteriol 2004;186(21):7196–204. https://doi.org/10.1128/JB.186.21.7196-7204.2004 PMid: 15489430.
[51] Sariaslani FS. Development of a combined biological and chemical process for production of industrial aromatics from renewable resources. Annu Rev Microbiol 2007;61:51–69. https://doi.org/10.1146/annurev.micro.61.080706.093248 PMid: 17456010.

[52] Kell DB, Swainston N, Pir P, et al. Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. Trends Biotechnol 2015;33(4):237–46. https://doi.org/10.1016/j.tibtech.2015.02.001.
[53] Tomas CA, Welker NE, Papoutsakis ET. Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol 2003;69(8):4951–65. https://doi.org/10.1128/AEM.69.8.4951-4965.2003.
[54] Foo JL, Jensen HM, Dahl RH, et al. Improving microbial biogasoline production in *Escherichia coli* using tolerance engineering. MBio 2014;5(6):e01932-14. https://doi.org/10.1128/mBio.01932-14.