Microbial Production of Caffeic Acid

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Contents

Abstract

Caffeic acid is a hydroxycinnamic acid mostly produced in plants although its microbial production has also been reported. This compound presents several biological activities and potential therapeutic properties. Additionally, it can be a precursor or intermediary of various relevant compounds. Current production methods include the inefficient, expensive, and not environmentally friendly extraction from plants that accumulate this compound in very low amounts. Therefore, highly efficient and environmentally friendly methods are needed. Microbial biosynthesis can potentially produce it in a purer, faster, and greener way. Since the establishment of caffeic acid heterologous production in *Streptomyces fradiae,* several studies have been published regarding its production in *Escherichia coli* and *Saccharomyces cerevisiae.* These studies include the production from supplemented tyrosine or *p*-coumaric acid but also glucose using

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tyrosine-overproducing strains. Presently, there are three different pathways to produce caffeic acid that have in common the first step that is catalyzed by a microbial tyrosine ammonia lyase that converts tyrosine to *p*-coumaric acid. The second step that synthesizes caffeic acid from *p*-coumaric acid was identified as the pathway bottleneck and can be performed by 4-coumarate 3-hydroxylase, hydroxyphenylacetate 3-hydroxylase (4HPA3H) complex or a cytochrome P450 CYP199A2 system. Although all these enzymes have been identified in bacteria, and caffeic acid has only recently been produced in *S. cerevisiae*, the productions in this host have almost reached the maximum productions reported for *E. coli* (569 mg/L vs. 767 mg/L, respectively). The maximum production was obtained from glucose using the 4HPA3H pathway. These developments on caffeic acid heterologous production are very promising.

Introduction

Caffeic acid (3,4 dihydroxycinnamic acid) is a polyphenolic acid produced by several plants as a secondary metabolite including in fruits, olives, potatoes, carrots, coffee, and various berries and herbs (El-Seedi et al. 2012). This polyphenolic acid is the most representative hydroxycinnamic acid (75%) found in fruits (El-Seedi et al. 2012) and, consequently, in the human diet. Caffeic acid has attracted growing attention due to its valuable properties, including anti-inflammatory (Zielińska et al. 2021), antioxidant (Kolgazi et al. 2021), anticancer (Park and Kim 2020), and antimicrobial (Perumal et al. 2017). Moreover, some athletes consume caffeic acid to enhance exercise tolerance and boost their performance (Al-Khelaifi et al. 2018).

In addition to its interesting biological activities, caffeic acid is a pathway intermediate of several relevant compounds also with potential therapeutic properties, including ferulic acid, caffeic acid phenethyl ester, esculetin, curcuminoids, piceatannol, and chlorogenic acid (Fig. 1) (Rodrigues et al. 2015a, 2020; Couto et al. 2017; Wang et al. 2017a; Li et al. 2021a; Rodrigues and Rodrigues 2021). Moreover, caffeic acid can also be a precursor of compounds commonly used as flavoring agents in the food industry and used in the production of plastics and rubbers (vanillin, hydroxystyrene analogous – 3,4-dihydroxystyrene, 4-hydroxy-3-methoxystyrene) (Rodrigues et al. 2015c; Rainha et al. 2020; Shen et al. 2020; Rodrigues and Rodrigues 2021). The increased demand for these specific compounds has been driving the increase in caffeic acid demand and market volume (Transparency Market Research 2021).

Due to caffeic acid's interesting biological activities, there is an established market for its production. Several fruits and vegetables contain caffeic acid in
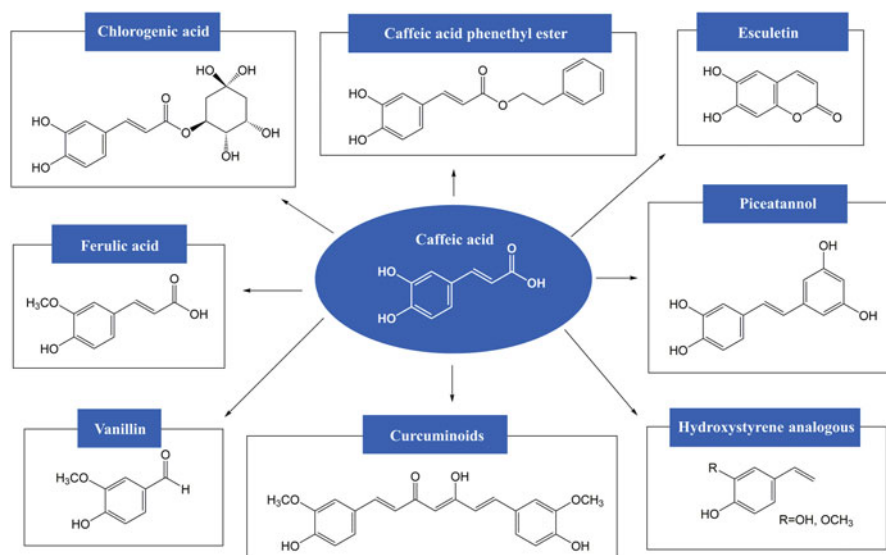
Fig. 1 Caffeic acid chemical structure and examples of industrially relevant molecules that can be produced in a pathway in which caffeic acid is an intermediate

significant amounts (El-Seedi et al. 2012); however, higher amounts are needed to further develop caffeic acid-based treatments and supplements. For these purposes, caffeic acid can be extracted from these plants. Nevertheless, extraction from fruits and vegetables is not ideal since they are essential for human/animal consumption. In addition, unfortunately, the parts of the plants that are not used as food, such as stalks and branches, are usually richer in lignin and do not accumulate significant amounts of caffeic acid as it is only an intermediary from the lignin pathway. Moreover, since plants contain several compounds with similar structures, the separation and purification processes are very complex, inefficient, expensive, and environmentally unfriendly (Rodrigues et al. 2015b; An et al. 2016). Therefore, caffeic acid extraction from plants is not advantageous.

Due to these reasons, more efficient and greener production processes have been explored, such as the use of microorganisms for caffeic acid heterologous production. The use of synthetic biology tools and approaches has been widely used to design microbes able to produce compounds with therapeutic and industrial interest (Rodrigues and Rodrigues 2017; Rodrigues et al. 2017b; Rainha et al. 2021). Microorganisms hold a number of advantages when compared to plants. They grow very fast, thus being possible to obtain the compounds of interest in significant amounts in a very short time regardless of the season, while plants take weeks to months to accumulate such compounds and most of the time in very low amounts. Additionally, since microbes generally do not produce structurally similar compounds, the separation and purification processes are easier and cheaper. Moreover, microorganisms are easy to genetically manipulate and generally do not have

competing pathways that may consume or degrade the target product (Rodrigues et al. 2015b, c). All of this makes them better hosts than plants. In addition, bioinformatic tools to predict and identify optimization strategies to improve the production yields and remarkable genome engineering tools such as CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-associated caspase 9 endonuclease) to implement those modifications are better developed in microorganisms (Oliveira et al. 2021; Rainha et al. 2021). Altogether, these tools and chassis will be crucial to develop a sustainable biotech process to produce caffeic acid towards a circular bioeconomy (Gudiña et al. 2020).

Caffeic Acid Biological Activity

Caffeic acid has been shown to have several interesting activities and was demonstrated to be safe for humans although high doses should be cautioned (Qin et al. 2015; Ekeuku et al. 2021). These activities include antioxidant, anti-inflammatory, anticancer, antiaging, antianxiety, antimicrobial, and antiviral (Dos Santos et al. 2018; Ogawa et al. 2018; Mudgal et al. 2020; Okada and Okada 2020; Kolgazi et al. 2021; Zielińska et al. 2021). Table 1 summarizes some examples of each biological activity and the corresponding mechanisms of action of caffeic acid.

Caffeic acid also demonstrated potential therapeutic activity in skeletal diseases such as osteoarthritis and osteoporosis (Ekeuku et al. 2021). In vitro studies suggest that caffeic acid has the potential to be used in bone regeneration as it enhanced osteoblasts proliferation and differentiation, and also increased calcium mineralization, inhibited osteoclastogenesis, bone resorption, and osteoblast apoptosis (Ekeuku et al. 2021; Liu et al. 2021).

Other activities such as immune-stimulatory have also been reported. For example, human clinical trials in immune thrombocytopenia have been completed and revealed that caffeic acid was an effective adjuvant in the treatment of the disease, leading to an increase in platelet count (ClinicalTrials.gov Identifier: NCT02351622, NCT02556814) (Qin et al. 2015).

Although caffeic acid has demonstrated tremendous potential to be used in the treatment of several diseases, in most cases, the activity in humans cannot be established due to the lack or diminished number of in vivo studies and clinical trials. Nevertheless, its positive pharmacokinetic profile and general lack of toxicity are very promising and should prompt further studies.

Caffeic Acid Production in Plants

Caffeic acid is an intermediate in the phenylpropanoids pathway linked to the lignin biosynthetic pathway in plants. These pathways are still not completely elucidated although recently the part of the pathway related to caffeic acid became less uncertain (Barros et al. 2019). The phenylpropanoids pathway starts with the conversion of phenylalanine to cinnamic acid by the enzyme phenylalanine

Table 1 Examples of caffeic acid biological activities

Biological activity	Mechanism of action	References
Antioxidant	Prevents gastric mucosal damage by preventing lipid peroxidation, neutrophil infiltration, and GSH (glutathione) depletion and inducing nitric oxide (NO) modulation	Kolgazi et al. (2021)
	Induces nitric oxide (NO) release and vasodilation due to moderate vasorelaxant activity, protecting tissues from ischemia-reperfusion injury	Silva and Lopes (2020)
Anti-inflammatory	Targets COX-2 (cyclooxygenase 2) and its products prostaglandin E2 (PGE2) and interleukin (IL)-8, and inhibits AGE (advanced glycation end product) formation which can have positive effects on intestinal inflammation	Zielińska et al. (2021)
	Reduces IL-6 and tumor necrosis factor-α (TNF-α), induces apoptosis of fibroblast-like synoviocytes in vitro and significantly represses matrix metalloproteinases and PGE2 that play a key role in rheumatoid arthritis, suggesting it can be effective in the treatment of this disease	Wang et al. (2017b)
Anticancer	Inhibits the GASC1/KDM4C (lysine demethylase 4C) oncogene, a gene amplified in squamous cell carcinoma 1. Clinical trials are ongoing (NCT04648917, NCT03070262)	Yuan et al. (2016)
	Inhibits the expression of genes from melanin production pathway and, consequently, melanin content and also tyrosinase activity which may decrease the risks of developing skin cancer	Park and Kim (2020)
	Induces, via apoptosis induction, cytotoxicity and morphological alterations in breast cancer cells in vitro	Rezaei-Seresht et al. (2019)
Antiaging	Increases clock genes expression by acting on NRF2 (nuclear factor E2-related factor 2) expression which indicates the possibility of an antiaging effect	Okada and Okada (2020)
	Increases life span and prevented oxidative damage in *Drosophila melanogaster* and *Caenorhabditis elegans* models which suggest that it can have potential in Alzheimer's disease treatment	Li et al. (2020a, 2021b)
	Stimulates glucose uptake, inhibits ATPase activity and cholinergic system dysfunctions, mitigates oxidative stress, and maintains tissue morphology in an ex-vivo study in rat brains, suggesting a neuroprotective effect	Salau et al. (2021)
Antianxiety/ antidepressant	Shows protection against behavioral and inflammation markers when mice are exposed to lipopolysaccharide, reducing anxiety in mice	Mudgal et al. (2020)
Antimicrobial / antiviral	Inhibits efflux pumps responsible for bacterial resistance of *Staphylococcus aureus* and could be used as an adjuvant in novel formulations using antibiotics	Dos Santos et al. (2018)
	Disintegrates the lipopolysaccharides layer of *Pseudomonas aeruginosa* outer membrane increasing the outer membrane permeability and facilitating the entry of hydrophobic antibiotics	Perumal et al. (2017)

Table 1 (continued)

Biological activity	Mechanism of action	References
	Inhibits hepatitis C virus replication in vitro via induction of interferon α (IFNα) antiviral response	Shen et al. (2018)
	Inhibits severe fever thrombocytopenia syndrome virus (SFTSV) spread in vitro by inhibiting the SFTSV binding to the human hepatoma cells	Ogawa et al. (2018)

ammonia lyase (PAL) (Fig. 2). Afterward, cinnamic acid is transformed to *p*-coumaric acid by cinnamate 4-hydroxylase (C4H). C4H is a cytochrome P450 (CYP) monooxygenase that is associated with plant cell endoplasmic reticulum. This enzyme uses NADH (nicotinamide adenine dinucleotide reduced)-CYP reductases (CPRs), which are flavoproteins, as electron donors. *p*-Coumaric acid can also be produced directly from tyrosine. This reaction is catalyzed by some PALs from monocots (e.g., grasses) that also has tyrosine ammonia lyase (TAL) activity (Barros et al. 2019). This pathway is simpler since it does not need C4H and CPR.

Recently, the gene encoding the 4-coumarate 3-hydroxylate (C3H) enzyme from *Arabidopsis thaliana,* that converts *p*-coumaric acid to caffeic acid, was identified (Barros et al. 2019). This enzyme is the only hydroxylase enzyme from the phenylpropanoids biosynthetic pathway which is not a membrane-bound CYP. The discovery of this gene allowed to elucidate the pathway since it was previously believed that caffeic acid was obtained in plants from *p*-coumaric acid by a trimeric complex of CYP enzymes, namely two C4H enzymes and one 4-coumaroyl shikimate 3′-hydroxylase (C3′H). C3′H is known to convert coumaroyl shikimate to caffeoyl shikimate (Fig. 2).

It has also been described that caffeic acid can be produced from caffeoyl shikimate, an intermediate in lignin biosynthetic pathway, by the action of caffeoyl shikimate esterase (CSE) (Vanholme et al. 2013; Saleme et al. 2017) (Fig. 2). However, CSE is not reported in all plants (Ha et al. 2016). For example, grass species lack this enzyme which confirms the existence of a different route to produce caffeic acid that later was found to include the C3H step.

Caffeic Acid Production in Microorganisms

As mentioned, although caffeic acid is mostly produced in plants, there are reports of its presence in microbes. The pathway identified in microbes to produce caffeic acid is simpler than the one in plants. Some microbes have a TAL enzyme that converts tyrosine directly to *p*-coumaric acid. Generally, TAL enzymes from microbes can convert tyrosine to *p*-coumaric acid, but also phenylalanine to cinnamic acid although with lower catalytic efficiency (Zhou et al. 2016). The first microbial TAL reported was identified in *Rhodobacter capsulatus* and its catalytic efficiency for tyrosine was up to 150 times higher than for phenylalanine (Xue et al. 2007b). Other microbial TALs (e.g., *Rhodobacter sphaeroides, Streptomyces* sp. Tü 4128,
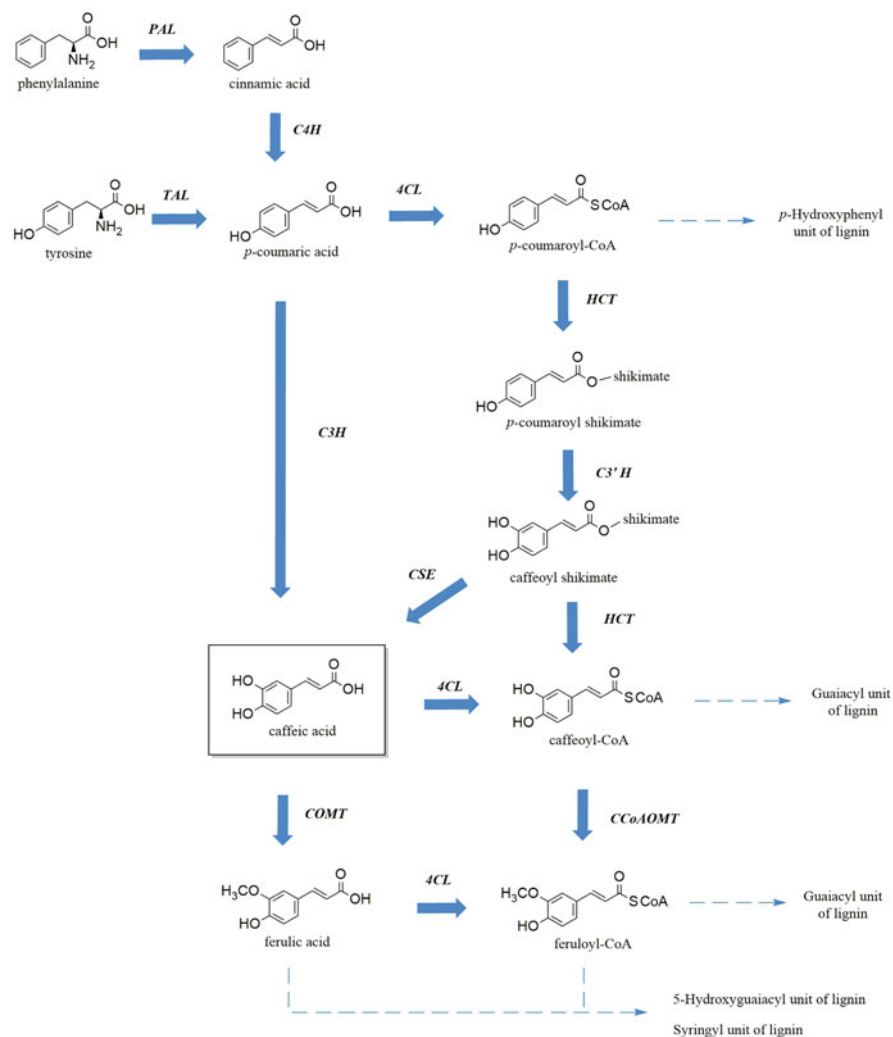
Fig. 2 Caffeic acid is an intermediate of phenylpropanoids biosynthetic pathway and is directly linked to lignin biosynthesis in plants. 4CL 4-coumarate-CoA ligase, C3H 4-coumarate 3-hydroxylase, C3'H 4-coumaroy shikimate 3'-hydroxylase, C4H cinnamate 4-hydroxylase, CCoAOMT caffeoyl-CoA 3-O-methyltransferase, COMT caffeic acid 3-O-methyltransferase, CSE caffeoyl shikimate esterase, HCT 4-hydroxycinnamoyl-CoA shikimate hydroxycinnamoyltransferase, PAL phenylalanine ammonia lyase, TAL tyrosine ammonia lyase (bifunctional). Dash arrows represent several steps of the pathway towards lignin production

Streptomyces clavuligerus) were also identified; however, the presence of a C3H and the product, caffeic acid, was not reported (Xue et al. 2007b; Zhu et al. 2012; Álvarez-Álvarez et al. 2015). Nevertheless, the pathway using TAL and a C3H enzyme was identified in bacteria such as *Saccharothrix espanaensis*, while the TAL

gene and caffeic acid production were reported in the yeast *Rhodotorula glutinis* (Berner et al. 2006; Xue et al. 2007a; Salar et al. 2013). The production of caffeic acid and other phenolic compounds in these microorganisms is a part of a protection mechanism and increases under stress conditions such as osmotic or oxidative stresses (Salar et al. 2013). In *S. espanaensis*, the production of caffeic acid is related to the production of saccharomicins, a class of antibiotics. Saccharomicins consist of 17 monosaccharide units linked to an aglycon, in which caffeic acid is connected to taurine (Berner et al. 2006). In this bacterium, TAL enzyme is encoded by the *sam8* gene and showed very low affinity for the substrate phenylalanine. Afterwards, C3H encoded by *sam5* converts *p*-coumaric acid to caffeic acid.

The conversion of *p*-coumaric acid to caffeic acid was also reported in other microorganisms such as *Streptomyces griseus*, *Streptomyces nigrifaciens, Streptomyces caeruleus, Gliocladium deliquescens*, and *Pycnoporus cinnabarinus* (Nambudiri et al. 1972; Torres y Torres and Rosazza 2001; Alvarado et al. 2003; Sachan et al. 2006). For example, *S. caeruleus* was able to produce 150 mg/L of caffeic acid from 5 mM of *p*-coumaric acid (Sachan et al. 2006). The fungus *P. cinnabarinus* is also considered a natural caffeic acid producer (Alvarado et al. 2003). It was able to produce 257 mg/L of caffeic acid from 450 mg/L of *p*-coumaric acid (supplemented in a phased manner in order to avoid toxicity). The pathway in these microorganisms was not elucidated. However, a tyrosinase was described to convert *p*-coumaric acid to caffeic acid in *Pycnoporus sanguineus* (Halaouli et al. 2005).

Heterologous Production of Caffeic Acid

Caffeic Acid Heterologous Production in Bacteria

Growth inhibition studies showed that *E. coli* can tolerate high concentrations of caffeic acid since concentrations between 100 and 1000 mg/L only affect growth in 20%. This suggests that *E. coli* is a suitable host for caffeic acid production (Zhang and Stephanopoulos 2013). Hence, in the last years, several studies focused on the use of this chassis for caffeic acid production. The caffeic acid heterologous production pathways in *E. coli* use TAL enzymes instead of PAL, as using this strategy, the use of C4H enzyme is not required (Rodrigues et al. 2015b, c) (Table 2). C4H, as a membrane-bound CYP enzyme, is not well expressed in prokaryotic organisms. In addition, prokaryotes lack CPR enzymes that are C4H redox partners. Therefore, the use of TAL makes the pathway simpler. In addition, it is highly efficient leading to high *p*-coumaric acid titers. The second part of the caffeic acid pathway is known to be the pathway bottleneck. Therefore, most of the studies related to caffeic acid heterologous production have been focused on finding an efficient enzyme to convert *p*-coumaric acid to caffeic acid. The identification of such enzyme is very important to avoid *p*-coumaric acid accumulation that is proved to be cytotoxic and to severely inhibit TAL activity (Sariaslani 2007; Xue et al. 2007a; Kang et al. 2012). So far, three different enzymes from different microorganisms have been described

Table 2 Heterologous production of caffeic acid in *Escherichia coli*

Pathway	*E. coli*	Pathway genes[a]	TYR modifications[b]	Conditions (precursor underlined when supplemented)	Titer (mg/L)	References
C3H	C41 (DE3)	SeTAL SeC3H	AroG^fbr/ TyrA^fbr ΔTyrR	Protein induction (1 mM IPTG – Isopropyl β-D-thiogalactopyranoside) in LB at 37 °C. after 5 h, cells were transferred to M9 medium (15 g/L glucose) with 1 mM IPTG at 26 °C for 36 h in shake flasks	150	Kang et al. (2012)
	BL21 (DE3)	SeTAL SeC3H	ΔTyrR/ ΔPheA AroG^fbr/ TyrA^fbr	M9 medium (1% yeast extract, 2% glucose) with 1 mM IPTG for 36 h at 30 °C	138.2	An et al. (2016)
	rpoA14 (DE3)	RgTAL SeC3H	AroG^fbr/ TyrA^fbr ΔTyrR/ ΔPheA	Synthetic medium with 5 g/L glucose and 0.1 mM IPTG at 37 °C for 4 d in bioreactor	106	Zhang and Stephanopoulos (2013)
	K-12 MG1655 (DE3)	RgTAL SeC3H	–	Protein induction (1 mM IPTG) in LB at 26 °C. Cells harvested after 5 h and transferred to M9 medium (40 g/L glucose) with 1 mM IPTG and 3 mM tyrosine at 26 °C for 63 h in shake flasks	180	Rodrigues et al. (2015b)
4HPA3H	BW25113	RcTAL Ec4HPA3H	ΔTyrR/ TyrA^fbr AroG^fbr/ PpsA/TksA	M9 medium (2.5 g/L glucose and 10 g/L glycerol) and 0.2 mM IPTG at 30 °C for 48 h	50.2	Lin and Yan (2012)

(continued)

Table 2 (continued)

Pathway	E. coli	Pathway genes[a]	TYR modifications[b]	Conditions (precursor underlined when supplemented)	Titer (mg/L)	References
	BW25113	Ec4HPA3H	–	M9 medium (0.5% yeast extract, 20 g/L glycerol) and 0.2 mM IPTG 3 h after inoculation at 37 °C in shake flasks. Supplementation of 20 mM of _p-coumaric acid 3 h after protein induction and 3 mM_ afterwards	3820	Huang et al. (2013)
	ATCC 31884	RgTAL Ec4HPA3H	ΔPheA/ ΔPheL ΔTyrA/ΔTyrR TyrA^{fbr}/ PheA^{fbr} AroH^{fbr}/ AroG^{fbr} AroF^{fbr}/PpsA/ TksA	M9 medium (2.5 g/L glucose and 10 g/L glycerol) with 20 mg/L phenylalanine supplementation and 0.2 mM IPTG at inoculation at 37 °C for 72 h in shake flasks	766.68	
	BL21 star (DE3)	Pa4HPA3H	–	Potassium phosphate buffer (200 mM, pH 7.5) with 10% glycerol and IPTG, at 30 °C for 24 h in shake flasks. 20 mM of _p-coumaric acid_ was supplemented four times	10,200	Furuya and Kino (2014)
	BL21 (DE3)	SsTAL Pa4HPA3H	ΔTyrR/ TyrA^{fbr} AroG^{fbr}/ PpsA/TksA	M9 medium with Kraft pulp (20 g/L glucan). Cellulase was added for saccharification for 6 h at 45 °C, afterward. E. coli was incubated at 26 °C for 320 h. IPTG (80 μM) was added after 4 h after inoculation	233	Kawaguchi et al. (2017)

CYP199A2	BL21 (DE3)	RpCYP199A2 PpPdr/RpPux	—	Potassium phosphate buffer (50 mM, pH 7.5) with 10% glycerol, 1 mM IPTG, and 20 mM of p-coumaric acid at 30 °C for 24 h in shake flasks	2800	Furuya et al. (2012)
	K-12 MG1655 (DE3)	RgTAL RpCYP199A2 PpPdr/RpPux	—	Protein induction (1 mM IPTG and 100 ng/mL aTc – Anhydrotetracycline) in LB at 26 °C. after 5 h, cells were transferred to M9 medium (40 g/L glucose) with inducers and 3 mM tyrosine at 26 °C for 63 h in shake flasks	280	Rodrigues et al. (2015b)
		FjTAL RpCYP199A2 PpPdR/RpPux	—	M9 medium (1 mM IPTG, 100 ng/mL aTc and 4% glucose) at 26 °C for 72 h in tubes. Production from glucose or supplemented tyrosine (3 mM)	~18 / ~324 (+tyr)	Haslinger and Prather (2020)
		FjTAL RpCYP199A2 RpPuR/RpPux	—	M9 medium (1 mM IPTG, 100 ng/mL aTc and 4% glucose) at 26 °C for 72 h in tubes	~27 / ~47[c]	

[a]Genes from caffeic acid pathway: 4HPA3H 4-hydroxyphenylacetate 3-monooxygenase complex, C3H 4-coumarate 3-hydroxylase, PdR putidaredoxin reductase, PuR palustrisredoxin reductase, Pux palustrisredoxin, TAL tyrosine ammonia lyase; Genes sources: Ec – Escherichia coli, Fj – Flavobacterium johnsoniae, Pa – Pseudomonas aeruginosa, Pp – Pseudomonas putida, Rc – Rhodobacter capsulatus, Rg – Rhodotorula glutinis, Rp – Rhodopseudomonas palustris, Se – Saccharothrix espanaensis, Ss – Streptomyces sp. WK-5344

[b]Some studies used a tyrosine-overproducing strain (TYR strain) in which: PheA (chorismate mutase/prephenate dehydratase), PheL (phe operon leader peptide), TyrA (chorismate mutase/prephenate dehydrogenase), and/or TyrR (tyrosine repressor) genes were deleted (ΔPheA/ΔPheL/ΔTyrA/ΔTyrR) to direct the pathway to tyrosine production; AroF/AroH/AroG (3-deoxy-D-arabino-heptolosonate-7-phosphate (DAHP) synthetases), PheA and/or TyrA feedback inhibition-resistant variants (AroF^fbr/AroH^fbr/AroG^fbr/PheA^fbr/TyrA^fbr) were overexpressed to eliminate tyrosine- and phenylalanine-mediated repression; PpsA (phosphoenolpyruvate synthetase) and TktA (transketolase 1) genes were overexpressed to increase the aromatic amino acids (AAA) precursors availability

[c]To obtain this caffeic acid concentration, an extra copy of pux gene was overexpressed

to perform this step and used in the construction of heterologous pathways in *E. coli*, namely C3H, 4-hydroxyphenylacetate 3-hydroxylase (4HPA3H), and CYP199A2.

Pathway Containing C3H

Caffeic acid heterologous production was first established in *Streptomyces fradiae* XKS (Berner et al. 2006). This bacterium was genetically modified to express the genes previously identified in *S. espanaensis*, *sam8* and *sam5,* and was able to produce caffeic acid. Later, these genes were expressed in *Escherichia coli* to produce ferulic acid, using caffeic acid as an intermediary (Choi et al. 2011; Kang et al. 2012). Choi et al. (2011) did not report the production of caffeic acid as an intermediary although the enzymes demonstrated activity, as they were able to produce ferulic acid from glucose, though in a low amount (7.1 mg/L). Afterward, Kang et al. (2012) studied the influence of using an *E. coli* codon-optimized TAL from *S. espanaensis* (*Se*TAL). The authors concluded that the codon-optimized gene improved *p*-coumaric acid production. However, the caffeic acid production resulting from the use of codon-optimized *Se*TAL and C3H also from *S. espanaensis* (*Se*C3H) was not improved. The accumulation of high amounts of *p*-coumaric acid led to a decrease in caffeic acid production (14 mg/L were produced instead of 42 mg/L). Therefore, it was demonstrated that C3H step may be a major bottleneck in the pathway. Afterward, a tyrosine overexpressing strain with modifications in the aromatic amino acids (AAA) pathway (Fig. 3) was tested. TyrR (tyrosine repressor) gene was deleted to eliminate the transcriptional control mediated by TyrR that can repress several genes that encode AroF/AroG (3-deoxy-D-arabino-heptolosonate-7-phosphate (DAHP) synthetases), TyrA (chorismate mutase/prephenate dehydrogenase), and TyrB (tyrosine aminotransferase) that are essential to produce tyrosine. In addition, since AroG and TyrA are inhibited by tyrosine (feedback inhibition), they overexpressed in a plasmid the feedback inhibition-resistant mutants AroG^{fbr} and TyrA^{fbr}. *p*-Coumaric acid production using this tyrosine-overproducing strain improved significantly; however, the production using a codon-optimized gene was lower than using the non-optimized version, thus demonstrating that TAL expression was not limiting the production. This was also observed for caffeic acid production. The maximum caffeic acid production was 150 mg/L, and it was obtained using *Se*TAL non-codon-optimized. Although codon-optimized TAL may enhance protein synthesis, it does not lead necessarily to higher *p*-coumaric acid production and consumption since C3H is the limiting step, not TAL. An et al. (2016) used the same genes (*Se*TAL and *Se*C3H) to produce caffeic acid in a tyrosine-overproducing strain (AroG^{fbr}/TyrA^{fbr} overexpressed in a plasmid; ΔTyrR/ΔPheA – PheA is also a chorismate mutase/prephenate dehydrogenase that deviates the pathway for phenylalanine production). The authors reported a caffeic acid production of 138.2 mg/L, which is in the same range of the production obtained by Kang et al. (2012) (Table 2).

TAL from *R. glutinis* (*Rg*TAL) is reported to have a much higher activity towards tyrosine when compared to other TAL, including *Se*TAL (k_{cat}/K_m = 860–298,000 and 968–3050 s^{-1} M^{-1}, respectively) (Berner et al. 2006; Xue et al. 2007a; Jendresen et al. 2015; Zhou et al. 2016). Therefore, Zhang and Stephanopoulos
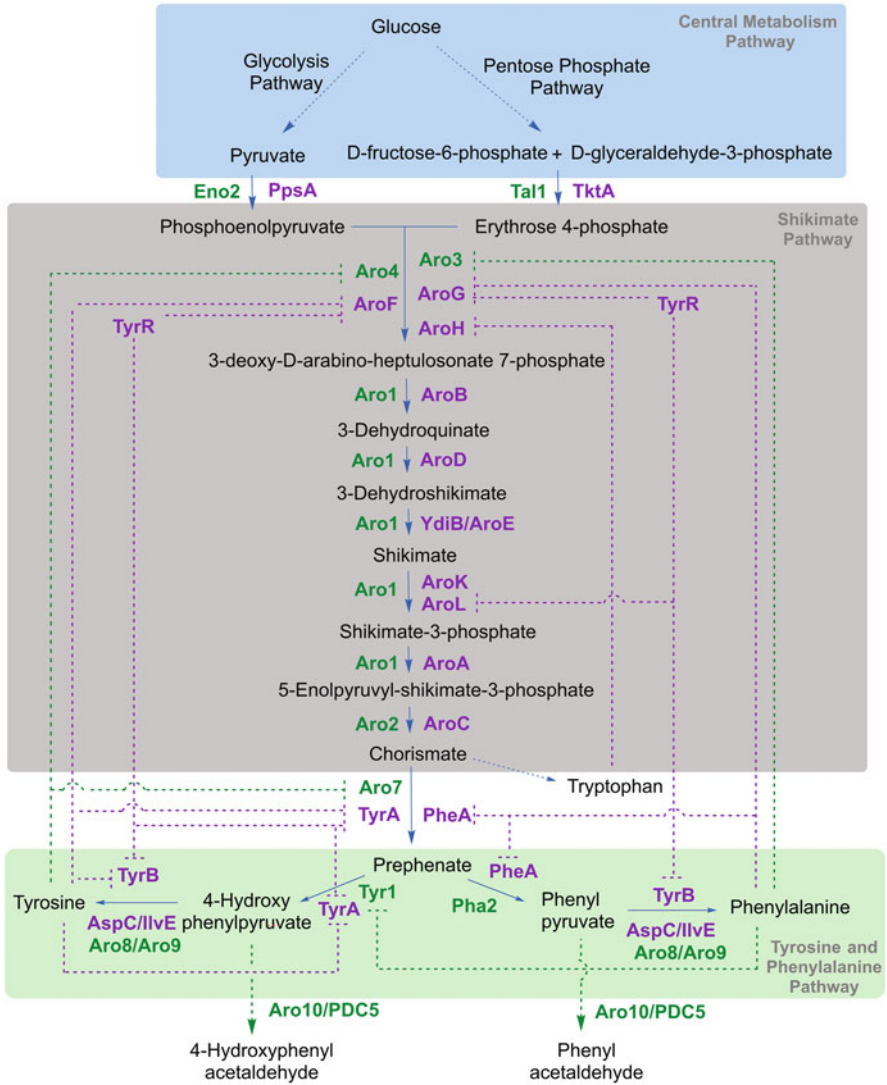
Fig. 3 Aromatic amino acid (AAA) biosynthesis in *Escherichia coli* and *Saccharomyces cerevisiae*. This pathway is regulated at transcriptional and allosteric levels. Several enzymes of the pathway are feedback regulated and inhibited by their end products and, consequently, are possible targets for genetic engineering. Dashed arrows represent multiple steps. Enzymes and regulations (represented as dashed lines) in purple and green correspond to *E. coli* and *S. cerevisiae*, respectively. Aro1 – pentafunctional AROM protein, Aro2 – chorismate synthase, Aro3/Aro4 – 3-deoxy-7-phosphoheptulonate (DAHP) synthases, Aro7 – chorismate mutase, Aro8 – aromatic aminotransferase I, Aro9 – aromatic aminotransferase II, Aro10 – phenylpyruvate decarboxylase, AroA – 5-enolpyruvoyl-shikimate 3-phosphate synthase, AroB – 3-dehydroquinate synthase, AroC – chorismate synthase, AroD – 3-dehydroquinate dehydratase, AroE – shikimate dehydrogenase, AroF/AroG/AroH – DAHP synthases, AroK/AroL – shikimate kinase 1/2, AspC – aspartate

(2013) used *Rg*TAL and *Se*C3H (both codon-optimized for *E. coli*) in a different pathway to produce caffeic acid. This pathway also contained a codon-optimized 4-coumarate-CoA ligase from *Petroselinum crispum* (*Pc*4CL) that converts *p*-coumaric acid to coumaroyl-CoA and an endogenous hydroxyphenylacetyl-CoA thioesterase from *E. coli* that can convert caffeoyl-CoA to caffeic acid. The authors assumed that C3H, in addition to converting *p*-coumaric acid to caffeic acid, also converted coumaroyl-CoA to caffeoyl-CoA (Kneusel et al. 1989) (Fig. 4). However, the highest caffeic acid production was obtained when only *Rg*TAL and *Se*C3H were expressed. Using only those genes, the authors were able to produce, in test tubes, 88 mg/L and 70 mg/L using a rich medium containing 5 g/L of glucose and xylose, respectively, in a tyrosine-overproducing strain (ΔTyrR/ΔPheA, AroG^{fbr}/TyrA^{fbr} integrated into the genome). Afterwards, the authors performed an experiment in a bioreactor only overexpressing *Rg*TAL and *Se*C3H. They were able to produce 106 mg/L of caffeic acid using glucose as a substrate. Additionally, they verified that the expression of *Rg*TAL in a high-copy plasmid did not improve caffeic acid production in most cases. Again, these results prove that high *p*-coumaric acid concentrations may not be favorable to caffeic acid production since C3H is not as efficient as the TAL enzyme. According to the authors, the alternative route using *Pc*4CL failed possibly due to the low activity of *E. coli* native thioesterase against caffeoyl-CoA and, in the future, it would be interesting to overexpress a thioesterase with known activity against caffeoyl-CoA. However, the benefits of the parallel route are questionable not only due to the possible lack of esterase activity, as reported by the authors, but also because *Pc*4CL is reported to have almost the same activity towards caffeic acid and to *p*-coumaric acid (Knobloch and Hahlbrock 1977). This means that besides converting *p*-coumaric acid to coumaroyl-CoA, *Pc*4CL is probably also able to convert the target caffeic acid to caffeoyl-CoA, which is a disadvantage. Therefore, the presence of caffeoyl-CoA is probably related to *Pc*4CL activity rather than to the C3H activity claimed by the authors. Moreover, the increase in the number of steps leading to intermediate accumulation and an increased metabolic burden also represents a disadvantage.

Caffeic acid was also produced using TAL and C3H codon-optimized for *E. coli* by direct supplementation of tyrosine (3 mM) (Rodrigues et al. 2015b). *Rg*TAL was able to efficiently convert the supplied tyrosine to *p*-coumaric acid (472 mg/L) and *Rg*TAL and SeC3H were able to produce 180 mg/L of caffeic acid with no accumulation of *p*-coumaric acid (Rodrigues et al. 2015b). Several plasmid combinations were tested and the one that allowed better results was the one with TAL in pCDFDuet-1 and C3H in pRSFDuet-1. This was expected, as more copies of C3H

Fig. 3 (continued) aminotransferase, Eno2 – enolase II, IlvE – branched-chain-amino-acid aminotransferase, PDC5 – pyruvate decarboxylase 5, Pha2 – prephenate dehydratase, PheA – fused chorismate mutase/prephenate dehydratase, PpsA – phosphoenolpyruvate synthetase, Tal1 – transaldolase, TktA – transketolase 1, Tyr1 – prephenate dehydratase, TyrA – fused chorismate mutase/prephenate dehydratase, TyrB – tyrosine aminotransferase, TyrR – tyrosine repressor, YdiB – shikimate dehydrogenase
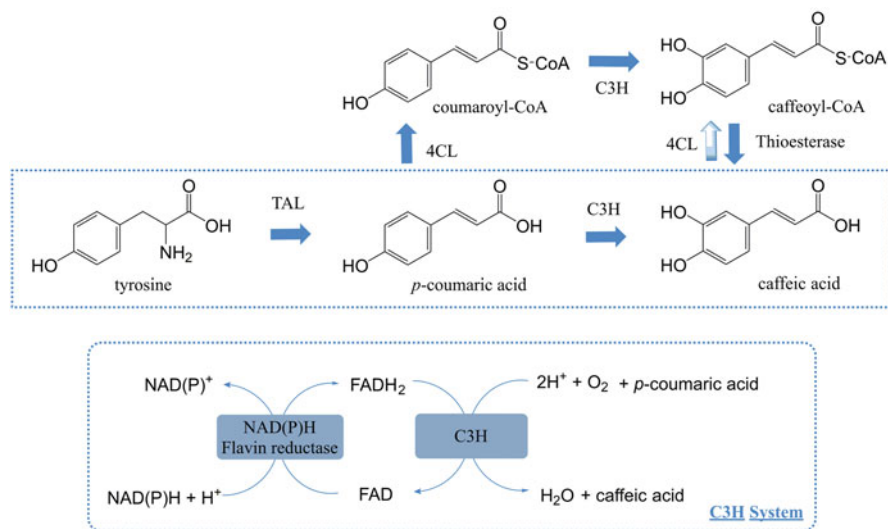
Fig. 4 Caffeic acid biosynthetic pathway in *Escherichia coli* containing TAL (tyrosine ammonia lyase) that converts tyrosine to *p*-coumaric acid and C3H (4-coumarate 3-hydroxylase) that converts *p*-coumaric acid to caffeic acid. The pathway proposed by Zhang and Stephanopoulos (2013) that in addition to TAL and C3H also contains 4CL (4-coumarate-CoA ligase) and a thioesterase is also presented. The faint arrow represents a known step not considered by the authors. The C3H system was proposed by Lee et al. (2014). NAD(P)H (nicotinamide adenine dinucleotide phosphate reduced) flavin reductase uses NAD(P)H to reduce FAD (flavin adenine dinucleotide) to FADH$_2$ (flavin adenine dinucleotide reduced) that reacts with O$_2$ that is activated and used in the reaction. NAD$^+$ nicotinamide adenine dinucleotide phosphate oxidized

are needed than TAL to obtain higher caffeic acid production since C3H represents the bottleneck step. The supplementation of 2 mM *p*-coumaric acid to the culture medium allowed to produce up to 168 mg/L of caffeic acid.

All these studies that used C3H to convert *p*-coumaric acid to caffeic acid did not overexpress redox partners. However, several studies classify C3H as a flavin-dependent monooxygenase (Lee et al. 2014; Heo et al. 2018). *E. coli* has in its genome several genes that encode a flavin reductase that can be supporting the C3H activity and its overexpression may not be needed. Nevertheless, it is unknown if the overexpression of a flavin reductase would help to improve caffeic acid titers as this approach was never attempted. Lee et al. (2014) overexpressed CPR from *Oryza sativa* with C3H to produce bioactive hydroxyflavones. This CPR is a NAD(P)H (nicotinamide adenine dinucleotide phosphate reduced) flavin reductase and was used to supply FADH$_2$ (flavin adenine dinucleotide reduced) since C3H uses NADP (H) and O$_2$ as co-substrates. FAD (flavin adenine dinucleotide) is reduced to FADH$_2$ by NAD(P)H and then, FADH$_2$ reacts with O$_2$ that is activated and used in the reaction (Fig. 4). The CPR overexpression allowed to increase the hydroxylation by 34–50%. In a different study, Heo et al. (2018) overexpressed and purified a NAD (P)H flavin reductase from *E. coli* K-12 (Fre enzyme) to combine with C3H in vitro

to perform the hydroxylation of several stilbenes (Heo et al. 2018). These results suggest that the overexpression of an NADP(H) flavin reductase may facilitate the electron transfer and allow to increase caffeic acid titers. More studies regarding bacterial and plant C3H characterization will help to improve caffeic acid titers in the future.

Recently, caffeic acid was again produced as an intermediary to produce ferulic acid in *E. coli* K-12 JM109 (DE3) (Lv et al. 2021). *R. capsulatus* (*Rc*TAL) and *Se*C3H were used in the biosynthetic pathway. However, the caffeic acid titers obtained with only these enzymes were not reported. Although these researchers did not overexpress redox partners, they evaluated the need to increase NADPH regeneration rate as NADPH is crucial in maximizing C3H reaction. To increase NADPH availability, several genes that encode NADPH regeneration enzymes were overexpressed. The production only improved using the *pntAB* gene encoding pyridine nucleotide transhydrogenase (37%) from *E. coli* K-12 MG1655 (NADP$^+$ + NADH → NADPH + NAD$^+$). All these results indicate that the overexpression of redox partners and also the increase of NADPH regeneration should be evaluated in future studies aiming to optimize caffeic acid production.

Pathway Containing 4HPA3H

In addition to the use of C3H to convert *p*-coumaric acid to caffeic acid, other enzymes have been described. One example is the 4HPA3H complex from *E. coli* (*Ec*4HPA3H) that can convert *p*-coumaric acid to caffeic acid. This complex has been described in some *E. coli* strains that can grow in 4-hydroxyphenylacetate (4-HPA), such as *E. coli* W, B, and C strains but not K-12 strains, and presents a broad substrate range (Prieto et al. 1993). 4HPA3H complex is involved in 4-HPA metabolism to 3,4-dihydroxyphenylacetate (3,4-DHPA) (Prieto et al. 1993). 4HPA3H is encoded by the *hpaBC* operon, known as 4-HPA hydroxylase operon, which contains *hpaB* gene that encodes the monooxygenase component (HpaB) and *hpaC* gene that encodes the flavin reductase component (HpaC). This *hpaBC* operon together with *hpaGEDFHI* (meta-cleavage operon) (that encodes enzymes that allow 3,4-DHPA further metabolism) and other regulatory genes *hpaA, hpaR,* and *hpaX* constitute the complete 4-HPA metabolic pathway. The promoter that transcribes the *hpaBC* operon is positively regulated by *hpaA* product whose expression is activated by the presence of 4-HPA, 3-HPA (3-hydroxyphenylacetic acid), or phenylacetate (Prieto and García 1997). Therefore, in normal growth conditions, these genes are not expressed and the *p*-coumaric acid that is heterologously produced in *E. coli* is not converted to caffeic acid.

Lin and Yan (2012) were the first ones to use 4HPA3H complex to convert *p*-coumaric acid to caffeic. In addition, they demonstrated that 4HPA3H, amplified from *E. coli* BL21 (*Ec*4HPA3H), is also able to convert tyrosine to *L*-DOPA (L-3, 4-dihydroxyphenylalanine), and *Rc*TAL and TAL from *R. sphaeroides* (*Rs*TAL) were able to convert not only tyrosine to *p*-coumaric acid but also *L*-DOPA to caffeic acid (Fig. 5). 4HPA3H activity in the presence of FAD and NADH (nicotinamide adenine dinucleotide reduced) was evaluated. Its activity towards *p*-coumaric acid was higher than towards tyrosine. Regarding TAL enzymes activity, they
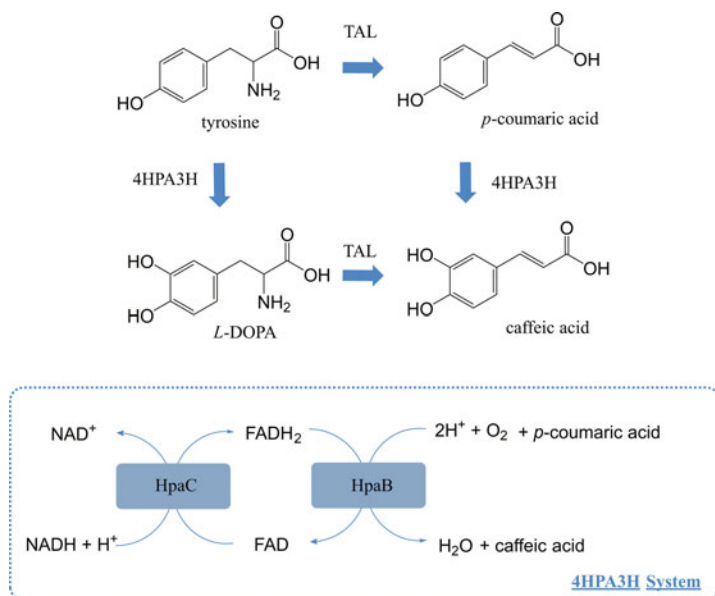
Fig. 5 Caffeic acid biosynthetic pathway in *Escherichia coli* using TAL (tyrosine ammonia lyase) and 4HPA3H (4-hydroxyphenylacetate 3-hydroxylase) complex. 4HPA3H complex is composed by HpaC (NADH-flavin oxidoreductase) that transfers reduced flavins to HpaB (4-hydroxyphenylacetate 3-monooxygenase). FAD flavin adenine dinucleotide, $FADH_2$ flavin adenine dinucleotide reduced, *L*-DOPA L-3, 4-dihydroxyphenylalanine, NAD^+ nicotinamide adenine dinucleotide oxidized, NADH nicotinamide adenine dinucleotide reduced

presented higher activity towards *L*-DOPA than tyrosine, and *Rc*TAL demonstrated higher activity towards both substrates than *Rs*TAL and therefore, it was chosen to be used together with 4HPA3H to produce caffeic acid. This pathway containing *Rc*TAL and 4HPA3H was able to produce 12.1 mg/L of caffeic acid in M9 minimal medium. The use of a tyrosine-overproducing strain allowed to increase the titer to 50.2 mg/L (Table 2). This strain included the ΔTyrR modification and the over-expression in a plasmid of TyrA^{fbr}, AroG^{fbr}, PpsA (phosphoenolpyruvate synthetase), and TktA (transkelotase 1). PpsA and TktA are responsible for the increase of AAA precursors availability (phosphoenolpyruvate and erythrose 4-phosphate, respectively).

Later, the same research group performed another study using *Ec*4HPA3H to produce caffeic acid (Huang et al. 2013). 4HPA3H counterpart from *Thermus thermophilus* HB8 was also evaluated but the *Ec*4HPA3H performance was far superior. *p*-Coumaric acid's possible toxicity to the cells was evaluated and it was concluded that a *p*-coumaric acid concentration of 5 g/L impaired growth. It was found that a smaller concentration of substrate (3 g/L *p*-coumaric acid) followed by additional feedings (0.5 g/L) after a decrease in the initial substrate concentration is a better approach to obtain high caffeic acid concentrations (3.82 g/L). Afterwards, a phenylalanine-overproducing strain (*E. coli* ATCC31884) converted into a tyrosine-

overproducing strain was used to produce caffeic acid using *Rg*TAL and *Ec*4HPA3H. This tyrosine-overproducing strain included the deletion of several genes (ΔTyrR, ΔPheA, ΔPheL (*phe* operon leader peptide), and ΔTyrA); and the overexpression of genes in the genome (PheA^{fbr} and AroH^{fbr}/AroF^{fbr} – DAHP synthetases) and in a plasmid (TyrA^{fbr}, AroG^{fbr}, PpsA, and TksA). Since the endogenous biosynthesis of phenylalanine was disrupted in this strain, 20 mg/L phenylalanine were supplemented. The authors reported that some of the culture media presented a darker color that might be related to a stop in the caffeic acid production and, consequently, to *L*-DOPA oxidation to melanin. To prevent *L*-DOPA accumulation, inoculants' optical density at 600 nm (OD$_{600}$) was optimized. The best de novo result (766.68 mg/L) was obtained when the inoculants' OD$_{600}$ was 2.4.

Recently, *Ec*HPA3H was also combined with *Se*TAL to produce caffeic acid as an intermediary in a heterologous pathway to produce chlorogenic acid in *E. coli* BW25113 (Li et al. 2021a). Using these enzymes, they were able to produce about 198 mg/L of caffeic acid in a ΔTyrR strain. They also overexpressed TktA, AroF, and AroE (shikimate dehydrogenase) in a plasmid but, surprisingly, the titers did not improve.

4HPA3H was also identified in *Pseudomonas aeruginosa* PAO1 (*Pa*4HPA3H). Furuya and Kino (2014) found that this enzyme had activity towards *p*-coumaric acid, converting it to caffeic acid. However, caffeic acid was also found to be oxidized to 3,4,5-trihydroxycinnamic (3,4,5-THCA) by the same enzyme. Although the enzyme has higher specificity towards *p*-coumaric acid, during substrate specificity assays, it was shown that it was able to convert 40% of 10 mM caffeic acid supplied to 3,4,5-THCA within 12 h. This can be highly disadvantageous. However, during in vivo production with *E. coli*, 3,4,5-THCA production was only observed after *p*-coumaric acid was almost totally converted. In addition, to avoid substrate inhibition, 20 mM of *p*-coumaric acid was supplied four times during fermentation. *E. coli* was able to produce 10.2 g/L of caffeic acid in 24 h in potassium phosphate buffer (PBS) supplemented with glycerol. Surprisingly, caffeic acid production was higher when no carbon source was added to PBS than in the presence of glucose. The authors concluded that the lower production could be related to the decrease in pH in the presence of glucose. Although the authors state that the enzyme converts caffeic acid into 3,4,5-THCA, surprisingly they do not evaluate caffeic acid consumption in this experiment.

*Pa*4HPA3H gene was also used to produce caffeic acid in a tyrosine-overproducing *E. coli* strain (ΔTyrR, TyrA^{fbr}/AroG^{fbr}/PpsA/TksA overexpressed in a plasmid) using kraft pulp as substrate (Kawaguchi et al. 2017). *Pa*4HPA3H and TAL from *Streptomyces* sp. WK-5344 (*fevV* gene) (highly specific for tyrosine) were combined to produce 233 mg/L of caffeic acid by simultaneous saccharification and fermentation. Separate hydrolysis and fermentation led to lower production (37.9 mg/L caffeic acid). Cellulase cocktail Cellic CTec2 was used to convert cellulose to glucan. In this study, it was again shown that glucose accumulation is detrimental to caffeic acid production. Glucose limitation is important to shift carbon metabolism from the production of organic acids (acetate, lactate by-products) towards caffeic acid (Kawaguchi et al. 2017). In addition, higher amounts of

cellulose/kraft pulp can inhibit caffeic acid production due to the presence of some compounds present in the kraft pulp hydrolysate.

The pathway containing TAL and 4HPA3H has the disadvantage of producing more intermediates (e.g., *L*-DOPA) than the pathway with TAL and C3H, and the aggravating of, for example, *Pa*4HPA3H being also known to convert caffeic acid to another hydroxycinnamic acid (3,4,5-THCA). However, this pathway (with *Ec*4HPA3H) allowed obtaining higher caffeic acid concentrations than the pathway with TAL and C3H. Nevertheless, it is important to point out that the growth conditions, tyrosine-overproducing strains, and TAL enzyme sources were different which can influence the final caffeic acid titers.

Pathway Containing CYP199A2

Another pathway that has been widely studied is the one that uses the TAL enzyme with the bacterial CYP monooxygenase CYP199A2. CYP199A2 was discovered in *Rhodopseudomonas palustris*, and Furuya et al. (2012) demonstrated that this enzyme exhibits activity towards *p*-coumaric acid, producing caffeic acid. In addition, the authors identified a residue (F185) that controls regioselectivity and substrate specificity. The F185L mutant allowed to obtain 5.5 times more caffeic acid than the wild-type enzyme. By using this mutant, it was possible to obtain 2.8 g/L of caffeic acid from 20 mM of *p*-coumaric acid. CYP199A2 mutant was co-expressed with palustrisredoxin gene (*pux*), an iron-sulfur cluster from *R. palustris* and putidaredoxin reductase gene (*pdR*), a flavoprotein from *Pseudomonas putida*. These genes are essential to provide CYP199A2 with the redox partners it needs. The redox partner's function is to support CYP199A2 catalytic activity by transferring electrons from NADH to CYP199A2 for oxygen activation (Fig. 6). The
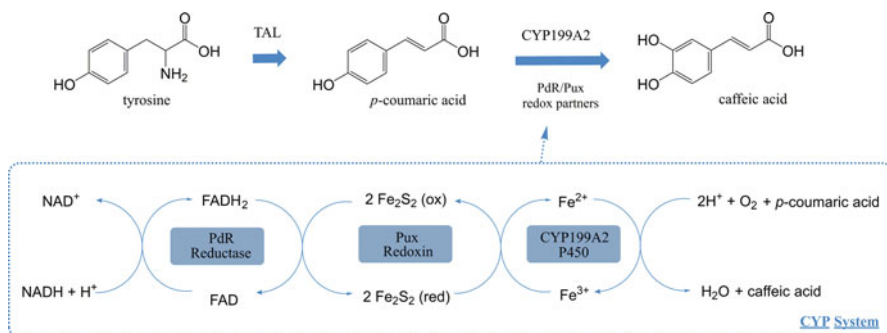
Fig. 6 Caffeic acid biosynthetic pathway in *Escherichia coli* using a cytochrome P450 (CYP) enzyme. TAL (tyrosine ammonia lyase), as in the other pathways, converts tyrosine to *p*-coumaric acid. The conversion of *p*-coumaric acid to caffeic acid is catalyzed by a Class I CYP system that is composed of CYP enzyme CYP199A2, an oxygenase-coupled NADH-dependent ferredoxin reductase (ONFR) (PdR – putidaredoxin reductase) and an iron-sulfur ferredoxin (Pux – palustrisredoxin). Other redox partners are also known to be able to assist CYP199A2 through electron transfer. FAD flavin adenine dinucleotide, $FADH_2$ flavin adenine dinucleotide reduced, NAD^+ nicotinamide adenine dinucleotide oxidized, NADH nicotinamide adenine dinucleotide reduced

putidaredoxin complex (Pdx – putidaredoxin/PdR/CYP101A1) from *P. putida* was first described (Peterson et al. 1990) than the one from *R. palustris*. Therefore, the putidaredoxin complex helped to elucidate CYP199A2 complex in *R. palustris*. After the discovery of Pux (palustrisredoxin) (Bell et al. 2006), it was concluded that this protein outperforms Pdx from *P. putida* and, consequently, this protein was always used for further studies with CYP199A2 together with PdR (Furuya and Kino 2010). PdR rare codon, GTG, was changed to ATG since it was proved that it results in an 18-fold increase in protein expression (Peterson et al. 1990). The CYP199A2 mutant, in addition to the F185L mutation previously described, also did not include the first 21 bp (Furuya et al. 2012). According to Furuya (personal communication), this decision was based on the results obtained using annotation software that annotated the 8th aa (GTG) from CYP199A2 as a start codon. Later, this enzyme mutant codon-optimized for *E. coli* (with or without the first 7 aa) was combined with *Rg*TAL to produce caffeic acid from tyrosine (Rodrigues et al. 2015b). It was found that CYP199A2 F185L without the first 7 aa (NΔ7) (from now on simply referred as CYP199A2) allowed to significantly improve the caffeic acid production. Moreover, it was demonstrated that the production improved when the redox partners (PdR/Pux) expression was induced 2.5 h after *Rg*TAL and CYP199A2 expression induction. This induction delay may alleviate the metabolic burden caused by the expression of several enzymes and numerous plasmids (Wu et al. 2013). The highest caffeic acid production in this study was 280 mg/L (3 mM tyrosine supplemented). This concentration was significantly superior to the one obtained with C3H (180 mg/L) in the same study. This demonstrated that the CYP199A2 system is a better option to produce caffeic acid although it needs the expression of more enzymes.

The use of C3H and CYP199A2 in caffeic acid production was also evaluated and compared using heat shock promoters. Chemical inducers frequently used at laboratory scale (e.g., IPTG – isopropyl β-D-1-thiogalactopyranoside, aTc – anhydrotetracycline) are expensive and potentially toxic and, consequently, their presence in the waste effluents or as contaminants in the final product should be eliminated at all costs. Therefore, their use at a larger scale should be avoided (Rodrigues and Rodrigues 2018). Heat shock promoters allow using the temperature as an induction mechanism. This method is cheap, safe, and easy to implement leading to fewer contaminations. The *E. coli* promoters from the heat shock proteins were thoroughly studied and have been used to induce the expression of heterologous pathways or as biosensors for environmental applications (Rodrigues et al. 2014; Chugunova et al. 2015). *E. coli* heat shock promoters from DnaK and IbpA were used to control the expression of the caffeic acid biosynthetic pathway (Rodrigues et al. 2017a). These two promoters were chosen because the *dnaK* gene is known to present a high expression at heat shock conditions and a considerable expression also at 37 °C, while *ibpA* is known to have a very high expression at heat shock temperatures and low expression at normal growth temperatures. In order to improve the translation initiation rates, the ribosome binding sites (RBS) were optimized using RBS calculator (https://salislab.net/software/). The conversion of tyrosine to *p*-coumaric acid was very high using *dnaK* promoter and an optimized RBS. It reached higher levels

than the ones obtained using T7 promoter (2.5 mM vs. 1.4 mM from 3 mM of tyrosine). However, caffeic acid production using C3H and *ibpA* promoter did not exceed 20 μM and 47 μM when expressed alone or combined with TAL, respectively. CYP199A2 expression under *dnaK* was significantly higher compared to C3H reaching production up to 370 μM (66.7 mg/L) from 2 mM of *p*-coumaric acid. The redox partners (Pux/PdR) were expressed under *dnaK* and *ibpA* promoters and no significant differences were observed in caffeic acid production. Again, these results suggest that the redox partners do not need to be available in high amounts to support the CYP199A2 activity. It was also concluded that the production was higher when the cultures were maintained at 37 °C for the whole time than when the heat shock was applied for 5 min (48 °C in water bath). This is possibly related to the fact that the exposure to heat is fast and the gene expression only increases temporarily and, at the same time, the heat can cause long-lasting adverse effects in the production of caffeic acid or other relevant metabolites by, for example, increasing the formation of inclusion bodies (Rodrigues et al. 2014). Although significant work still needs to be done to optimize the induction of heterologous pathways using these promoters, the results demonstrated that these types of promoters and RBS design can be extremely helpful in the fine-tuning of heterologous pathways with multiple steps.

More recently, Haslinger and Prather (2020) studied in more detail the CYP199A2 pathway for caffeic acid production in *E. coli* MG1655. First, they evaluated different TAL enzymes (*Rg*TAL, *Se*TAL, and TAL from *Flavobacterium johnsoniae* – *Fj*TAL) and CYP199A2 (F185L NΔ7)/Pux/PdR performance regarding caffeic acid production with or without tyrosine supplementation. In these studies, no tyrosine-overproducing strain was used. *Rg*TAL has very high activity towards tyrosine but it has an even higher activity towards phenylalanine (Xue et al. 2007b; Zhou et al. 2016). *Fj*TAL and *Se*TAL also exhibit very high catalytic efficiency but higher selectivity for tyrosine than for phenylalanine (2400 and 1200 times more, respectively) (Jendresen et al. 2015). When high amounts of tyrosine were available, all enzymes were able to efficiently produce caffeic acid, but at low concentrations of tyrosine, *Fj*TAL outperformed all the other TAL. Using *Fj*TAL, up to ~0.1 mM (18 mg/L) and ~1.8 mM (324 mg/L) of caffeic acid was produced in the absence or presence of tyrosine (3 mM), respectively. Therefore, *Fj*TAL was selected to perform the other tests regarding CYP199A2 redox partners. The authors started by testing alternative redox partners. As previously mentioned, PdR is not part of CYP199A2 redox natural system but it was used in previous studies with CYP199A2 given that it was identified earlier than palustrisredoxin reductase (PuR) from *R. palustris* (Xu et al. 2009). Haslinger and Prather (2020) compared Pux/PdR and Pdx/PdR redox systems with the natural one containing Pux/PuR. The highest titer (1.6 mM – 288 mg/L caffeic acid) was obtained with the natural redox system after *p*-coumaric acid supplementation (3 mM). It was four times higher than the hybrid redox system Pux/PdR. Caffeic acid production was residual using Pdx/PdR system. These results show that the natural redox system is a better option to produce caffeic acid. In addition, the combination of *Fj*TAL and CYP199A2/Pux/PuR allowed obtaining ~0.15 mM (27 mg/L) of caffeic acid from

glucose, 1.5 times more than when Pux/PdR system was used. Next, the authors doubled the number of copies of *pux* by expressing it in two different plasmids. The increase in the number of copies allowed to obtain almost 0.2 mM of caffeic acid from glucose after 72 h and 0.26 mM (47 mg/L) after 96 h. This demonstrated that the availability of Pux was limiting the CYP199A2 performance. The titers achieved using this pathway (*Fj*TAL combined with CYP199A2/Pux/PuR with an extra copy of *pux*) where slightly higher than the ones obtained using other pathways previously described without L-tyrosine supplementation (43 mg/L using C3H and 12.1 mg/L using 4HPA3H pathways). However, the fermentation time was lower in the other cases. Nevertheless, this strain is able to produce high amounts of caffeic acid when tyrosine is supplemented, thus suggesting that it will be able to produce high amounts of this compound in a tyrosine-overproducing *E. coli* strain.

Since the redox partners proved to highly influence caffeic acid production, Haslinger and Prather (2020) also applied a tethering strategy to improve the CYP199A2 system. In this strategy, the proteins of the CYP199A2 system were fused to the DNA sliding clamp PCNA (proliferating cell nuclear antigen) from *Sulfolobus solfataricus* P2 archaeon. This PCNA complex is a heterotrimer of three subunits (PCNAI, PCNAII, and PCNAIII) that acts as a scaffold and stimulates the activity of DNA ligase, DNA polymerase, and flap endonuclease of *S. solfataricus* (Dionne et al. 2003). The fusion of CYP systems to these PCNA subunits has proven in the past to improve the catalytic efficiency of several CYP proteins in vitro. Due to this fusion, CYP enzymes and their redox partners are very close to each other, enabling an efficient electron transfer and an increase in the catalytic activity without the need of using excess ferredoxin (Hirakawa and Nagamune 2010). Hence, the authors used this strategy for the first time in whole cells. They explored two fusion designs: PuR or PdR fused to PCNAI, Pux or Pdx to PCNAII, and CYP199A2 to PCNAIII (design I); and CYP199A2 fused to PCNAI, Pux or Pdx to PCNAII, and PuR or PdR to PCNAIII (design II). It was observed that the caffeic acid production from *p*-coumaric acid for the tethered redox systems was, in most cases, higher than for the respective free enzymes. The systems that benefited the most from the fusions were the ones that presented lower production in the free state. The highest caffeic acid production from *p*-coumaric acid, 2.3 mM, was obtained with the tethered Pux/PdR system using design I, followed by 2.1 mM obtained with tethered Pdx/PdR system using design II. Afterward, they combined *Fj*TAL with the tethered systems to produce caffeic acid from glucose but the production was lower than the ones obtained using free enzymes probably due to the increased metabolic burden related to the expression of several enzymes and the tethering domains. Consequently, the authors concluded that the tethering strategy should only be used in cases that the CYP/redox partners expression is low or if the natural redox partners are unknown and surrogate redox partners (such as Pdx/PdR for CYP199A2) need to be used for the reconstitution of CYP activity.

Caffeic Acid Heterologous Production in Yeast

In the last 2 years, the heterologous production of caffeic acid in *Saccharomyces cerevisiae* started to emerge. Currently, three different studies report different pathways for caffeic acid production (Liu et al. 2019, 2020b; Zhou et al. 2021) (Table 3), while *p*-coumaric acid production was previously thoroughly studied (Vannelli et al. 2007; Rodriguez et al. 2015; Mao et al. 2017; Borja et al. 2019). The first study tested caffeic acid production using TAL from *Rhodosporidium toruloides* (*Rt*TAL) and different enzymes to convert *p*-coumaric acid to caffeic acid (C3H, CYP199A2, 4HPA3H) (Liu et al. 2019). *Rt*TAL was previously used in pathways to produce naringenin and resveratrol in *S. cerevisiae* and was found to be very efficient (Rodriguez et al. 2015; Zhang et al. 2017). *Se*C3H and CYP199A2, codon-optimized for *S. cerevisiae*, did not demonstrate activity in this organism. Afterward, Liu et al. (2019) compared the production using different 4HPA3H enzymes. The production was obtained in *S. cerevisiae* BY4741 from 500 mg/L of tyrosine. The combination of *Rt*TAL and *Ec*4HPA3H allowed to produce 6.3 mg/L of caffeic acid. In order to increase the titers, other 4HPA3H that presented high similarity to *Ec*4HPA3H (>90%) were tested, namely 4HPA3H from *Klebsiella pneumoniae* (*Kp*4HPA3H), 4HPA3H from *Photorhabdus luminescens* (*Pl*4HPA3H), *Pa*4HPA3H and 4HPA3H from *P. putida* (*Pp*4HPA3H). Although the production with *Kp*4HPA3H, *Pl*4HPA3H, and *Pp*4HPA3H only reached 1.1, 2.1, and 16.8 mg/L of caffeic acid, respectively, *Pa*4HPA3H was shown to be promising as it allowed to produce 68.2 mg/L. To evaluate if the combination of genes encoding 4HPA3H from different organisms could improve the titers, the authors combined the over-expression of *hpaC* or *hpaB* from *P. aeruginosa* with *hpaB* or *hpaC* from the other organisms, respectively. The highest titer using the gene *hpaC* from *P. aeruginosa* was 12.8 mg/L when combined with *hpaB* from *E. coli*. The other titers were even lower (1.3–6.8 mg/L) which suggested that HpaB could be the bottleneck enzyme. In addition to the other *hpaB* from the referred organisms, they also combined *hpaC* from *P. aeruginosa* with *hpaB* from *Sulfobacillus acidophilus* TPY and *Escherichia cloacae*. The titers obtained were also low, 5.7 and 22.7 mg/L, respectively. Regarding the combinations of *hpaB* from *P. aeruginosa* with other *hpaC* genes, it was concluded that the results in general were improved when compared with the natural combinations. When combined with *hpaC* from *E. coli*, *P. putida*, *K. pneumoniae*, and *P. luminescens*, the production was 37.6, 68.49, 175.86, and 241.3 mg/L, respectively. The *hpaC* from *Salmonella enterica* was also tested and 289.4 mg/L were obtained (from 500 mg/L of tyrosine), the highest titer reported in this study. Here it was demonstrated that is important to find a good combination of *hpaB* and *hpaC* genes in order to obtain high titers of caffeic acid using the 4HPA3H. To understand why HpaB from *P. aeruginosa* allowed obtaining higher production than the other HpaB, the authors compared its amino acid sequence to the ones from *P. luminescens, K. pneumoniae, E. coli, E. cloacae*, *S. acidophilus* TPY, and *P. putida*. The authors concluded that HpaB sequence from *P. aeruginosa* was unique. It has six key amino acids different from the sequences from other organisms that are positioned around the FAD binding site. The theory that these residues

Table 3 Heterologous production of caffeic acid in *Saccharomyces cerevisiae*

Pathway	S. cerevisiae	Pathway genes[a]	TYR modifications[b]	Conditions (precursor underlined when supplemented)	Titer (mg/L)	References
4HPA3H	BY4741	RtTAL/Ec4HPA3H	–	Synthetic complete medium (20 g/L glucose) with 500 mg/L tyrosine at 30 °C for 96 h in shake flasks	6.3	Liu et al. (2019)
		RtTAL/Pa4HPA3H			68.2	
		RtTAL/PaHpaB/PpHpaC			68.49	
		RtTAL/PaHpaB/KpHpaC			175.86	
		RtTAL/PaHpaB/PlHpaC			241.3	
		RtTAL/PaHpaB/SenHpaC			289.4	
	BY4741	RgTAL[c]/PaHpaB/SenHpaC[c]	–	YPD medium (yeast-extract, peptone, dextrose) (20 g/L glucose) at 30 °C for 72 h	222.7	Zhou et al. (2021)
			Aro4^{fbr}/ΔAro3		330.4	
			Aro4^{fbr}/ΔAro3/Aro7^{fbr}		341.8	
			Aro4^{fbr}/ΔAro3/Aro7^{fbr}/ΔAro10		435.2	
			Aro4^{fbr}/ΔAro3/Aro7^{fbr}/ΔAro10TyrC		569.0	

C3H	BY4742	RcTAL AtC3H AtCPR1	—	Synthetic complete medium (20 g/L glucose) at 30 °C for 120 h in shake flasks. Production from glucose or supplemented tyrosine (500 mg/L)	0.183 1.5 (+ tyr)	Li et al. (2020b)
			Aro4^{fbr}/Aro7^{fbr} ΔAro10 /ΔPDC5	Synthetic complete medium (20 g/L glucose) at 30 °C for 120 h in shake flasks	2.78	
				YPD medium (40 g/L glucose) at 30 °C for 120 h in shake flasks	11.4	

[a]Genes from caffeic acid pathway: 4HPA3H – 4-hydroxyphenylacetate 3-monooxygenase complex, C3H – 4-coumarate 3-hydroxylase, CPR1 – cytochrome P450 reductase 1, HpaB – 4-hydroxyphenylacetate 3-monooxygenase, HpaC – NADH-flavin oxidoreductase, TAL – tyrosine ammonia lyase; Genes sources: At – Arabidopsis thaliana, Ec – Escherichia coli, Kp – Klebsiella pneumoniae, Pa – Pseudomonas aeruginosa, Pl – Photorhabdus luminescens, Pp – Pseudomonas putida, Rc – Rhodobacter capsulatus, Rg – Rhodotorula glutinis, Rt – Rhodosporidium toruloides, Sen – Salmonella enterica

[b]Some studies used a tyrosine-overproducing strain (TYR strain) in which: Aro10 (phenylpyruvate decarboxylase), PDC5 (pyruvate decarboxylate), and Aro3 (3-deoxy-D-arabino-heptolosonate-7-phosphate (DAHP) synthetase – inhibited by phenylalanine) genes were deleted (ΔAro10/ΔPDC5/ΔAro3) to direct the pathway to tyrosine production; Aro4 (DAHP synthetase) and Aro7 (chorismate mutase) feedback inhibition-resistant variants (Aro4^{fbr}/Aro7^{fbr}) were overexpressed to eliminate tyrosine- and phenylalanine-mediated repression. TyrC (prephenate dehydrogenase) from Zymomonas mobilis was overexpressed since it is not feedback inhibited by phenylalanine

[c]Genes controlled by galactose promoters in a strain with GAL80 (galactose metabolism regulatory protein) deleted

impact HpaB catalytic activity should be evaluated in future studies via point mutagenesis.

The caffeic acid pathway using C3H was also recently reconstructed in *S. cerevisiae* BY4742 (Li et al. 2020b). For that purpose, the authors used codon-optimized C3H from *Arabidopsis thaliana* (*At*C3H). To aid *At*C3H, they also overexpressed a codon-optimized CPR, CPR1 from *A. thaliana* (*At*CPR1). By adding 500 mg/L of *p*-coumaric acid, they were able to observe a peak correspondent to caffeic acid production by HPLC (high-performance liquid chromatography). According to the authors, this was the first report on the successful expression of a plant-derived C3H in *S. cerevisiae*. Afterwards, they compared the production when using different *At*CPR enzymes (*At*CPR1 and *At*CPR2). The caffeic acid production with *At*CPR1 reached 17.7 mg/L while with *At*CPR2 only reached 4.4 mg/L, which is consistent with previous studies that used these CPRs for C4H expression and concluded that *At*CPR1 is the best option (Koopman et al. 2012). They also compared BY4742 and BY4741 strains and concluded BY4742 was a better choice (17.5 mg/L vs. 5.7 mg/L, respectively, from *p*-coumaric acid). The supplementation of *p*-coumaric acid concentration was also optimized (100–1000 mg/L). They concluded that the caffeic acid production increased until 600 mg/L of *p*-coumaric acid was supplemented (18.1 mg/L caffeic acid) but higher concentrations led to a decrease in the production. As in *E. coli*, very high concentrations of *p*-coumaric acid proved to inhibit *S. cerevisiae* growth significantly. The *p*-coumaric acid addition time was also studied and when it was added at 0, 4, or 8 h, the production was higher (~18 mg/L) than when added at 12 or 16 h (~10 mg/L)

To produce caffeic acid from tyrosine, Li et al. (2020b) combined *At*C3H/*At*CPR1 with codon-optimized *Rc*TAL. Through the supplementation of tyrosine (500 mg/L), 1.5 mg/L of caffeic acid was obtained. The authors believe that the differences achieved between their study and Liu et al. (2019) may be related to the use of C3H but also the selected TAL, since *Rt*TAL was previously reported to be more efficient in producing *p*-coumaric acid in yeast than *Rc*TAL (Jendresen et al. 2015). The production of *p*-coumaric acid from supplemented tyrosine using *Rc*TAL was only 29.6 mg/L. Subsequently, the authors decided to evaluate if *p*-coumaric acid and then caffeic acid could be produced from endogenous tyrosine. However, only 3.85 and 0.183 mg/L, respectively, were produced after 5 d. Therefore, to increase the titers from glucose, they engineered *S. cerevisiae* BY4742 to overproduce tyrosine. Since Aro4 (DAHP synthetase) and Aro7 (chorismate mutase) are inhibited by tyrosine, they co-expressed feedback inhibition-resistant mutants (Aro4^{fbr}/Aro7^{fbr}). The authors integrated the overexpression cassettes into PDC5 (pyruvate decarboxylase) and Aro10 (phenylpyruvate decarboxylase) gene positions. These genes perform the decarboxylation of *p*-hydroxyphenylpyruvate and deviate the flux from the AAA pathway to the Ehrlich pathway which is not desired, whereby their knockout is favorable (Fig. 3). Using this tyrosine-overproducing strain, the production of *p*-coumaric acid and then caffeic acid increased to 49.8 and 2.78 mg/L, respectively (corresponding to an improvement of 12.9 and 15.2-fold). These concentrations were not only higher than the ones obtained in the BY4742 strain from glucose, but also from 500 mg/L of tyrosine. In addition to genetic optimizations, the

fermentation medium was also optimized. The authors compared the production in synthetic medium (2% glucose) with the production using rich medium YPD (yeast extract-peptone-dextrose) with 4% glucose. They were able to produce 11.4 mg/L of caffeic acid using this medium without any selective pressure (the same plasmids were used for comparison). According to the authors, 78.7% of the cells were able to maintain the plasmids during the fermentation, whereby this strategy was more favorable than using antibiotics for selection. Nevertheless, pathway integration in the genome will be considered in the future. The higher production obtained using the rich medium may be related to the higher cell density reached as the specific caffeic acid titers were very similar – 0.630 and 0.528 mg/L/OD (optical density) for growth in synthetic and rich medium, respectively. Since the rich medium allows to obtain higher production and is cheaper, it is considered a more viable option to produce caffeic acid.

Li et al. (2020b) also found that the *p*-coumaric acid consumed was not completely converted to caffeic acid. This was more notorious when a rich medium was used possible due to the higher cell density attained. This consumption is related to the *p*-coumaric acid decarboxylation in *S. cerevisiae*. This yeast contains phenylacrylic acid decarboxylase (PAD1) and ferulic acid decarboxylase (FDC1) that can convert *p*-coumaric acid to 4-vinylphenol and ferulic acid to 4-vinylguaiacol. When PAD1 and/or FDC1 are deleted, *p*-coumaric acid consumption is not observed (Mukai et al. 2010). Therefore, these can be suitable targets to knock out in order to improve caffeic acid production in *S. cerevisiae*. However, despite *p*-coumaric acid being converted to other compounds, there is still a large amount that is not converted which, once again, proves that C3H is the rate-limiting enzyme of caffeic acid pathway. The authors suggested that this problem may be solved by integrating several copies into *S. cerevisiae* genome using a delta integration strategy (Song et al. 2017).

More recently, Zhou et al. (2021) also used 4HPA3H to produce caffeic acid in BY4741 strain. They used codon-optimized *Rg*TAL combined with codon-optimized HpaB from *P. aeruginosa* and HpaC from *S. enterica* (that were reported as being the highest production in the Liu et al. (2019) study). To express these genes, the authors used a modified GAL system. To avoid adding galactose, which is expensive, to the medium, they replaced GAL80 gene for HpaB and HpaC genes under control of GAL1 and GAL10 promoters, respectively. GAL80 gene encodes a repressor of GAL4 that represses expression when galactose is absent. The gene *Rg*TAL was also integrated into the genome into DPP1 (diacylglycerol pyrophosphate phosphatase) site. Using this strategy, 222.7 mg/L of caffeic acid was produced from glucose (YPD) after 72 h. The caffeic acid production started after glucose was completely consumed. This titer is almost as high as the one obtained by Liu et al. (2019) (289.4 mg/L) using a similar pathway but with the supplementation of 500 mg/L of tyrosine. In order to improve the production, the authors constructed a tyrosine overexpressing strain. They integrated Aro4[fbr] under control of GAL1 promoter into Aro3 site (Aro3 – DAHP synthase – is inhibited by phenylalanine). This strain allowed to produce 330.4 mg/L of caffeic acid. However, it was observed a decrease in biomass by 10.2%. Afterwards, they also overexpressed Aro7[fbr]

(controlled by GAL10 promoter and also integrated into Aro3 site) and produced 341.8 mg/L of caffeic acid. Finally, they modified the strain to redirect the flux to the AAA pathway by eliminating competing pathways. They deleted Aro10 gene to reduce the consumption of *p*-hydroxyphenylpyruvate and the deviation to the Ehrlich pathway as performed by Li et al. (2020b). With this deletion, the strain accumulated 435.2 mg/L of caffeic acid. Afterward, they integrated TyrC (cyclohexadienyl dehydrogenase) gene from *Zymomonas mobilis* into Aro10 gene loci. Since TyrC is not feedback inhibited by phenylalanine as Tyr1 (prephenate dehydrogenase) from *S. cerevisiae*, it allows increasing the flux to *p*-hydroxyphenylpyruvate, the direct precursor of tyrosine. This allowed increasing caffeic acid titer to 569.0 mg/L in 72 h. The authors also tested if PDC5 deletion could improve the production but it was not possible to observe an improvement. Therefore, the highest production of caffeic acid from glucose in this study was 569.0 mg/L, which is also the highest titer by far reported in *S. cerevisiae*. As in *E. coli*, the highest titer was obtained using a tyrosine-overproducing strain carrying the 4HPA3H pathway for de novo caffeic acid biosynthesis.

Conclusions and Future Perspectives

Caffeic acid heterologous production has been extensively studied in the last decade not only due to its several promising therapeutic activities but also because it is a precursor of other relevant compounds with therapeutic and industrial potential. *E. coli* has been the most studied chassis to carry the caffeic acid pathway. However, in the last 2 years, caffeic acid production in *S. cerevisiae* emerged and the maximum titers obtained (569 mg/L) are almost as high as those obtained in *E. coli* (around 767 mg/L) from simple carbon sources in tyrosine-overproducing strains. This demonstrated that these two hosts can be good chassis to optimize caffeic acid production at a larger scale. Although the pathway is not complex, it has proved to be challenging as the enzyme that performs the second step that converts *p*-coumaric acid to caffeic acid has limited catalytic efficiency. Three enzymes/enzyme complexes have been thoroughly studied and the one that allowed to obtain higher titers was the 4HPA3H complex, both in *E. coli* and *S. cerevisiae*, which suggests that new studies should be carried out with this enzymatic complex that can be found in different microbes. The first step performed by the TAL enzyme that converts tyrosine to *p*-coumaric acid is highly efficient. In general, all the TAL enzymes evaluated exhibited high efficiency. However, this results in the accumulation of high amounts of *p*-coumaric acid that inhibit the TAL enzyme activity and affect the productivity and the strain's growth due to its toxicity. This problem can be slightly attenuated in a tyrosine-overproducing strain since tyrosine is made available at a slower rate than when tyrosine is directly supplemented to the culture medium, which can lead to a lower accumulation of *p*-coumaric acid and, consequently, lower toxic effect if in the presence of a minimally efficient enzyme responsible for the second step. The discovery of a more catalytically efficient enzyme/enzyme complex for this step is essential to witness advances in caffeic acid heterologous production.

The research regarding 4HPA3H complex is still in its infancy and there are several enzymes from unexplored microorganisms that can catalyze this step. However, until a more efficient enzyme to perform this step is discovered, other strategies can be used to minimize *p*-coumaric acid toxic effect and improve the titers. One of these strategies includes the use of tolerance engineering to increase strain resistance to *p*-coumaric acid. Several studies concluded that transporter engineering (modifications or overexpression of genes that encode transporter proteins involved in AAA catabolism) can confer tolerance to *p*-coumaric acid (and caffeic acid) by improving the secretion of the aromatic acids (Van Dyk et al. 2004; Pereira et al. 2020) and, consequently, increase the titers. Another promising strategy is the overexpression of heat shock and membrane-associated genes whose transcription is highly induced in the presence of toxic compounds such as hydroxycinnamic acids (Lee et al. 2012). The overexpression of proteins related to stress response when hydroxycinnamic acids are accumulated can increase strain tolerance to these compounds by helping the cells to maintain membrane integrity and to shift from "survival mode" to "growth and production mode" (Rodrigues and Rodrigues 2018). Additionally, the use of coculture engineering can also help reducing the *p*-coumaric acid toxicity and the metabolic burden (Rodrigues et al. 2020). All of these strategies provide a starting point to increase *E. coli* or *S. cerevisiae* chassis tolerance to *p*-coumaric acid and to further enhance caffeic acid production.

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