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Published in:
Biotechnology and Bioengineering

Link to article, DOI:
10.1002/bit.28292

Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Virklund, A., Jensen, S. I., Nielsen, A. T., & Woodley, J. M. (2023). Combining genetic engineering and bioprocess concepts for improved phenylpropanoid production. *Biotechnology and Bioengineering*, *120*(3), 613-628. https://doi.org/10.1002/bit.28292

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Check for updates

Received: 17 May 2022 | Revised: 2 November 2022 | Accepted: 22 November 2022

DOI: 10.1002/bit.28292

REVIEW

Combining genetic engineering and bioprocess concepts for improved phenylpropanoid production

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Funding information
Novo Nordisk Foundation, Grant/Award Number: NNF17SA0031362

Abstract

The group of natural aromatic compounds known as phenylpropanoids has diverse applications, but current methods of production which are largely based on synthesis from petrochemicals or extraction from agricultural biomass are unsustainable. Bioprocessing is a promising alternative, but improvements in production titers and rates are required to make this method profitable. Here the recent advances in genetic engineering and bioprocess concepts for the production of phenylpropanoids are presented for the purpose of identifying successful strategies, including adaptive laboratory evolution, enzyme engineering, in-situ product removal, and biocatalysis. The pros and cons of bacterial and yeast hosts for phenylpropanoid production are discussed, also in the context of different phenylpropanoid targets and bioprocess concepts. Finally, some broad recommendations are made regarding targets for continued improvement and areas requiring specific attention from researchers to further improve production titers and rates.

KEYWORDS
aromatic compounds, bioprocessing, genetic engineering, phenylpropanoids

1 | INTRODUCTION

Phenylpropanoids are a diverse group of aromatic compounds naturally synthesized by plants from the amino acid phenylalanine. They serve diverse functions in nature as structural components in lignin, giving protection from microorganisms, attracting pollinators, and scavenging free radicals. These chemicals are also valuable for the production of polymers, as food ingredients, cosmetic ingredients, and health products, driving interest in efficient production methods. Current production methods are based on chemical synthesis, agricultural biomass extraction, and bioprocessing (Flourat et al., 2020). Chemical synthesis often relies on petroleum-derived starting materials and is neither sustainable nor considered natural for use in consumer products. While some types of biomass extraction are considered natural, low yields make the method unsustainable, and the extracts contain a variety of phenylpropanoids which may be difficult to separate from one another. This leaves bioprocessing, which can be considered natural and potentially sustainable depending on the feedstock, and with which it is possible to increase yields and reduce by-product formation. The fermentation of phenylpropanoids from sugars relies on the shikimic acid and aromatic amino acid pathways to produce L-phenylalanine and L-tyrosine. Phenylalanine and tyrosine can be de-aminated by phenylalanine/tyrosine ammonia lyase (PAL/TAL) to produce cinnamic acid and *p*-coumaric acid, respectively, from which other phenylpropanoids are derived. However, production can be limited by the toxicity of phenylpropanoids towards the production hosts (Lou et al., 2012; Vestergaard & Ingmer, 2019), as well as flux

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limitations through certain key reactions (Gu et al., 2020; Moore et al., 2021). Genetic engineering strategies have been used to improve tolerance of production hosts towards phenylpropanoids, improve flux through rate-limiting steps, and increase the supply of metabolic precursors and co-factors. Bioprocess concepts have also been used with success to reduce phenylpropanoid toxicity and to compensate for flux limitations. These efforts have led to an increase in production metrics, and several heterologous pathways have been established in microorganisms as proof-of-concept to expand the diversity of target phenylpropanoids (J. Li et al., 2019; Yang et al., 2020). Especially the combination of genetic engineering and bioprocess concepts seems promising and maybe even necessary to achieve commercial realization of phenylpropanoid production via bioprocessing.

1.1 | Applications of phenylpropanoids

Phenylpropanoids as a group have many potential applications, and we shall briefly review some of the individual phenylpropanoids and their main applications. Styrene or 4-vinylphenol can undergo free radical polymerization to create their respective polymers, polystyrene or poly(4-vinylphenol) (Barclay et al., 1998). Polystyrene is one of the most widely used plastics in the world with uses including packaging, insulation, and more familiarly disposable laboratory products. Poly(4-vinylphenol) is used mainly in electronics (I. Y. Lee et al., 2014), but also has applications in photoresist materials and antimicrobial coatings. While it has not itself been polymerized, zosteric acid can be incorporated into surface coatings to prevent biofilm formation (Newby et al., 2006), with promising applications in the maritime industry.

Many phenylpropanoids are active ingredients in traditional medicines and herbal supplements, as well as precursors to modern drugs. The flavonoid baicalein is approved as traditional medicine in Japan, and acts as an allosteric modulator of the $GABA_A$ receptor (De Carvalho et al., 2011). Mandelic acid is a precursor to various drugs including the cough suppressant homatropine, and bioprocessing is attractive to produce optically pure mandelic acid, rather than the racemic mixtures produced by chemical synthesis. Taxifolin and coniferyl alcohol can be combined to produce silybin and its' steroisomers, drugs which are used to treat liver disease (Saller et al., 2008). The flavonoids naringenin and apignein are being studied for the treatment of Alzheimer's disease (Ghofrani et al., 2015; Salehi et al., 2019), as well as for antimicrobial and antiviral activity, including against SARS-CoV-2 (Clementi et al., 2021). The stilbenoid resveratrol is commonly found in red wine, and is a popular supplement, while its' derivative pterostilbene exhibits increased bioavailability (Z. B. Yan et al., 2021).

Several phenylpropanoids are flavor compounds that can be used as food additives or in cosmetics. Vanillin is the primary flavor compound in vanilla, and bioprocessing is attractive for producing natural vanillin since the demand for vanilla flavor is greater than the supply of vanilla beans (Dignum et al., 2007). Likewise for raspberry ketone, the primary aroma compound of raspberries, since the extraction yields are only a few milligram per kilogram of fruit (Beekwilder et al., 2007). Cinnamyl acetate is yet another flavor compound used as a food additive, and also in cosmetics. Curcumin and rosmarinic acid are most commonly used as dietary supplements, but they can also be used as a food coloring and a flavoring respectively.

1.2 | Economics of phenylpropanoid production

Titer, rate, and yield (TRY) are widely used metrics to characterize and compare the economics of different bioprocesses. The titer determines the cost of downstream processing, the production rate determines the capital costs related to the production equipment, while the yield determines the raw material costs (Krömer et al., 2020). Examples of TRY reported in recent years for the production of phenylpropanoids are listed in Table 1.

While bioprocessing may in principle be more sustainable than chemical synthesis or agricultural biomass extraction, it must also be profitable to be a viable alternative. As an example we give styrene, which costs around US$ 1.3/kg in bulk. Considering that glucose costs around US$ 0.25/kg, and downstream processing typically costs at least US$ 0.5/kg product, a styrene bioprocess would need to yield more than 0.31 kg styrene/kg glucose to make any profit, while actual yields were 0.21 kg/kg (Liang et al., 2020). Low downstream processing costs are contingent on a high titer, say 100 g/L (Van Dien, 2013), while fermentation titers have only reached 5.3 g/L styrene (K. Lee et al., 2019). Capturing just 1% of the approximately 26 million tons/year global styrene market (Dickson & Mitrajit, 2012; Market Data Forecast, 2022) via a production plant consisting of 10 aerobic 250 m^3 fermenters would require production rates of about 13 g/L*h, while actual rates were less by two orders of magnitude (K. Lee et al., 2019; Liang et al., 2020). Even with additional research and development, bioprocessing is unlikely to reach the target metrics to be competitive with chemical synthesis specifically for styrene production. Generally bulk chemical (<US$ 10/kg) phenylpropanoids are difficult targets, because the titer is limited by product toxicity, (Lou et al., 2012; Vestergaard & Ingmer, 2019), the rate is limited by PAL/TAL (Jendresen et al., 2015; Sáez-Sáez et al., 2020), and the yield is reduced from the theoretical yield through byproduct and biomass formation. Borderline cases include *p*-coumaric acid, ferulic acid, and so forth, which are not easily chemically synthesized, but which can be extracted from a variety of agricultural biomass waste streams in yields of grams per kilogram via direct extraction or alkaline hydrolysis (J. Zhao et al., 2011; S. Zhao et al., 2014). Alkaline hydrolysis is effective but considered nonnatural in the EU, and bulk preparations are of limited purity due to the presence of multiple phenylpropanoids in the raw materials. The main potential of bioprocessing is therefore in the area of fine chemical phenylpropanoids (>US$ 10/kg), encompassing those that are difficult to chemically synthesize and are naturally scarce. Even when a phenylpropanoid can be produced in

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TABLE 1 Production of phenylpropanoids and aromatic amino acid precursors from the period under review

Product	Feedstock	Host	Mode	Titer g/L	Rate g/(L*h)	Yield g/g	Strategy	Source
Phenylalanine	Glucose	*Escherichia coli*	Fed-batch bioreactor	61	1.3	22%	Dynamic regulation via modified *aroK* promoter	J. Wu et al. (2019)
Phenylalanine	Glucose	*E. coli*	Fed-batch bioreactor	73	1.3	26%	*tyrR* T495I, Δ*ptsG*, Δ*tyrA*, *galP*, *glk*, *aroD* overexpression	Y. Liu et al. (2018)
Phenylalanine	Glycerol	*Yarrowia lipolytica*	Shake flask	1	0.0083	0.25%	*aro1*, *aro2*, $aro3^{fbr}$, $aro4^{fbr}$, $aro7^{fbr}$, *aro8*, *aro10 overexpression*	Larroude et al. (2021)
Tyrosine	Glucose	*E. coli*	Repeated batch bioreactor	40	2.50	30%	Repeated batch fermentations	G. Li et al. (2020)
Tyrosine	Glucose	*E. coli*	Repeated batch bioreactor	39	1.70	29%	Centrifuge bioreactor with recovery	G. Li et al. (2021)
Tyrosine	Glucose	*E. coli*	Fed-batch bioreactor	56	1.4	25%	Heat-inducible promoters for $aroG^{fbr}$ and $tyrA^{fbr}$ expression	Xu et al. (2020)
Tyrosine	Glucose	*E. coli*	Fed-batch bioreactor	43	0.54	11%	Δ*tyrP*, $aroG^{fbr}$, *tyrC* overexpression, fed-batch optimization	Kim et al. (2018)
Cinnamic acid	Phenylalanine	*Corynebacterium glutamicum*	Repeated batch bioreactor	2.0	0.68	68%	Whole cell biocatalysis and cell recycling	Son et al. (2021)
Cinnamic acid	Glycerol	*Pseudomonas taiwanensis*	Fed-batch bioreactor	5.0	0.022	19%	Solvent tolerant host, deletion of phenylalanine degradation	Otto et al. (2019)
p-Coumaric acid	Glucose	*Saccharomyces cerevisiae*	Fed-batch bioreactor	13	0.13	14%	Increased E4P supply, dual phenylalanine/tyrosine pathways	Q. Liu et al. (2019)
p-Coumaric acid	Tyrosine	*E. coli*	Microtiter plate	2.9	2.9	53%	Novel *StsTAL*, dewatering	Cui et al. (2020)
p-Coumaric acid	Glucose	*E. coli*	Shake flask	0.39	0.0082	2.0%	Directed evolution of *RgTAL*	Huo et al. (2020)
p-Coumaric acid	Glucose	*E. coli* and *E coli*	Batch and repeated batch bioreactors	39	0.54	23%	Two-stage production and biocatalyst immobilization	Patnaik et al. (2008); Trotman et al. (2007)
Caffeic acid	Glucose	*E. coli*	Culture tube	0.047	4.9e-4	-	Functional bacterial cytochrome P450 expression	Haslinger and Prather (2020)
Caffeic acid	Glucose	*S. cerevisiae*	Fed-batch biorактor	5.5	0.056	-	Improved co-factor supply and recycling	Chen et al. (2022)
Ferulic acid	Glucose. Glucose	*Y. lipolytica Y. lipolytica*	Fed-batch shake flask. Fed-batch shake flask	3.8	0.031	-	Increased E4P and PEP supply, pathway overexpression, and byproduct deletion	Gu et al. (2020)
Ferulic acid	Tyrosine	*E. coli*	Shake flask	0.21	0.0018	21%	Host, promoter, and copy number tuning, and NAPDH regeneration	Lv et al. (2021)

(Continues)

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TABLE 1 (Continued)

Product	Feedstock	Host	Mode	Titer g/L	Rate g/(L*h)	Yield g/g	Strategy	Source
Zosteric acid	Glucose	*E. coli*	Fed-batch bioreactor	0.78	-	-	Sulfotransferase screening and increasing sulfate supply	Jendresen and Nielsen (2019)
Zosteric acid	Tyrosine	*E. coli*	Fed-batch bioreactor	5.0	~0.068	~74%		
Styrene	Glucose	*E. coli*	Fed-batch bioreactor	5.3	0.088	-	ISPR with solvent extraction	K. Lee et al. (2019)
Styrene	Glucose	*E. coli*	Fed-batch bioreactor	3.2	0.033	47%	Directed evolution and gas stripping	Liang et al. (2020)
4-Vinylphenol	*p*-Coumaric acid	*C. glutamicum*	Microtiter plate	50	1.0	66%	Host optimization and solvent extraction	Rodriguez et al. (2021)
(*S*)-Mandelic acid	Glycerol	*E. coli* and *E coli*	Fed-batch bioreactor and shake-flask	10	0.19	-	Two-stage production, enzyme cascade, and biphasic system	Lukito et al. (2019)
(*R*)-Mandelic acid	Glycerol	*E. coli* and *E coli*	Fed-batch and batch bioreactors	0.76	0.015	-	Two-stage production, enzyme cascade, and biphasic system	Lukito et al. (2021)
Resveratrol	Glucose	*Y. lipolytica*	Fed-batch bioreactor	12	0.14	5.4%	Gene copy number and media optimization	Sáez-Sáez et al. (2020)
Resveratrol	Glucose	*E. coli and S. cerevisiae*	Shake flask	0.036	5.0e-4	0.18%	Coculture decoupling tyrosine and resveratrol production	Yuan et al. (2020)
Resveratrol	*p*-Coumaric acid	*S. cerevisiae*	Shake flask	0.040	6.7e-4	57%	Comparing six stilbene synthases	Villa-Ruano et al. (2020)
Pinosylvin	Cinnamic acid	*E. coli*	Shake flask	0.053	0.0027	12%	Increasing malonyl-CoA supply	Salas-Navarrete et al. (2018)
Pterostilbene	Glucose	*E. coli*	Shake flask	0.080	0.0017	0.53%	Directed evolution of pathway enzymes	Z. B. Yan et al. (2021)
Resveratrol	Glycerol	*P. pastoris*	Fed-batch bioreactor	1.8	0.015	2.41%	Overexpression of $ARO4^{fbr}$ and $ARO7^{FBR}$	Kumokita et al. (2022)
Naringenin				1.1	0.011	1.41%		
Naringenin	*p*-Coumaric acid	*S. cerevisiae*	Fed-batch bioreactor	1.2	0.017	48%	Promoter screening	Gao et al. (2020)
Naringenin	*p*-Coumaric acid	*E. coli*	Fed-batch bioreactor	0.26	0.0026	-	High throughput screening of promoters and 5' untranslated regions	Hwang et al. (2021)
Naringenin	Glucose	*Y. lipolytica*	Fed-batch bioreactor	0.90	0.0027	-	Increasing malonyl-CoA supply	Palmer et al. (2020)
Apigenin	Tyrosine	*Streptomyces albus*	Batch bioreactor	3.4e-4	7.1e-6	34%	Pre-inoculum and fermentation process parameter optimization	Ferraiuolo et al. (2021)
Eriodyctiol	Glucose. Malonate	*E. coli* and *E. coli*	Shake flask	0.052	0.0011	0.35% 2.6%	Coculture and fermentation process optimization	Thuan et al. (2022)
Taxifolin	Glucose	*S. cerevisiae*	Fed-batch bioreactor	0.34	0.0035	-	Pathway optimization, NADPH regeneration	Yang et al. (2020)
Coniferyl alcohol				0.20	0.0028	-		

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TABLE 1 (Continued)

Product	Feedstock	Host	Mode	Titer g/L	Rate g/(L*h)	Yield g/g	Strategy	Source
Baicalein	Phenylalanine	*E. coli*	Shake flask	0.0085	1.8e-4	1.7%	Screening heterologous proteins	J. Li et al. (2019)
Scutellarein	Tyrosine	*E. coli*	Fed-batch shake flask	0.047	9.8e-5	9.4%		
Cinnamyl acetate	Glucose	*E. coli*	Shake flask	0.63	0.0022	0.31%	Synthetic metabolic pathway	Pan et al. (2020)
Chlorogenic acid	Glucose	*E. coli*	Batch	0.11	0.0024	1.1%	Pathway optimization	H. L. Lee et al. (2021)
p-Coumaroyl shikimate				0.71	0.014	7.1%		
Rosmarinic acid	Caffeic acid and danshensu	-	Batch	0.32	0.32	85%	Cell-free system with ATP and CoA regeneration	Y. Yan et al. (2019)
Rosmarinic acid	Glucose (polysaccharide)	*S. cerevisiae*	"Fed-batch" deep-well plate	0.0059	8.2e-5	0.0098%	Factorial design of pathway	Babaei et al. (2020)
Raspberry ketone	*p*-Coumaric acid	*E. coli*	Batch	0.091	0.0013	91%	Gene copy number and media optimization	C. Wang et al. (2019)
Raspberry ketone	Tyrosine (yeast extract)	*E. coli*	Microtiter plate	0.013	8.1e-4	-	High throughput promoter screening	Moore et al. (2021)
Raspberry ketone	Glucose	*E. coli*	Fed-batch	0.062	5.0e-4	0.12%	Increasing *p*-coumaric acid and malonyl-CoA supply	Masuo et al. (2022)
Vanillin	Ferulic acid	*E. coli*	Fed-bath bioreactor	4.3	0.18	80%	Process condition optimizations	Luziatelli et al. (2019)
Vanillin	Ferulic acid	*A.* sp. ATCC 39116	Repeated batch bioreactor	4.2	0.46	69%	Repeated batch fermentations	Valério et al. (2021)
Ferulic acid	Tyrosine	*E. coli*	Shake flask	0.26	0.0041	46%	Expression vector optimization and coculture	Rodrigues et al. (2020)
Curcumin	Ferulic acid	*E. coli*	Fed-batch shake flask	0.56	0.0089	97%		
Curcumin	Tyrosine	*E. coli* and *E. coli*	Shake flask	0.016	2.5e-4	0.047%		

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bulk via chemical synthesis or alkaline hydrolysis, bioprocessing may still be competitive for the market of natural flavors. 'Natural flavor' is a label regulated by the US Food and Drug Administration (2012) and the European Commission (2008), which can therefore be used to market value-added consumer products. The definition of natural may also expand to products beyond flavors in the future (US Food and Drug Administration, 2015), or it may be possible to market products as natural even where the definition of the term is not regulated.

2 | METABOLIC ENGINEERING OF THE SHIKIMATE AND AROMATIC AMINO ACID PATHWAYS

The production of phenylpropanoids first requires either phenylalanine or tyrosine precursors (Figure 1). These come from the shikimic acid pathway, which is made up of seven enzymatic reactions that combine D-erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) to make 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP), and modify DAHP to make chorismate. Chorismate can subsequently be converted to the aromatic amino acids phenylalanine, tyrosine, and tryptophan. A key aspect of metabolic engineering is to balance the carbon flux towards E4P and PEP for DAHP synthase (*aroF*, *aroG*, and *ARO4*). In yeasts the available pool of E4P is supposed to be at least one order of magnitude lower than that of PEP, and so a phosphoketalose-based pathway was introduced to divert carbon from PEP towards E4P via fructose-6-phosphate (Gu et al., 2020; Q. Liu et al., 2019). In *Escherichia coli* PEP is considered the limiting precursor for *aroF* and *aroG*, because PEP is required for glucose uptake via the phosphotransferase system (PTS). One approach to avoid this is to delete and replace the PTS with an alternative sugar transporter, such as the galactose:H$^+$ symporter (*galP*) (Choi et al., 2019). A different option is to grow the cells on pentoses, which are transported by ATP-driven permeases in *E. coli*. PEP is also a substrate for pyruvate kinase, so deletion or downregulation of the main pyruvate kinase (*pykA, PYK1*) has also been shown to increase PEP availability in *E. coli* and yeasts (Choi et al., 2019; Gu et al., 2020; Hassing et al., 2019).

Another focus of the metabolic engineering effort has been to relieve bottlenecks through the shikimic acid pathway by deleting negative regulators and overexpressing gene variants that are resistant to feedback inhibition (fbr). Expressing *aroG^{fbr}* has been shown to increase flux through the shikimic acid pathway in *E. coli*, *Pseudomonas taiwanensis* and *Yarrowia lipolytica* (Choi et al., 2019; Gu et al., 2020; Otto et al., 2019), as has expressing *aro4^{fbr}* in yeasts (Kumokita et al., 2022; Larroude et al., 2021; Q. Liu et al., 2019; Lv et al., 2021; Sáez-Sáez et al., 2020). Dynamically regulating shikimate kinase (*aroK*) in *E. coli* prevented excessive metabolic flux through the shikimic acid pathway during the growth phase and increased flux during the stationary phase, thereby increasing titer and yield (J. Wu et al., 2019). Expressing fbr chorismate mutase (*tyrA^{fbr}*, *pheA^{fbr}*, and

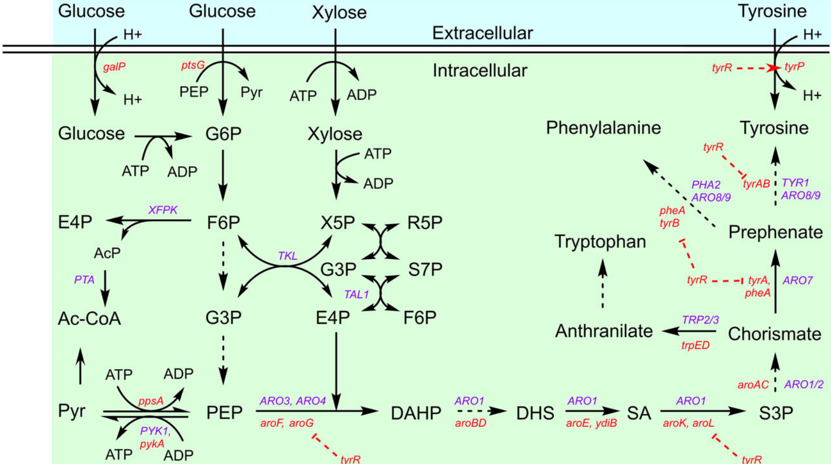

FIGURE 1 Engineering targets under review for phenylalanine and tyrosine overproduction. Purple text, genes expressed in yeasts; red text, genes expressed in *Escherichia coli*. Ac-CoA, acetyl-CoA; ADP, adenosine diphosphate; aroF, aroG, aroH, ARO3, ARO4, DAHP synthase; aroB, 3-dehydroquinate synthase; *aro7*, chorismate mutase; *aroD*, 3-dehydroquinate dehydratase; *aroE*, ydiB, dehydroshikimate reductase; *aroK*, aroL, shikimate kinase 1 and 2; *aroA*, 5-enolpyruvylshikimate 3-phosphate synthase; *aroC*, ARO2, chorismate synthase; *ARO8*, ARO9, aromatic aminotransferase I and II; *ARO1*, pentafunctional AROM polypeptide; ATP, adenosine triphosphate; E4P, D-erythrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHS, 3-dehydroshikimate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G3P, glyceraldehde-3-phosphate; *galP*, galactose:H$^+$ symporter; PEP, phosphoenolpyruvate; *pheA*, chorismate mutase/prephenate dehydratase, tyrA, chorismate mutase/prephenate dehydrogenase; *PHA2*, prephenate dehydratase; *ppsA*, phosphoenolpyruvate synthase; *pykA*, PYK1, pyruvate kinase 1; *pta*, phosphotransacetylase; *ptsG*, major glucose transporter; Pyr, pyruvate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; SA; shikimic acid; S3P, shikimate-3-phosphate; *trpE*, TRP2, anthranilate synthetase component I; *trpD*, TRP3, anthranilate synthetase component II; *TYR1*, prephenate dehydrogenase; *tyrR*, DNA-binding transcriptional dual regulator *tyrB*, tyrosine aminotransferase; X5P, xylulose-5-phosphate; *xfpk*, phosphoketolase.

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ARO7^{fbr}) also increases flux from chorismate towards tyrosine or phenylalanine in both bacteria and yeasts (Gu et al., 2020; Kumokita et al., 2022; Larroude et al., 2021; Q. Liu et al., 2019; Otto et al., 2019; Sáez-Sáez et al., 2020; Xu et al., 2020). Notably, a superior homolog of *tyrA*^{fbr} was found in *tyrC* from *Zymomonas mobilis* (Kim et al., 2018).

Preventing the production of the two unwanted aromatic amino acids is another strategy for increasing flux towards the desired aromatic amino acid, but the auxotrophs grow at a reduced rate and require media supplementation. In *E. coli* a knockout of *trpD* reduced the titers of tyrosine (Xu et al., 2020), and in *Y. lipolytica TRP2 and TRP3* deletion increased the titer but reduced growth (Gu et al., 2020), indicating that these targets may also benefit from dynamic regulation. TyrR is a regulator of genes essential for aromatic amino acid production and transport in *E. coli* (Figure 1), where it typically functions as a repressor of gene expression (Pittard et al., 2005). Deletion of *TyrR* is thus a common strategy of relieving negative feedback inhibition, but TyrR can also function as an activator for the expression of the tyrosine and tryptophan transporters TyrP and mtr respectively. Overproduction of tyrosine was achieved with a knockout of *TyrP* instead of *TyrR* (Kim et al., 2018), indicating that a *TyrR* knockout is not essential. Remarkably, the best phenylalanine-producing *E. coli* strain reported to date also did not have a *TyrR* knockout, but rather a point mutation in the domain responsible for negative feedback inhibition (Y. Liu et al., 2018). Flux analyses of existing *E. coli* overproducing strains in combination with systems-based metabolic engineering offers to identify bottlenecks and byproducts, which may be relieved or reduced when targeted with enzyme engineering and dynamic regulation. This could still yield significant cumulative benefits but with diminishing returns, also considering that tyrosine titers are already limited by foaming rather than insufficient metabolic flux (Patnaik et al., 2008). While precursor supply is rarely a limiting factor for phenylpropanoid production in bacteria, optimizing it can still be relevant for use in processes that decouple precursor production and phenylpropanoid production between two different hosts.

Yeasts are not serious contenders for bulk amino acid production, and thus less research has been invested in this area. However, the interest in yeasts as hosts for phenylpropanoid production has sparked interest in improving their aromatic amino acid precursor supply (Larroude et al., 2021; Y. Li et al., 2020; Y. Wu et al., 2020). The most common metabolic engineering strategies in yeasts have been to try and copy the strategies which were successful in *E. coli*, although this does not always pan out. For one thing, the carbon metabolism of yeasts supplies different ratios of PEP and E4P than *E. coli* (Q. Liu et al., 2019). For another, the regulation of amino acid biosynthesis and transport by the global transcription factor Gcn4 (Natarajan et al., 2001), is arguably more complex than in *E. coli*. Supplementation of amino acid precursors often drastically increases the production of phenylpropanoids in yeasts (J. Li et al., 2019), and the best-performing strains were designed to improve amino acid supply (Q. Liu et al., 2019; Sáez-Sáez et al., 2020), indicating that this is typically the limiting factor for TRY.

2.1 | Sustainable feedstocks for phenylpropanoid production

Substantial improvements in phenylpropanoid production could also come through the utilization of cheaper and more sustainable feedstocks, such as lignocellulosic hydrolysate. *E. coli* is notable in its' ability to efficiently metabolize all of the sugars in lignocellulose; glucose, xylose, arabinose, galactose, arabinose, and mannose. The sugars are consumed stepwise rather than simultaneously, which may actually be a benefit in terms of aromatic amino acid production, by triggering a two-stage fermentation. First glucose is transported through *ptsG* and consumed to produce biomass, and then the remaining sugars are transported through *GalP* or ATP-driven permeases, allowing for PEP accumulation and product formation (K. Li & Frost, 1999). In comparison, *Corynebacterium glutamicum* and most yeasts cannot naturally metabolize xylose, making them poor choices for the valorization of lignocellulosic biomass. The main drawback of using *E. coli* for metabolizing lignocellulose-derived sugars is its' low tolerance towards the inhibitors in these feedstocks (Lou et al., 2012; Rau et al., 2016), but this is also an issue for other hosts such as *C. glutamicum* (X. Wang et al., 2018) and yeasts (Konzock et al., 2021; Unrean et al., 2018). Crude glycerol from biodiesel production is also a potentially sustainable feedstock for production, which was recently used to produce phenylpropanoids in *Pichia pastoris* (Kumokita et al., 2022).

3 | GENETIC ENGINEERING FOR PHENYLPROPANOID PRODUCTION

Phenylalanine or tyrosine need to be converted via PAL/TAL to produce cinammic acid or *p*-coumaric acid respectively, from which other phenylpropanoids derive (Figure 2). PAL/TAL is often rate-limiting for phenylpropanoid production due to its' low turnover number (k_{cat}) under physiological conditions, so increasing its' activity has been the subject of much research. Also, improving the substrate specificity of PAL/TAL towards either tyrosine or phenylalanine is of interest to prevent byproduct formation. Bioinformatic screening and characterization of genes with sequence homology to known *TAL* genes has yielded *TAL* variants with higher substrate affinity (K_M) and specificity than previously characterized variants (Jendresen et al., 2015). A directed evolution approach was developed for *Anabaena variabilis* PAL, relying on the ammonia released by PAL from phenylalanine to serve as the sole nitrogen source, and screening for improved growth (Mays et al., 2020). This approach revealed 12 mutational hot spots, which through different molecular mechanisms increased k_{cat} twofold, reduced K_M, and reduced product inhibition (Trivedi et al., 2022). Historically, the catalytic efficiency (k_{cat}/K_M) of *Rhodotorula glutinis* PAL was also shifted in favor of tyrosine via directed evolution, by selecting for the reverse reaction TAL activity in a tyrosine auxotroph grown on minimal media containing *p*-coumaric acid (Gatenby et al., 2002). Beyond PAL/TAL, comparisons have been made to help identify superior natural homologs within sulfotransferases (Jendresen & Nielsen, 2019) and stilbene

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FIGURE 2 Production of phenylpropanoids in the period under review. ADP, adenosine diphosphate; AldO, alditol oxidase; AlkJ, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ATP, adenosine triphosphate; ATR2, cytochrome P450 reductase; ATF1, alcohol acetyltransferase; BAS, benzalacetone synthase; BAR, benzalacetone reductase; CAR, carboxylic acid reductase; COMT, caffeic acid O-methyltransferase; CoA, coenzyme A PAL/TAL, phenylalanine/tyrosine ammonia lyase; C4H, cinnamic acid hydroxylase; 4CL, 4-coumaryl CoA-ligase; C3H, p-coumaric acid 3-hydroxylase; 4CL, feruloyl-CoA synthetase; CAD, cinnamyl alcohol dehydrogenase; CHI, chalcone isomerase; CCR, cinnamoyl-CoA reductase; CHS, chalcone synthase; CPR, cytochrome P450 reductase; CURS, curcumin synthase; CYB5, cytochrome B5 reductase; CYP98A14, 4-coumaroyl-4′-hydroxyphenyllactate 3/3′-hydroxylase; DCS, diketide-CoA synthase; ECH, enoyl-CoA aldolase; F3′H, flavonoid 3'-monooxygenase; F3H, flavanone 3-hydroxylase; FAD, flavin adenine dinucleotide; FNS, flavone synthase; F6H, flavone C-6 hydroxylase; FDC, ferulic acid decarboxylase; HST, hydroxycinnamate-CoA shikimate transferase; HQT, hydroxycinnamate-CoA quinate transferase; NAD, Nicotinamide adenine dinucleotide; PAPS, 3'-Phosphoadenosine-5'-phosphosulfate; PAD, phenolic acid decarboxylase; RAS, rosmarinic acid synthase; ROMT, resveratrol O-methyltransferase; Sfp, phosphopantetheinyl transferase S; SMO, styrene monooxygenase; STS, stilbene synthase; YjgB, aldehyde reductase; SULT1A1, sulfotransferase 1A1.

synthases (Villa-Ruano et al., 2020). Finally, there have been campaigns to optimize expression of the genes specific to phenylpropanoid biosynthesis via their copy numbers or promoters. Increasing the integrated copy numbers of the resveratrol pathway from 1 to 6 increased the titer fivefold (Sáez-Sáez et al., 2020), and the optimal combination of plasmid copy numbers produced fourfold more raspberry ketone than the worst combination in a dual-plasmid expression system (C. Wang et al., 2019). A high-throughput screen of a library containing different promoter combinations driving naringenin biosynthesis improved the titer fivefold in *S. cerevisiae* (Gao et al., 2020), and a similar strategy improved the naringenin titer threefold in *E. coli* (Hwang et al., 2021).

3.1 | Co-factor supply and additional substrates for derivative phenylpropanoids

Apart from the supply of phenylalanine and/or tyrosine, many phenylpropanoids require additional substrates and co-factors to supply the enzymes. One of the enzymes most commonly required is 4-coumarate ligase (4CL), which uses ATP to activate the cinnamic acids with coenzyme A (CoA) to form *p*-coumaroyl-CoA, caffeoyl-CoA, and so forth before inter- or intramolecular reactions catalyzed by synthases. Malonyl-CoA is another common substrate required for the production of many phenylpropanoids including the stilbenoids, curcuminoids, flavonoids, and raspberry ketone. While several hosts and strategies have been attempted for stilbenoid production, the highest reported resveratrol titer to date was produced using the oleaginous yeast *Y. lipolytica*, which naturally has a large metabolic flux towards malonyl-CoA (Sáez-Sáez et al., 2020). Furthermore, *Y. lipolytica* was engineered to improve its' malonyl-CoA supply for the production of naringenin (Palmer et al., 2020).

The hydroxylation of phenylpropanoids in plants requires cytochrome P450 (CYP) enzymes using a heme prosthetic group, while some of the same hydroxylations are catalyzed by flavin-dependent monooxygenases (FMOs) in prokaryotes (Di Gennaro et al., 2011). As both groups of enzymes ultimately require NADPH as an electron donor, the more practical distinction is that the plant

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CYPs are membrane bound and poorly soluble when expressed in bacteria, while the prokaryotic FMOs are soluble in the bacterial cytosol. A successful alternative to *pheA* deletion for *p*-coumaric acid production in *Saccharomyces cerevisiae* was to convert cinnamic acid to *p*-coumaric acid via a cinnamic acid hydroxylase, (*C4H*), cytochrome B5 reductase (*CYB5*), and cytochrome P450 reductase (*ATR2*) (Q. Liu et al., 2019), which mimics the native pathway of plants. This strategy is attractive because the PAL k_{cat} is higher than the TAL k_{cat} for most PAL/TAL enzymes, and this also removes the need for phenylalanine supplementation in the growth media otherwise required by phenylalanine auxotrophs. Another common modification is the methylation of hydroxyl groups, which require *o*-methyl transferases (OMTs) and *S*-adenosyl methionine (SAM) as a co-factor. Finally, reductases and dehydrogenases catalyze the formation of phenylpropanoid aldehydes, alcohols, and ketones using the electron acceptors NAD^+ or FAD. In an example of co-factor engineering, increasing the NADPH/$NADP^+$ ratio by 46% in *S. cerevisiae* improved the cinnamic acid titer by 45%, and engineering SAM metabolism improved the ferulic acid titer by 180% (Chen et al., 2022). An NADPH-dependent mutant of 2,3-butanediol dehydrogenase was used as an engine for NADPH recycling in *S. cerevisiae*, which improved the efficiency of flavanone 3-hydroxylase (F3H) and flavonoid 3'-monooxygenase (F3'H) for the production of taxifolin (Yang et al., 2020).

3.2 | Genetic engineering for improved host tolerance

Product toxicity tends to be the limiting factor for high phenylpropanoid titers in fermentations with bacteria. Meanwhile, yeasts have attained higher titers of phenylpropanoids in fermentations than bacteria (Q. Liu et al., 2019; Sáez-Sáez et al., 2020), indicating that yeasts have a relatively higher tolerance towards these compounds. Yet yeasts still suffer reduced growth rates at elevated phenylpropanoid concentrations (Konzock et al., 2021; Pereira et al., 2020), contributing to low production rates. Here it should be noted that the solubility of many phenylpropanoids in water tends to be quite low at acidic pH near their isoelectric points (Ji et al., 2016), so that product toxicity will plateau as the solubility limit is reached. Product toxicity can be mitigated by using hosts which are natively tolerant to phenylpropanoids, and by improving host tolerance to phenylpropanoids. Host tolerance can be improved through adaptive laboratory evolution or semi-rational strain engineering, such as engineering efflux pumps with increased specificity and activity (Fisher et al., 2014).

S. cerevisiae evolved in the presence of *p*-coumaric acid or ferulic acid at low pH increased its' tolerance to either chemical fivefold by overexpressing an exporter, and reconstructing the phenotype in a *p*-coumaric acid production strain increased the titer by 47% (Pereira et al., 2020). It is important to test for tolerance in production strains, as an increased tolerance to extracellular phenylpropanoids does not necessarily correlate with an increase in titer. *E. coli* evolved in the presence of *p*-coumaric acid increased its' tolerance to the chemical fourfold, around half of which could be reconstructed by introducing causative knockouts in three regulatory genes, although the mechanism of increased tolerance could not be explained (Lennen et al., 2019). When a targeted mutant library of *E. coli* transcriptional regulators was selected for styrene tolerance, isolates were consistently enriched with mutations in four genes regulating the stress response, and the styrene titer also increased by 31% in the best mutant (Liang et al., 2020). *E. coli* evolved in the presence of vanillin showed a twofold increase in cell density after 6 h of growth in 10 mM vanillin, and all four evolved replicates had mutants of citrate synthase with increased activity, implicating the enzyme in supporting vanillin tolerance (Pattrick et al., 2019). *Pseudomonas putida* KT2440 has been shown to tolerate approximately double the concentration of *p*-coumaric acid compared to *E. coli* (Calero et al., 2018), making it an interesting candidate for phenylpropanoid production. *P. putida* KT2440 evolved in the presence of *p*-coumaric or ferulic acid yielded a decrease in lag phase in the former and an increase in growth rate in the latter, and one of the key genes found to be mutated was an aromatic transporter (Mohamed et al., 2020). As a side note, improving the general stress response and tolerance towards phenylpropanoids is likely to have the added benefit of improving tolerance towards inhibitors in lignocellulose-derived feedstocks or crude glycerol.

4 | BIOPROCESS CONCEPTS FOR PHENYLPROPANOID PRODUCTION

4.1 | In-situ product removal for phenylpropanoid production

Bioprocess concepts can also offer improvements to the TRY of phenylpropanoids and precursors, by mitigating product toxicty, increasing biocatalyst loading, and through biocatalyst recycling. For example, repeated batch fermentations increased the rate and yield of tyrosine by 44% and 74% compared with a single fed-batch fermentation (G. Li et al., 2020). A novel differential centrifugation bioreactor set-up building on this concept was developed, which separates tyrosine crystals and biomass to integrate product recovery with repeated batch fermentations (G. Li et al., 2021), also offering a possible solution to low tyrosine titers caused by foaming. Another technology which might perform the same function is high frequency untrasound separation (Juliano et al., 2017), although this has not been tested. In situ product removal (ISPR) is a viable strategy for mitigating product toxicity and product inhibition, but many phenylpropanoids are amphipathic, poorly soluble in water due to their hydrophobic phenyl groups (Alevizou & Voutsas, 2013; Ji et al., 2016), as well as in nonpolar organic solvents due to their hydrophilic groups (Combes, Clavijo Rivera, et al., 2021). ISPR methods based on extraction with medium-low polarity solvents, adsorption, or crystallization are therefore expected to be generally favored (Salas-Villalobos et al., 2021). When 10 polar solvents were

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compared for in-situ *p*-coumaric acid extraction, oleyl alcohol, butyl acetate, and hexyl acetate were found to be biocompatible with *S. cerevisiae* and able to extract *p*-coumaric acid at acidic conditions (Combes, Clavijo Rivera, et al., 2021). However, when oleyl alcohol was implemented in a biphasic fermentation, the titer of *p*-coumaric acid did not increase compared to the control without ISPR, presumably because the engineered strain did not produce inhibitory levels of product (Combes, Imatoukene, et al., 2021). (*R*)-Mandelic acid was produced from glycerol in *E. coli*, and a biphasic system using *n*-hexadecane increased the titer threefold by reducing the toxicity of the intermediates styrene and (*S*)-styrene oxide (Lukito et al., 2019). A high titer of 4-vinylphenol was produced from *p*-coumaric acid in *C. glutamicum* expressing phenolic acid decarboxylase (*PAD*) and combined with solvent extraction (Rodriguez et al., 2021). Styrene was also produced from glucose in *E. coli* by the expression of *PAL* and ferulic acid decarboxylase (*FDC*), and combined with solvent extraction and/or gas stripping (K. Lee et al., 2019; Liang et al., 2020).

4.2 | Decoupling phenylpropanoid production

Decoupling microbial growth from phenylpropanoid production can have several advantages, including mitigating product toxicity and increasing biocatalyst loading. To illustrate the former, the minimum inhibitory concentration (MIC) of vanillin against *E. coli* was found to be 2.2 g/L, but the effect was merely bacteriostatic, and some metabolic activity was maintained at the maximum tested concentration of 7.6 g/L (Fitzgerald et al., 2004). Indeed, 4.3 g/L vanillin was produced from ferulic acid in resting cells of *E. coli* expressing 4CL and enoyl-CoA aldolase (ECH) (Luziatelli et al., 2019). This demonstrates that resting cells may still be able to regenerate co-factors and catalyze phenylpropanoid biosynthesis, even when their growth is inhibited. Going a step further in terms of decoupling growth, rosmarinic acid was produced from caffeic acid and danshensu using a cell-free system, including 4CL and rosmarinic acid synthase (RAS) together with a double regeneration system for ATP and CoA (Y. Yan et al., 2019). As for biocatalyst loading, increasing the concentration of a whole-cell *E. coli StsTAL* biocatalyst tenfold led to an almost sevenfold increase in the volumetric rate (Cui et al., 2020). Decoupling phenylpropanoid production can also improve the reaction rate not only through increasing biocatalyst loading, but also by optimizing the process conditions in terms of pH, temperature, and substrate loading. For example, PAL/TAL and benzalacetone synthase enzymes have much higher activity at alkaline pH (Abe et al., 2007; Jendresen et al., 2015), and thermostable PAL/TAL variants can maintain higher activities at elevated temperatures (Xue et al., 2007). Historically, DuPont developed a two-stage bioprocess for sequential tyrosine fermentation and *p*-coumaric acid biocatalysis which greatly outcompeted the best one-stage *p*-coumaric acid fermentation process reported to date in TRY (Q. Liu et al., 2019; Patnaik et al., 2008; Trotman et al., 2007). However, this direct comparison is somewhat unfair, as it does not take into account the extra steps involving recovery of tyrosine or production of the immobilized whole-cell TAL biocatalyst. Even so, it may be desirable to extend such a two-stage bioprocess to other phenylpropanoids via biocatalytic cascades, rather than introducing more stages and increasing process complexity. This approach does require compromises in terms of the process conditions, so that they are compatible with all the enzymes in the cascade. A high titer of enantiopure (*S*)-mandelic acid was produced from phenylalanine by expressing a four-enzyme cascade of PAL, PAD, styrene monooxygenase (SMO), epoxide hydrolase (EH) and alditol oxidase (AldO) (Lukito et al., 2019). A similar enzyme cascade was used for (*R*)-mandelic production, and it was demonstrated that a bioprocess decoupling phenylalanine fermentation and the enzyme cascade in two stages improved the titer more than threefold compared to using a single strain (Lukito et al., 2021). Coculturing has also been attempted to decouple the biosynthetic pathways for the production of resveratrol, curcumin, and eriodictyol from glucose, and while Coculturing was superior to mono-culturing, the TRY were still relatively modest (Rodrigues et al., 2020; Thuan et al., 2022; Yuan et al., 2020). Coculturing can be used to improve the supply of aromatic amino acids via a bacterial host in combination with functional CYP expression in a yeast host, but it does little to mitigate product toxicity. In theory the toxicity towards the bacteria is somewhat mitigated in a bacterial/yeast coculture compared to a bacterial monoculture, if the phenylpropanoids are not produced intracellularly in the bacteria.

The development of robust hosts which can tolerate high concentrations of phenylpropanoids and extreme process conditions is also required for whole-cell biocatalysis to be economical, especially when co-factor regeneration is required. When *E. coli*, *C. glutamicum*, and *Bacillus subtilis* were compared as *PAD* whole-cell biocatalysts, *C. glutamicum* produced the highest titer of 4-vinylphenol (Rodriguez et al., 2021). *C. glutamicum* was also successfully used as a *PAL* whole-cell biocatalyst for the production of cinnamic acid, where it remained catalytically active for 12 h at alkaline pH and elevated temperature (Son et al., 2021). Beyond these conventional hosts, the use of extremophiles as hosts in biocatalysis for phenylpropanoids would be an interesting avenue of research. Finally, whole-cell immobilization such as in calcium-alginate beads can be used to increase the lifetime of the biocatalysts (Trotman et al., 2007). The implementation of bioprocess concepts for phenylpropanoid production is illustrated in Figure 3.

4.3 | Choice of bioprocess

The preferred bioprocess will depend on several factors including the target phenylpropanoid. For relatively nontoxic products such as zosteric acid, fermentation with bacterial hosts may be preferred. Yeast fermentation may be sufficient for some moderately toxic products, especially if an effective method of ISPR can be developed. However, it is probably not economical to produce either phenylalanine or tyrosine directly in yeasts, when they can be produced much more efficiently in bacteria and supplied through a coculture or two-stage process with biocatalysis. For highly toxic products,

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FIGURE 3 Implementing bioprocess concepts for phenylpropanoid production.

TABLE 2 Comparison of bioprocesses for phenylpropanoid production

	Bacterial fermentation	Yeast fermentation	Bacterial/yeast coculture	Biocatalysis
Aromatic amino acids	High	Low	Moderate	High
Product toxicity	High	Moderate	Moderate	Low
Catalytic activity	Moderate	Low	Moderate	High
Co-factor regeneration	High	Moderate	Moderate	Low
Functional CYP expression	Low	High	High	Host-dependent
Process complexity	Low	Low	Moderate	High

biocatalysis may be necessary to fully decouple growth and production. Whole-cell catalytic activity should be greater in bacteria than in yeasts, due to generally higher levels of recombinant protein expression and better metabolic control. On the other hand, yeasts may compensate with better supply of key precursors like malonyl-CoA, and their ability to express functional CYP enzymes. While all the products discussed in this review can be produced without CYP enzymes, CYP enzymes may provide higher rates, and allow for the use of phenylalanine rather than tyrosine as a precursor. Phenylalanine is more soluble and easier to overproduce (and cheaper to purchase) than tyrosine, making it the more attractive precursor for biocatalysis. Added process complexity is the major drawback of these bioprocesses, because it entails increased capital costs. The different bioprocesses discussed here are compared in Table 2.

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5 | CLOSING REMARKS

The two general problems facing phenylpropanoid production through bioprocessing are the toxicity of phenylpropanoids towards microorganisms and the poor reaction rate of PAL/TAL and other enzymes in the biosynthetic pathways, which limit the production titers and rates, respectively. Development of ISPR methods and improved host tolerance will likely be aided by technology transfer from lignocellulosic biomass detoxification and fermentation, as the toxic compounds in lignocellulosic biomass are related to phenylpropanoid products (Bhatia et al., 2020). At the same time, general advances in genomics, protein engineering, and biofoundries will make it easier to improve PAL/TAL and other rate-limiting enzymes in phenylpropanoid production. The strategy which requires specific attention is that of decoupling phenylpropanoid production, particularly the development of biocatalytic cascades and robust whole-cell biocatalysts. Bioprocessing is the only method which promises to meet the increasing industry and consumer demands for a variety of pure, sustainable, and natural phenylpropanoids. Combining genetic engineering and bioprocess concepts is a promising approach to overcoming the challenges facing phenylpropanoid production through bioprocessing, while making the method profitable.

6 | METHODOLOGY

The approximate cost of specific chemicals was estimated by searching their names and/or CAS numbers on Alibaba.com and assessing the relevant search results. The titers, rates, and yields presented in Table 1 are presented as given in the original publications. When any of the TRY metrics were not given directly in the original publications, they were calculated from the data, in some cases approximately in plots.

ACKNOWLEDGMENT

This work was funded by the Novo Nordisk Foundation within the framework of the Fermentation-based Biomanufacturing Initiative (FBM), grant number NNF17SA0031362.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Virklund, A., Jensen, S. I., Nielsen, A. T., & Woodley, J. M. (2023). Combining genetic engineering and bioprocess concepts for improved phenylpropanoid production. *Biotechnology and Bioengineering*, 120, 613–628. https://doi.org/10.1002/bit.28292

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