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REVIEW

Yeast factories for the production of aromatic compounds: from building blocks to plant secondary metabolites

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Abstract The aromatic amino acid biosynthesis pathway is a source to a plethora of commercially relevant chemicals with very diverse industrial applications. Tremendous efforts in microbial engineering have led to the production of compounds ranging from small aromatic molecular building blocks all the way to intricate plant secondary metabolites. Particularly, the yeast *Saccharomyces cerevisiae* has been a great model organism given its superior capability to heterologously express long metabolic pathways, especially the ones containing cytochrome P450 enzymes. This review contains a collection of state-of-the-art metabolic engineering work devoted towards unraveling the mechanisms for enhancing the flux of carbon into the aromatic pathway. Some of the molecules discussed include the polymer precursor muconic acid, as well as important nutraceuticals (flavonoids and stilbenoids), and opium-derived drugs (benzylisoquinoline alkaloids).

Keywords Metabolic engineering · Aromatic amino acids · Shikimic acid · Flavonoids · Stilbenoids · Benzylisoquinoline alkaloids · *Saccharomyces cerevisiae*

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Introduction

During the past two decades, the biotechnological production of petroleum-derived compounds has attracted worldwide attention, leading to extraordinary research for the construction and optimization of microbial factories capable of utilizing renewable feedstocks as starting materials [3, 7, 42]. Given the richness and variety of biological pathways, bio-based production by engineered microorganisms has expanded from just biofuels to the realm of specialty chemicals and secondary metabolites [14, 19]. This group of chemicals has garnered special attention due to the cumbersome processes required for their production through classical chemical synthesis or the immense amounts of plant tissues required for extraction. Amongst the core metabolic pathways that lead to the production of specialty compounds, the aromatic amino acid biosynthetic pathway stands out for its unique diversity.

The aromatic, or arene, chemicals are recognized as cyclic six-carbon structures with delocalized electrons that generally possess a sweet or pleasant aroma, the property from which this class of molecules gets its name. This vast family of compounds is of great importance to the chemical industry; benzene, for instance, serves as the aromatic building block of many other chemicals, such as toluene, phenol, and other polycyclic aromatics. Unfortunately, using petroleum-derived benzene as the starting molecule compromises the sustainability and the safety of the process. Acute and chronic exposure to benzene has proven to be carcinogenic [64]; furthermore, the synthesis of benzene derivatives usually entails the release of toxic gases into the environment [53]. For these reasons, the production of aromatic compounds in microbial systems has become more attractive.

The family of aromatic compounds comprises a vast number of molecules. In nature, the aromatic amino acids,

namely, L-tryptophan (L-trp), L-phenylalanine (L-phe), and L-tyrosine (L-tyr), serve as building blocks for the biosynthesis of polypeptides. In several plants, these amino acids also serve as the precursors for the production of highly sought-after nutraceuticals, fragrances, and drugs. Although these secondary metabolites encompass a market in the billion–dollar range [55], extraction from plant tissues requires high amounts of biomass and cumbersome separation processes, leading to very low yields. Hence, the implementation of microbial factories, engineered with the heterologous biosynthetic pathways of aromatic secondary metabolites, represents a great solution to this problem. This ensures not only higher yields but also sustainable and greener processes. With the advent of recombinant DNA technology, genome editing tools, and synthetic biology, this solution has proven to be feasible [3, 7, 42], albeit optimization of the microbial hosts to increase the carbon yields still remains as a limiting factor.

This review summarizes the metabolic engineering efforts to manipulate the biosynthesis of aromatic compounds in microbial systems with exclusive attention directed to *Saccharomyces cerevisiae*. Yeast hosts have a greater potential in the production of secondary aromatic compounds due to their greater capability of expressing membrane-bound cytochrome P450 oxidases, which are key catalysts in these metabolic pathways [39, 62, 71].

The aromatic amino-acid biosynthetic pathway

The aromatic amino-acid biosynthetic pathway is present in many microorganisms and plants; it is responsible for the de novo synthesis of L-tyr, L-phe, and L-trp. This pathway is sectioned into three main parts: (1) the shikimic acid pathway, (2) the L-trp branch, and (3) the L-tyr and L-phe branches (Fig. 1). The energetic expenditure of synthesizing aromatic acids requires twice the ATP cost than most of other amino acids [5]. Therefore, it is expected to encounter strict regulation at the transcriptional and allosteric levels throughout the pathway.

Initiation, shikimic acid pathway, and aromatic amino acid branches

The initiation of the synthesis of aromatic amino acids requires two precursors from distinct, yet closely related pathways. Erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) are the two starter units; the former derives from the pentose phosphate pathway (PPP) and the latter from glycolysis. A balanced ratio between these two molecules would be optimal to enhance the carbon entrance into the aromatic pathway. However, given the intrinsic functional differences between these parallel pathways, the available fluxes of these individual precursors differ drastically. Metabolic flux analysis in *S. cerevisiae* has shown that the available E4P flux is at least an order of magnitude lower than the PEP flux [67]. Hence, as described in the subsequent sections, efforts have been directed towards engineering of PPP as well as the lower end of the glycolytic pathway to enhance the production of precursors to enter the aromatic pathway.

The enzyme 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (isoenzymes *ARO3/4)* is in charge of condensing the two precursors to initiate the biosynthesis of aromatic compounds. Following this reaction, the pentafunctional *ARO1* enzyme catalyzes five out of the six steps in the shikimic acid pathway, from DAHP to 5-enolpyruvyl-3-shikimate phosphate (EPSP). This enzyme is exemplary in yeast, since all five subunits are encoded within one open reading frame (ORF), giving a single multi-domain protein, unlike its homologues in bacteria, where each subunit is encoded separately. Finally, the enzyme chorismate synthase *ARO2* converts EPSP into chorismate, which is the common precursor to the biosynthesis of L-trp, L-phe, and L-tyr [8]. Chorismate can enter the L-trp branch composed of five steps (*TRP1* to *TRP5)* or enter the L-tyr/L-phe branches which share the first catalytic step, from chorismate to prephenate, catalyzed by the enzyme chorismate mutase *ARO7*. Following this step, the pathway branches into the synthesis of phenylpyruvate (PP), catalyzed by prephenate dehydratase (*PHA2*); and the synthesis of 4-hydroxyphenylpyruvate, (4-HPP) catalyzed by prephenate dehydrogenase (*TYR1*). Finally, two aromatic amino transferases, *ARO8* and *ARO9,* catalyze the transfer of an amine group to each of the previous molecules to yield L-phe and L-tyr [8] (Fig. 1).

Transcriptional regulation

It is well known that transcription factors (TFs) can globally regulate the transcription rates of a network of genes. TFs bind to specific promoter regions of structural genes to up- or down-regulate the transcription of such genes [79]. GCN4p is a TF that has been associated with transcriptional activation of amino acid biosynthesis genes in response to nutrient starvation [36, 37]. This regulatory protein contains a leucine-zipper structure that is commonly found in DNA-binding polypeptides. As expected, several GCN4p-binding sequences have been found in the promoter sequences of the aromatic amino acid biosynthesis genes, such as *ARO1*, *ARO3, ARO4, ARO9, TRP2,* and *TRP3* [8]. It has been demonstrated that certain metabolites can control the transcription rates of specific genes in the pathway. For instance, the transcription of *TYR1* can be regulated by the concentrations of L-phe [50]. Another example is the influence of excess L-trp in the activation of *ARO9* and *ARO10,* which can initiate the process of aromatic amino acid degradation

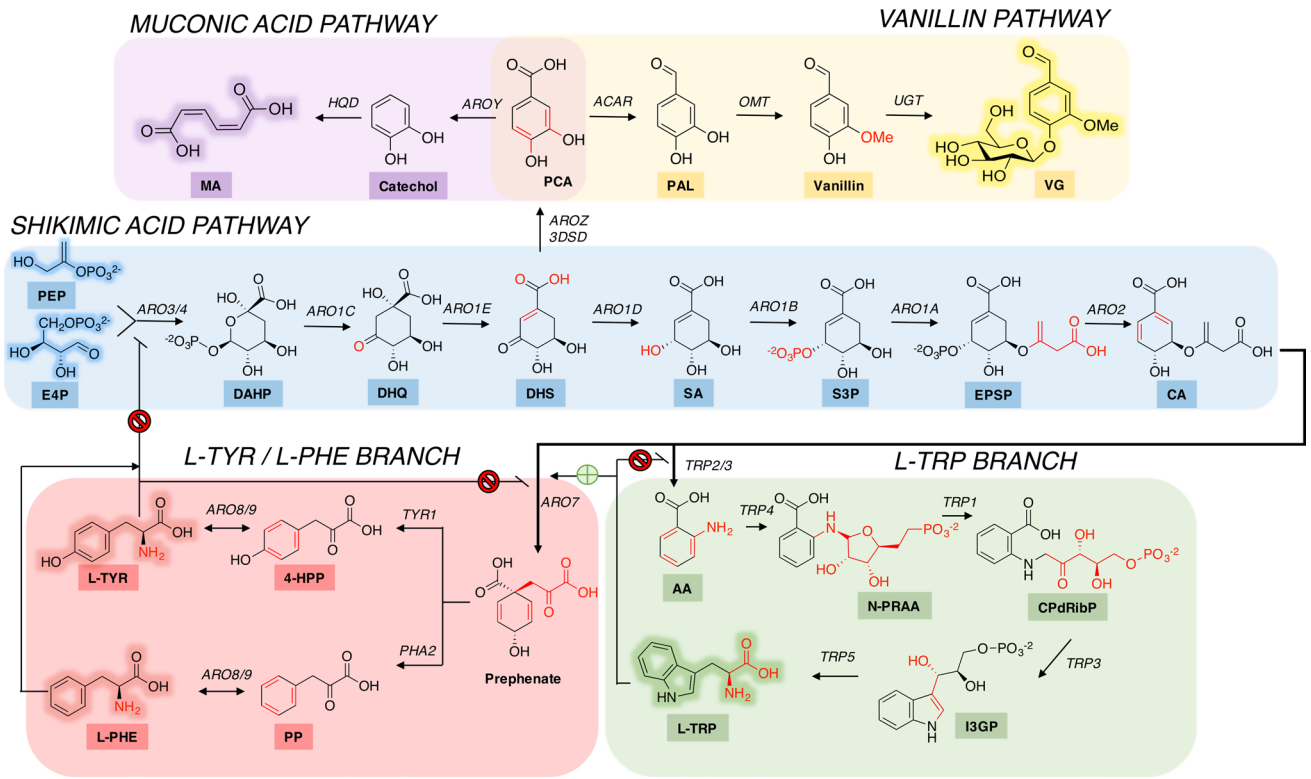

Fig. 1 Aromatic amino-acid biosynthetic pathway, the heterologous muconic acid (MA) pathway, and the heterologous vanillin pathway. Abbreviation of metabolites—E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabinoheptulosonate-7-phosphate; DHQ, dehydroquinoate; DHS, dehydroshikimate; SA, shikimic acid; S3P, shikimate-3-phosphate; EPSP, 5-enolpyruvyl-3-shikimate phosphate; CA, chorismic acid; PP, phenylpyruvate; 4-HPP, p-hydroxyphenylpyruvate; L-PHE, L-phenylalanine; L-TYR, L-tyrosine; AA, anthranilic acid, N-PRAA, N-(5-phospho-D-ribosyl)-anthranilate; CPdRibP, 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate; I3GP, indole-3-glycerol phosphate; L-TRP, L-tryptophan; PCA, protocatechuic acid; PAL, protocatechuic aldehyde; VG, vanillin-D-glucoside. Abbreviation of enzymes—*ARO3/4*, DAHP synthase isoenzymes; *ARO1*, aromatic pentafunctional enzyme; subunits—C: DHQ synthase, E: DHQ dehydratase, D: DHS dehydrogenase, B: SA kinase, A: ESPS synthase; *ARO2*, chorismate synthase; *ARO7*, chorismate mutase; *PHA2*, prephenate dehydratase; *TYR1*, prephenate dehydrogenase; *ARO8/9*, aromatic amino transferases; *TRP2*, anthranilate synthase; *TRP3*, indole-3-glycerol-phosphate synthase; *TRP4*, anthranilate phosphoribosyl transferase; *TRP1*, phosphoribosylanthranilate isomerase; *TRP5*, tryptophan synthase; *AROZ* and 3DSD, 3DHS dehydratase; *AROY*, PCA decarboxylase; *HQD*, catechol 1,2-dioxygenase; *ACAR*, aromatic carboxylic acid reductase; *OMT*, O-methyl transferase; *UGT*, UDP glycotransferase

[46]. The implementation of a global transcriptional regulation approach to increase the production of aromatic compounds, although promising, is naturally challenging. For instance, the starvation of aromatic amino acids will not only trigger a GCN4p-dependent transcriptional increase response on the genes from the corresponding pathway, but other genes involved in the biosynthesis of histidine, arginine, isoleucine, leucine, and other amino acids will also be activated, preventing the possibility of conducting a controllable localized up-regulation [37]. This creates intricate regulatory networks that have to be explored from a global perspective [52].

Allosteric regulation

There are three major allosteric checkpoints occurring at the branching points of the aromatic pathway. Engineering these control points in *S. cerevisiae* is the most straightforward approach and, thus, has enabled the heterologous production of aromatic compounds from the three main tiers in the pathway. The first one occurs at the level of the DAHP synthase isoenzymes *ARO3* and *ARO4* [49]. These catalyze the first committed step in the biosynthesis of aromatic amino acids, and both *ARO3* and *ARO4* are sensitive to the feedback inhibition posed by L-phe or L-tyr, respectively (Fig. 1). Thus, the presence of these two amino acids can block the entrance of carbon into the pathway, dramatically decreasing the production of any of the downstream metabolites.

The second allosteric control occurs on the enzymes anthranilate synthase (*TRP2*) and indole-3-glycerol-phosphate synthase (*TRP3*), whose activities are repressed by the presence of L-trp. Simultaneously, L-trp can act as an activator of the enzyme chorismate mutase (*ARO7*), which

catalyzes the conversion of chorismate into prephenate, initiating the production of L-tyr and L-phe (Fig. 1). A third recognized allosteric control occurs at high concentrations of L-tyr. This causes feedback inhibition of *ARO7*, potentially switching the carbon flux towards production of L-trp to maintain a balanced production of aromatic amino acids [8].

Metabolic engineering strategies for production of aromatic compounds

Engineering the shikimic acid pathway

The shikimic acid pathway has been a focal point in *S. cerevisiae* to engineer the production of three important metabolites: muconic acid, shikimic acid, and vanillin.

Muconic acid

Muconic acid is the unsaturated precursor of adipic acid and terephthalic acid, which are the building units for the plastics Nylon 6,6 and polyethylene terephthalate (PET) with an annual market value greater than $22 million [48, 53, 57]. The classic chemical processes to produce these derivatives are not environmentally friendly, thus stressing the need for the development of microbial fermentation alternatives. Curran et al. [13] engineered the first *S. cerevisiae* strain capable of accumulating this compound by introducing a 3-gene heterologous pathway which diverted carbon from the shikimate pathway at the node of dehydroshikimate (DHS) (Fig. 1). The metabolic engineering rationale included the removal of the feedback inhibition loop by overexpressing the L-tyr insensitive DAHP synthase variant, $ARO4_{K229L}$ (Fig. 2a). Furthermore, to increase the precursor availability, flux balance analysis (FBA) was performed. The results suggested that the PPP had to be rewired to force the carbon entrance through the non-oxidative PPP branch, allowing a higher E4P pool to enter the aromatic pathway. The overexpression of the transketolase gene (*TKL1*) and the deletion of glucose-6-phosphate-1-dehydrogenase (*ZWF1*) were the key predicted manipulations to achieve this goal (Fig. 2b). These manipulations translated into an increase in muconic acid titer from 40 to ~60 mg L^{-1} [13]. Overexpression of the rate-limiting step, protocatechuic acid (PCA) decarboxylase (*AROY*), led to a final strain producing 141 ± 8 mg L^{-1} (3.9 mg g^{-1}glucose) (Table 1). A second study demonstrated that overexpressing the mutant $ARO1_{D1409A}$, capable of halting the conversion of DHS to shikimic acid, increased the titer to 235 mg L^{-1} (Fig. 2c). Coupling this strategy with a controlled oxygen fermentation to relieve the oxygen sensitivity of the *AROY* isolated from the anaerobic bacterium *Klebsiella pneumoniae* increased the muconic acid titer to 559.5 mg L^{-1}, the highest reported titer and yield to date in *S. cerevisiae* [68] (Table 1).

Shikimic acid

Shikimic acid is the fourth intermediate in the shikimate pathway, and serves as the precursor of the commercial anti-influenza drug Tamifu® [1]. It is commonly extracted from plants (the genus *Illicium*), however, the low yield, long cultivation cycle, and susceptibility of open-field crop growth to environmental factors pose the microbial production as a preferred alternative.

The engineering key to enable the accumulation of shikimic acid in *S. cerevisiae* is the overexpression of the mutant enzyme $ARO1_{D920A}$, which has the shikimate kinase subunit disrupted and, hence, cannot convert shikimic acid into shikimate-3-phosphate (S3P) (Figs. 1 and 2c). A recent study compared the capacity of four distinct *S. cerevisiae* strains to accumulate SA as an approach to model the carbon entrance into the aromatic amino acid pathway (Fig. 2d). It was demonstrated that the four strains, containing the same set of episomal plasmids for gene overexpression, differed dramatically in their titers. Metabolic flux analysis (MFA) coupled with quantitative PCR (qPCR) enabled the establishment of an appropriate engineering rationale that allowed titer increases in all four strains. This strategy consisted of grouping the genes encoding *TKL1*, $ARO4_{K229L}$, and $ARO1_{D920A}$ together in a low-copy plasmid, demonstrating that the aromatic amino-acid pathway is sensitive to the burden of excessive protein expression [67]. The highest shikimic acid titer in batch fermentation reached 380 mg L^{-1} in the diploid strain INVSc1 (Table 1).

Vanillin

The production of aromatic flavors or aromas is predominant in several industries. The two most popular benzenoid flavors are vanillin and 2-phenylethanol (derived from L-phe, "Monoterpene indole alkaloids"); the market of these two compounds together make 44.4 % of the total benzenoid market, which as to 2014 was $2.8 billion, and is expected to increment to $3.4 billion by 2019 [55]. Vanillin is the most extensively used flavoring compound in industry. Its global market in 2019 is expected to reach $800 million, which represents the highest increase among all the benzenoid flavors. This plant-derived secondary metabolite can be extracted from the seedpods of the flower *Vanilla planifolia*. However, due to extremely low yields and laborious cultivation schemes that lead to a cost-ineffective process, nearly 99 % of vanillin is obtained from a biochemical synthesis processes involving the breakdown of lignocellulosic biomass or from a petroleum-derived precursor [58].

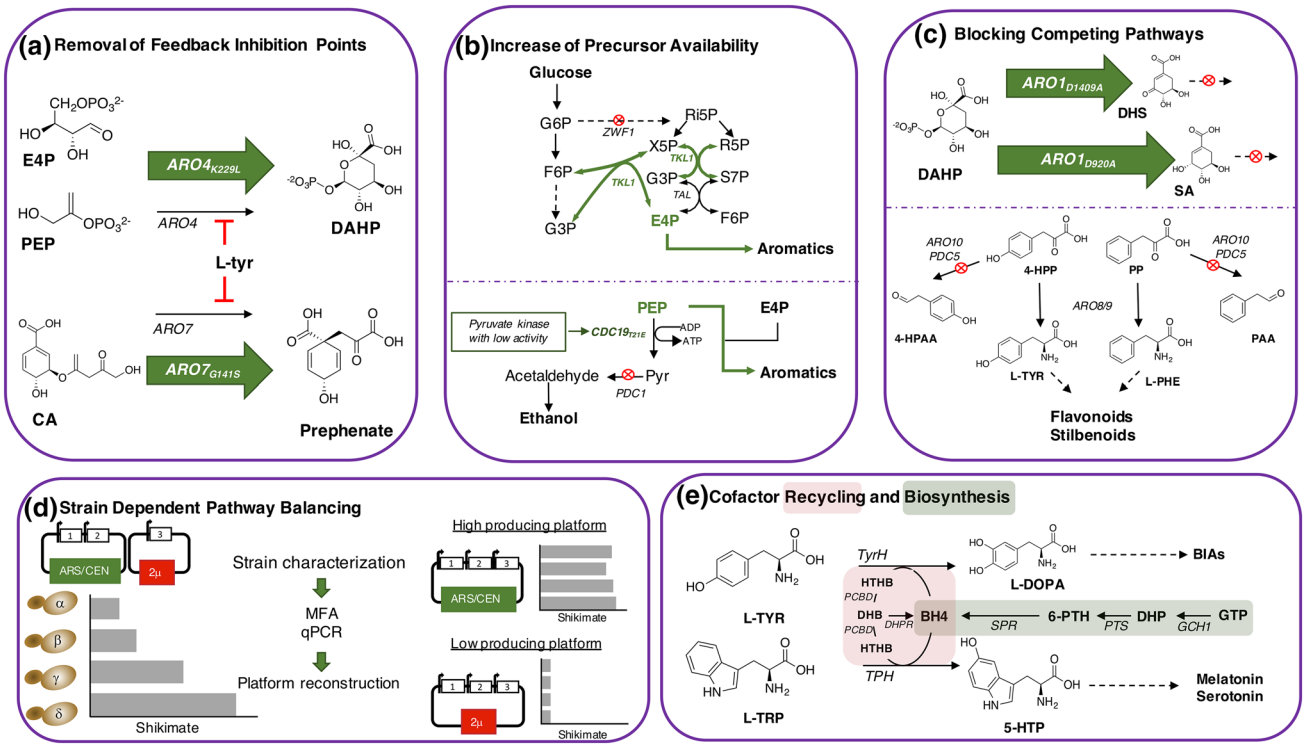

Fig. 2 Common metabolic engineering strategies implemented in *S. cerevisiae* to increase the production of compounds deriving from the aromatic amino acid biosynthetic pathway. **a** Removal of feedback inhibition points has been achieved by overexpression of the tyrosine insensitive enzymes $ARO4_{K229L}$, which deregulates the first committed step in the aromatic biosynthesis pathway; and $ARO7_{G141S}$, which controls the lower branching point to the production of the L-phe and L-tyr. **b** Increasing the availability of the main precursor E4P has been achieved by overexpression of the transketolase 1 gene (*TKL1*) in combination with deleting the glucose-6-phosphate-1-dehydrogenase gene (*ZWF1*) to force the entrance of carbon into the non-oxidative section of PPP. Reducing the activity of the catalytic steps in the lower portion of glycolysis (pyruvate kinase mutant $CDC19_{T21E}$ and *PDC1*) has been attempted to increase the availability of PEP. **c** Blocking competing pathways has been demonstrated by overexpressing mutant versions of the ARO1 enzyme to halt the conversion of intermediates in the shikimate pathway. Deletion of *ARO10* and *PDC5* increases precursor availability. **d** A strain-dependent pathway balancing approach was implemented to establish a strong yeast platform for the production of the common precursor shikimic acid. **e** Recycling of the cofactor BH4 (tetrahydrobiopterin) is essential in the production of secondary aromatic compounds. Abbreviations—DHB, dihydrobiopterin; 6-PTH, 6-pyruvoyl-tetrahydropterin; DHP, dihydroneopterin triphosphate; GTP, guanosine triphosphate; *PCBD*, pterin-4-alpha-carbinolamine dehydratase; *DHPR*, dihydropteridine reductase; *SPR*, sepiapterin reductase; *PTS*, 6-pyruvoyl-tetrahydropterin synthase; *GCH1*, GTP cyclohydrolase I. The *dash lines* represent multiple steps

Microbial production of vanillin from glucose has also been reported in *S. cerevisiae* via expression of a heterologous pathway that diverts the carbon from the shikimic acid pathway at the node of DHS. This pathway consisted of four steps, namely, DHS dehydratase (*3DSD*) from *Podospora pausiceta*, an aromatic carboxylic acid reductase (*ACAR*) from *Nocardia sp.*, a phosphopantetheinyl transferase (*PPTase*) from *Escherichia coli* to activate *ACAR*, and an *O*-methyl transferase (*OMT*) from *Homo sapiens* (Fig. 1). The introduction of this pathway in *S. cerevisiae* resulted in the accumulation of 45 mg L^{-1} of vanillin after 48 h of small-scale batch fermentation [31]. A further study addressed the toxicity of vanillin by overexpression of a glycotransferase (*UGT*) from *Arabidopsis thaliana* in the previously reported strain, yielding a strain capable of producing 100 mg L^{-1} of vanillin-β-D-glucoside (VG) after 90 h of fermentation [9]. Furthermore, the same group investigated engineering strategies to increase the carbon flux into the aromatic pathway by means of in silico metabolic modeling. The FBA prediction suggested that the knockout of *GHD1* encoding glutamate dehydrogenase could enhance the production of VG. This was supported by the fact that deletion of this gene increased the pool of NADPH, thermodynamically favoring the activity of *ACAR*. However, this prediction did not result in higher vanillin titers due to a reduced biomass yield on glucose. A second knockout target was the gene *PDC1* encoding pyruvate decarboxylase, whose deletion could reduce the overall pyruvate decarboxylase activity by ~30 %, ensuring that less carbon was directed towards production of ethanol while maintaining cellular growth (Fig. 2b). This resulted in a twofold increased production of VG compared with the

Table 1 De novo production of the most significant aromatic amino acids-derived compounds in *S. cerevisiae*

	Compound	Strain	Substrate	Titer mg L^{-1}	Yield mg g^{-1}	Fermentation type	References
Shikimic acid pathway	Shikimic acid	INVSc1-SA3	Glucose	380	19	Batch	[67]
	Muconic acid	MA-12	Glucose	141	3.9	Batch	[13]
		INVSc1-MA2	Glucose	559.5	14	Batch (mini reactor with 10–20 % dissolved oxygen)	[68]
	Vanillin	VG2	Glucose	500	32	Continuous chemostat (dilution rate = 0.015 h^{-1})	[9]
L-tyr/L-phe branch	L-tyrosine	TY1041M	Glucose	350 (cytosolic)	17	Batch	[29]
	Coumaric acid	ST4058	Glucose	1930	*NS*	Fed-batch (FIT media)	[60]
	Naringenin	IMX106	Glucose	108	5.4	Batch	[45]
	trans-Resveratrol	ST4152	Glucose	415.6	*NS*	Fed-batch	[47]
	trans-Resveratrol	ST4152	Ethanol	531.4	*NS*	Fed-batch	[47]
	Thebaine	CSY1064	Glucose	0.0064	0.00032	Batch	[25]
	Hydrocodone	pCS2765	Glucose	0.0003	0.000015	Batch	[25]
L-trp branch	*N*-Acetylserotonin	SCE-iL3-HM-60	Glucose	~45	*NS*	Fed-batch (FIT media)	[27]
	Melatonin	SCE-iL3-HM-60	Glucose	14.5	*NS*	Fed-batch (FIT media)	[27]

NS not specified

parental strain from 250 to 500 mg L^{-1} in a very low dilution rate chemostat fermentation [9] (Table 1).

Engineering the L-tyr and L-phe branches

In plants, the L-tyr and L-phe metabolic branches provide precursors to a great amount of aromatic secondary metabolites, such as flavonoids, stilbenoids, and alkaloids. The application of these molecules ranges from the food and nutraceutical industries to the pharmaceutical industry. For instance, the pain management pharmaceuticals, a group of drugs that contain the L-tyr-derived alkaloids, are expected to reach a global market of $40.8 billion by 2020 [17]. Extraction of these compounds from plant tissues, however, is strongly dependent on environmental factors that can cause production instability in the farming processes. Hence, the production of secondary metabolites derived from L-tyr and L-phe has been intensively studied in microbial platforms in the past decade.

To enable its microbial production, research has been directed mostly towards engineering the aromatic amino-acid pathway for the accumulation of L-tyr. The work by Gold et al. [29] described a metabolomics approach aided by the in silico analysis for the construction of a yeast platform for overproduction of L-tyr. To construct the base strain, the feedback inhibition points were removed by overexpression of the *S. cerevisiae* feedback insensitive enzymes $ARO4_{K229L}$ and $ARO7_{G141S}$ (Fig. 2a), and the byproduct formation was removed by knocking out phenylpyruvate decarboxylase *ARO10* (Fig. 2c). Special attention was directed towards engineering the central carbon metabolism to enhance the production of precursors. Deletion of *ZWF1* encoding glucose-6-phosphate dehydrogenase was attempted with the hypothesis of linking NADPH deficiency and subsequent regeneration in the L-tyr pathway by the action of overexpressed $NADP^+$-dependent prephenate dehydrogenase *TYR1*. The L-tyr concentrations were improved to 192 mM (~350 mg L^{-1}) when L-methionine was added into the medium to recover the growth deficiency caused by deletion of *ZWF*1, because L-methionine reduces the cellular demand for NADPH during amino acid synthesis (Table 1). Finally, to increase availability of PEP, a mutant version of the major pyruvate kinase ($CDC19_{T21E}$) (Fig. 2b), with impaired activity was knocked into substitute the wild-type gene. However, no increase of carbon flux into the aromatic amino acid pathway was observed. Even though the mutant still possessed low pyruvate kinase activity, the decreased cellular fitness made the cells unviable in the absence of L-methionine and caused the L-tyr production to drop significantly.

2-Phenylethanol

Phenethyl alcohol (2-PE) is a benzene-derived chemical with an ethylic group widely used in industry due to its particular floral odor. Altogether, its market is expected to reach $700 million by 2019 [55], yet its current production process is based on chemical synthesis (alkylation of benzene) which yields undesired side products, making the separation processes costly [18]. Production of 2-PE

has also been studied in microbial systems. As a naturally occurring molecule in *S. cerevisiae,* it provides organoleptic features to fermented beverages like sake and beer. This compound can be produced de novo through the conversion of glucose to L-phe. The latter amino acid undergoes transamination, decarboxylation, and dehydrogenation to finally yield 2-PE (a mechanism known as the Ehrlich pathway) (Fig. 3). Work has been directed towards increasing the de novo production of 2-PE. For instance, Fukuda et al. [23] subjected *S. cerevisiae* to inhibitory concentrations of fluorophenylalanines, which act as competitive inhibitors of L-phe synthesis. The recovered spontaneous mutants, those with higher tolerance to the inhibitors, could also accumulate sixfold higher 2-PE (1.3 g/L) than the parental strain [24]. Analysis of the mutant strain revealed a mutation on the L-tyr-inhibited DAHP synthase (*ARO4*) causing its release from the feedback inhibition and resulting in higher carbon flux towards production of L-tyr, L-phe, and 2-PE [22].

Biotransformation of L-phe to produce 2-PE has also been extensively studied in *S. cerevisiae* as an alternative to overcome the low yields of de novo synthesis. This process requires the addition of L-phe into the fermentation medium, usually accompanied by a poor nitrogen source, forcing the cells to catabolize the aromatic amino acid. Concentrations as high as 4.8 g L^{-1} have been obtained in yeast [44], albeit product toxicity remained as the limiting factor. In situ product recovery has thus been crucial in the production of 2-PE, with the most successful technique being two-phase extraction using oleic acid as the extractant, which elevated the production to 12.6 g L^{-1} [65, 66].

Coumaric acid

The production of the important nutraceutical group of flavonoids and stilbenoids initiates with the common precursor p-coumaric acid (pHCA). This compound can be derived directly from L-tyr through the enzyme L-tyr ammonia lyase (*TAL*), or from L-phe, through the sequential action of two enzymes: phenylalanine ammonia lyase (*PAL*) and cinammate-4-hydroxylase (*C4H*) [74] (Fig. 3).

Recently, Jendresen et al. [38] characterized several *TAL*s from various microbial sources, resulting in the finding of two enzymes, from the bacteria Herpetosiphon aurantiacus and Flavobacterium johnsoniae, with increased catalytic activity and high product specificity (barely any cinnamic acid was observed). The latter enzyme (*FjTAL*) was incorporated in the subsequent research by Rodriguez et al. [60] with the goal of constructing a *S. cerevisiae* platform for high accumulation of pHCA. Unlike the work described by Gold et al. [29], the focus was only directed to manipulate the aromatic aminoacid pathway without altering precursor supply through tailoring the central carbon metabolism. Similar strategies were implemented: removal of L-tyr feedback inhibition (overexpression of $ARO4_{K229L}$ and $ARO7_{G141S}$), removal of competitive pathways leading to phenylacetaldehyde (PAA) and 4-hydroxyphenylacetaldehyde (4-HPAA) (*ARO10* and pyruvate decarboxylase *PDC5*; Fig. 2c), and channeling of carbon flux towards L-tyr (overexpression of *TYR1*, Fig. 1). Finally, to study the push of carbon flux through the pathway, the complete shikimate pathway from *S. cerevisiae* (*ARO1* and *ARO2*) and the orthologous genes from *E. coli* were independently integrated into the genome under the control of strong constitutive promoters. Overexpression of the *E. coli* SA kinase II alone (*aroL*) demonstrated the biggest improvement, indicating that this step could be rate limiting in the pathway. The highest producing strain accumulated 1.93 g L^{-1} of pHCA in microplate fermentation and 1.89 g L^{-1} in bioreactors, both using synthetic Feed-In-Time (FIT) fed-batch media that gradually release glucose from higher polysaccharides within the medium solution under the action of supplemented hydrolase (Table 1).

Flavonoids and stilbenoids

Jiang et al. reported the production of the flavanone nutraceutical naringenin in *S. cerevisiae* [40]. This work was further extended by Trantas et al. [72] via constructing a series of strains capable of converting L-phe to a palate of flavonoids and stilbenoids, namely, trans-resveratrol, naringenin, genistein, kaempferol, and quercetin. To convert L-phe into the aforementioned products, initially, a strain overexpressing *PAL*, *C4H* (cytochrome P450 enzyme) and *CPR* (cytochrome P450 reductase) was constructed to accumulate pHCA. Although *S. cerevisiae* contains a copy of CPR, overexpression of a Glycine max (soybean) *CPR* increased the accumulation of pHCA by fourfold. All of the subsequent strains contained a 4-coumaric acid ligase gene (*4CL*) to convert pHCA to 4-coumaroyl CoA. To enter the stilbenoids pathway, the type III polyketide synthase (resveratrol synthase, *RS*) was overexpressed to produce trans-resveratrol. To construct the flavonoid-producing strains, the chalcone synthase (*CHS*) and the chalcone isomerase (*CHI*) were overexpressed, leading to production of naringenin. Further strains diverting naringenin into genistein (isoflavone) or flavonols (kaempferol and quercetin) were obtained by expression of the isoflavone synthase (*IFS*), or the flavanone 3-hydroxylase (*F3H/F3′H*) together with the flavonol synthase (*FLS*) (Fig. 3). Although low titers were obtained, all the products were observed in the medium when L-phe or any of the downstream metabolites were fed into the medium. This indicated that all the plant genes were functional in *S. cerevisiae*, but that pathway optimization was required to remove bottlenecks and enable higher conversion efficiencies.

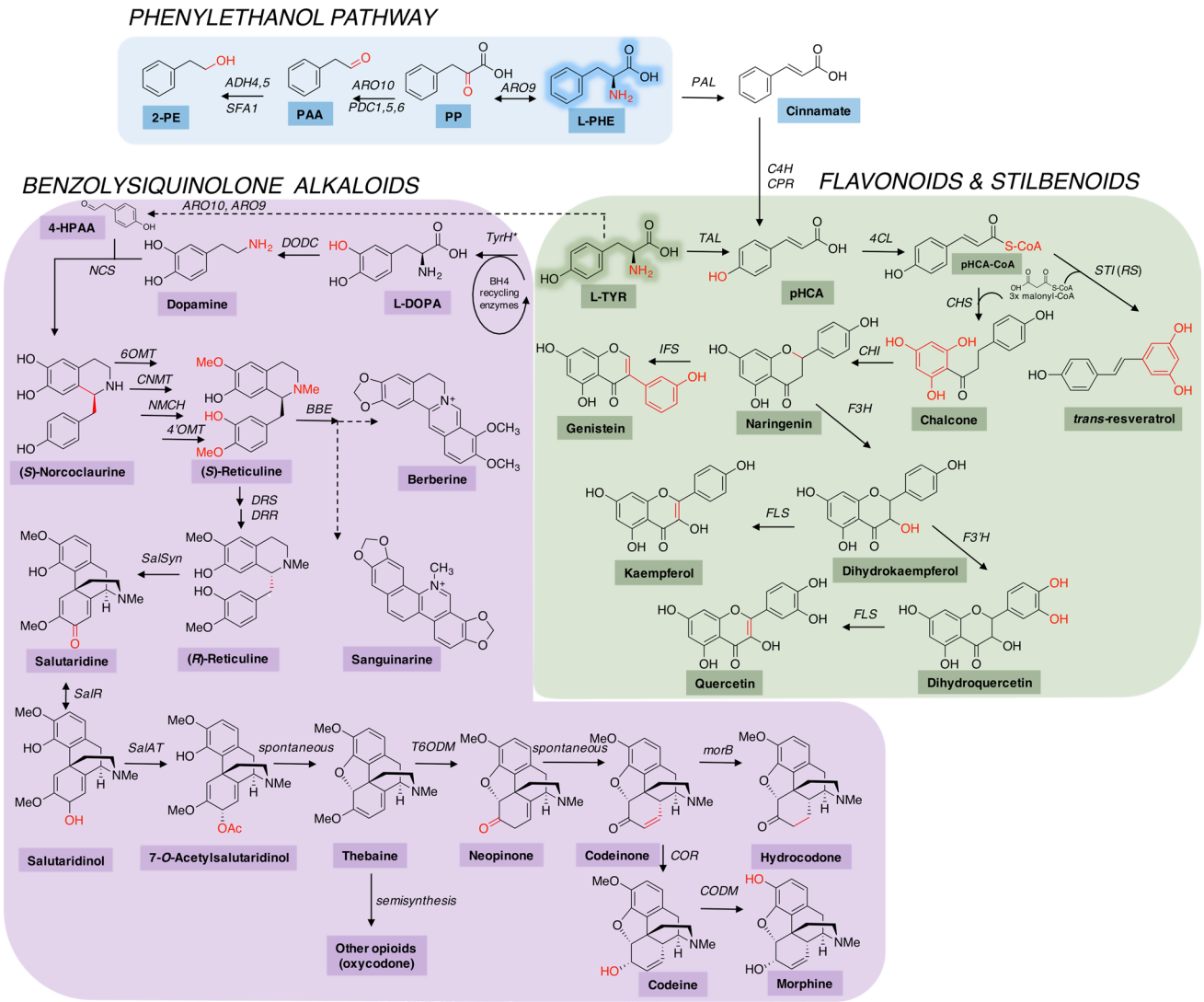

Fig. 3 Metabolic pathways (native and heterologous) for production of L-tyr- or L-phe-derived aromatic compounds in *S cerevisiae*. Abbreviation of metabolites—PAA, phenylacetaldehyde; 2-PE, phenylethanol; pHCA, coumaric acid; L-DOPA, L-3,4-dihydroxyphenylalanine; 4-HPAA, 4-hydroxyphenylacetaldehyde. Abbreviation of enzymes—*ARO10*, phenylpyruvate decarboxylase; *PDC1,5,6,10*, pyruvate decarboxylase isoenzymes; *ADH4,5*, alcohol dehydrogenase isoenzymes; *SFA1*, bifunctional ADH and formaldehyde dehydrogenase; *PAL*, phenylalanine ammonia lyase; *TAL*, tyrosine ammonia lyase; *4CL*, 4-coumaric acid-CoA ligase; *RS*, resveratrol synthase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *IFS*, isoflavone synthase; *F3H/F3'H*, flavanone 3-hydroxylase; *FLS*, flavonol synthase; *TyrH**, tyrosine hydroxylase repression insensitive mutant, *DODC*, *L-DOPA* decarboxylase; *morB*, morphine reductase. The following enzymes require cytochrome P450 reductase (CPR): *C4H*, cinammate-4-hydroxylase; *NCS*, norcoclaurine synthase; *6OMT*, norcoclaurine 6-O-methyltransferase; *CNMT*, coclaurine N-methyltransferase; *4′OMT*, 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase; *NCMH*, N-methylcoclaurine hydroxylase; *BBE*, berberine bridge enzyme; *DRS*, 1,2-dehydroreticuline synthase; *DRR*, 1,2-dehydroreticuline reductase; *SalSyn*, salutaridine synthase; *SalR*, salutaridine reductase; *SalAT*, salutaridinol acetyltransferase; *T6ODM*, thebaine 6-O-demethylase; *COR*, codeinone reductase; *CODM*, codeine demethylase. The *dash lines* represent multiple steps

Metabolic engineering strategies for de novo production of naringenin were investigated by Koopman et al. [45]. The common strategies for removal of feedback inhibition and byproduct formation were implemented in this work. In addition, expressing the corresponding ammonia lyase enzymes (*TAL* or *PAL*) to increase precursor availability enhanced the production of pHCA. The bottleneck step downstream was partially removed by integrating into the genome additional copies of *CHS*, leading to a production of 134.5 μM naringenin (36 mg L^{-1}) in batch fermentation. Optimization of the fermentation process in a bioreactor led to a maximal production of 400 μM (108 mg L^{-1}) (Table 1).

Resveratrol is a stilbenoid that is highly sough-after due to its nutraceutical properties. The production in *S. cerevisiae* has been achieved by whole-cell conversion

of pHCA [4, 72] or in complex media containing L-tyr [63, 75]. Although one of the highest producing strains on record could accumulate up to 391 mg L^{-1} resveratrol, it also required the supplementation of pHCA [70]. Recently, a *S. cerevisiae* strain was constructed for the de novo production of resveratrol in minimal media without the supplementation of L-tyr or L-phe [47]. This entailed the expression, under strong constitutive promoters, of the heterologous *TAL* gene (tyrosine ammonia lyase) from *Herpetosiphon aurantiacus*, the *ST1* gene (resveratrol synthase) from *Vitis vinifera*, the *4CL* from *Arabidopsis thaliana*, and the acetyl-CoA carboxylase variant $ACC1_{S659A,\ S1157A}$ (post-translational de-regulated mutant) from *S. cerevisiae* to increase the availability of malonyl-CoA. These manipulations, together with the removal of L-tyr feedback inhibition, led to the production of 235 mg L^{-1} resveratrol. Furthermore, production in fed-batch mode using glucose or ethanol as feeding substrates after the initial carbon depletion led to the production of 415.65 and 531.41 mg L^{-1} resveratrol, respectively, representing the highest productions reported to date (Table 1).

Benzylisoquinoline alkaloids

Benzylisoquinoline alkaloids (BIAs) compose a large group of plant secondary metabolites that include the opioid chemicals. Used for the treatment of severe pain, this group of molecules, which also contains codeine, morphine, oxycodone, and hydrocodone, represents the largest global market of pain management pharmaceuticals [17]. Other BIAs, such as berberine and sanguinarine, can be implemented as antibacterial agents [21]. *Papaver somniferum*, commonly named opium poppy, is the primary source of natural and semisynthetic opioids. Industrial farming of this plant to satisfy its huge demand, however, is prone to commercial instabilities due to its dependence on environmental factors. Hence, efforts to decipher the natural enzymatic pathways and construct microbial factories to produce these important metabolites to ensure a more controllable production scheme have been a work in progress for the last couple of decades [2, 30, 81]. Eukaryotic microorganisms like *S. cerevisiae* have been central in this research, given their outstanding ability to express challenging heterologous enzymes, especially cytochrome P450 s, whose activities require the presence of an electron donor protein (*CPR*), as well as a compartmentalized cellular environment provided by endoplasmic reticulum membranes [59].

One of the biggest breakthroughs of 2015 was the demonstration of the complete biosynthesis of BIAs from glucose in yeast. This work, published by Galanie et al. [25], is a product of over a decade of work from several research groups that intensively worked on reconstructing the entire pathway leading to the production of thebaine (an important precursor of morphinan opioids) and hydrocodone (an important semisynthetic opioid) from glucose. The biosynthesis of the initial intermediate (*S*)-reticuline was targeted first, which started from the heterologous expression of a repression insensitive L-tyr hydroxylase variant ($TyrH_{R37E,\ R38E,\ W166Y}$) from *Rattus norvegicus* coupled with four enzymes from the same source for the biosynthesis of the electron carrier co-substrate tetrahydrobiopterin (BH4) and its recycling and salvage enzymes to synthesize L-DOPA (Figs. 2e, 3). Central carbon metabolism was further modified following the examples presented in the previous sections, namely, derepression of feedback inhibition by expressing L-tyr insensitive *ARO4* and *ARO7* ($ARO4_{Q166K}$ and $ARO7_{T226I}$), expression of *TKL1*, and deletion of *ZWF1*. To convert L-DOPA to (*S*)-reticuline, seven heterologous genes and the endogenous *ARO10* for production of 4-HPAA were overexpressed. The genes were isolated from different plants and bacteria, including the L-DOPA decarboxylase (*DODC*), a norcoclaurin synthase (*NCS*), three methyltransferases characterized in the previous works [32, 51], and one cytochrome P450 enzyme (*NMCH*) associated with *CPR* [73].

Completion of the pathway to produce thebaine required the determination of the key enzymatic step responsible for the stereochemical conversion of (*S*)-reticuline to (*R*)-reticuline. This goal was achieved by three independent groups, which opened the gate to the complete biosynthesis of morphine [20, 25, 76], leading to the discovery of 1,2-dehydroreticuline synthase-1,2-dehydroreticuline reductase fusion enzymes (*DRS-DRR*) (Fig. 3). The conversion of (*R*)-reticuline to thebaine was followed by the expression of an engineered salutaridine synthase (*SalSyn*, bottleneck step), in which the *N*-linked glycosylation was avoided by replacement of the *SalSyn* α-helices with those from cheilanthifoline synthase (*CFS*), which is homologous to *SalSyn*, but will not be glycosylated when expressed in yeast [25]. Furthermore, codon-optimized salutaridine reductase (*SalR*) and salutaridinol acetyltransferase (*SalAT*) [35, 80] were expressed to complete the pathway to thebaine. Finally, the expression of thebaine *6-O*-demethylase (*T6ODM*) and morphine reductase (*morB*) led to the construction of a strain capable of accumulating hydrocodone at a titer of ~0.3 g L^{-1} after 120 h of fermentation directly from sugars (Table 1, Fig. 3).

Engineering the L-trp branch

The biosynthesis of L-trp initiates with the conversion of chorismate to anthranilate by action of the enzymatic complex *TRP2* and *TRP3*. The entrance of carbon into this branch of the aromatic amino acid pathway can be regulated by the concentrations of L-tyr and L-trp. L-tyr feedback

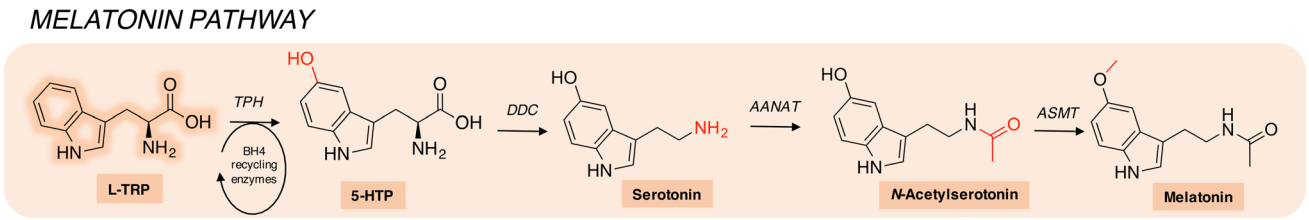

Fig. 4 Heterologous metabolic pathway for production of L-trp-derived melatonin in *S. cerevisiae*. Abbreviation of metabolites—5-HTP, 5-hydroxytryptophan. Abbreviation of enzymes—*TPH*, tryptophan hydroxylase; *DDC*, 5-hydroxy-L-tryptophan decarboxylase; *AANAT*, serotonin N-acetyltransferase; *ASMT*, *N*-acetylserotonin *O*-methyltransferase

inhibits *ARO7*, whereas L-trp counteracts this inhibition but feedback inhibits *TRP2* and *TRP3*, therefore, allowing carbon flux to be appropriately split between two branches [10] (Fig. 1). Although L-trp is an important metabolite and key in the production of natural products (e.g., indole alkaloids) as well as mammalian neurotransmitters like melatonin and serotonin, so far there are no reports of a *S. cerevisiae* strain capable of overproducing L-trp.

Monoterpene indole alkaloids

Monoterpene indole alkaloids (MIAs) are a group of secondary metabolites found in many plants with diverse medicinal properties [56]. Two common MIAs are the *Catharanthus roseus*-derived vinblastine and vincristine, which have been approved for anticancer treatment [28]. Engineering the main MIA precursor strictosidine in yeast has been achieved by direct supplementation of the precursors tryptamine (a derivative of L-trp) and secologanin in a strain carrying the *C. roseus* strictosidine synthase, leading to a titer of 2 g L^{-1} strictosidine [26]. De novo production of strictosidine has also been recently demonstrated. This work was devoted to engineering the mevalonate pathway for increased availability of geranyl pyrophosphate (GPP) followed by heterologous expression of 14 plant genes encoding the synthesis of secologanin. No evident engineering work was done on the aromatic amino acid pathway to increase the supply of L-trp for production of tryptamine (L-trp was supplemented in the amino acid mix of the growth medium) [11]. To date, engineering the production of L-trp has yet to be demonstrated.

Melatonin

Melatonin is a naturally occurring, L-trp-derived hormone in mammals that regulate the circadian cycle, and it is popularly known due to its nutraceutical properties as a sleeping aid as well as an antioxidant compound [12, 43]. Although melatonin is commonly extracted from the pineal gland of mammalian organisms, it can also be naturally produced by *S. cerevisiae* during alcoholic fermentation. The production is highly dependent on the concentration of L-trp in the media, the different nitrogen sources, and the strain used [61]. As a GRAS (generally recognized as safe) microorganism, de novo production of melatonin in yeast could be preferred over extraction from animal tissues or from chemical synthesis which involved very toxic compounds [33].

Two groups have recently demonstrated the production of 5-hydroxytryptophan (5-HTP), which is the first intermediate in the synthesis of melatonin. Zhang et al. [78] studied the action of three types of hydroxylases, i.e., L-trp hydroxylase (*T3H* or *T5H*), and L-phe hydroxylase (*P4H*) on the conversion of L-trp to 5-HTP. Expression of *Oryctolagus cuniculus T5H* in combination with the biosynthesis pathway of the cofactor BH4 (Fig. 2e) resulted in a strain producing around 7.3 g L^{-1} starting from 2 g L^{-1} of L-trp.

A second group achieved de novo production of 5-HTP and also demonstrated the production of melatonin [27]. The highest producing strain was constructed by integrating into the yeast's Ty2 retrotransposon sites multiple copies of L-trp hydrolysase from *Schistosoma mansoni* (*smTPH*), *H. sapiens* 5-HTP decarboxylase (*HsDDC*), *Bos taurus* serotonin acetyltransferase (*BsAANAT*), and finally, *H. sapiens* acetylserotonin O-methyltransferase (*HsASMT*) (Fig. 4). BH4 was also recycled by the expression of *P. aeruginosa* pterin-4-alpha-carbinolamine dehydratase (*PaPCBD1*) and *R. norvegicus* 6-pyruvoyl-tetrahydropterin synthase (*RnDHPR*) (Fig. 2e). The highest producing strain accumulated melatonin at a titer of 14.5 mg L^{-1} in fed-batch mode (Table 1). It is important to mention, however, that several strategies implemented in this work could not elevate the production of melatonin; for instance, the external supplementation of L-trp did not show a significant increase. A potential approach suggested by the success in *E. coli* could be expression of the novel salicylate 5-hydroxylase, which can convert anthranilate into 5-hydroxyanthranilate. This molecule could further be processed through the promiscuous L-trp biosynthesis genes in *S. cerevisiae* leading to the direct production of 5-HTP, eliminating the usage of the unstable *T5H* and the requirement of expressing the enzymes for BH4 recycling (Fig. 2e) [69]. Given the

minuscule titers observed from these previous examples, it is evident that engineering of the L-trp biosynthesis branch in *S. cerevisiae* is still in its infancy.

Remarks and future direction

The advent of synthetic biology, inexpensive DNA sequencing tools, and various bioinformatics tools has enabled the elucidation of complex aromatic amino acid-derived natural product pathways as well as the establishment of such pathways into microbial hosts for more efficient and sustainable production processes. However, it is evident that the yields and titers in *S. cerevisiae*, using a renewable sugar as the sole carbon source, are still far from meeting the market demands. As discussed in this review, the manipulations on the pathways to increase the carbon flow are merely limited to the removal of the feedback inhibition points (*ARO4* and *ARO7*) (Fig. 2). Thus, far, engineering availability of the precursor E4P has been modestly demonstrated by expression of *TKL1*, but altering the lower glycolytic pathway to enable higher accumulation of PEP for the production of DAHP remains as a great challenge. Furthermore, the examination of potential transcriptional regulators that could help rewire the carbon distribution to boost the carbon entrance into the aromatic pathway remains uninvestigated.

The most updated market analyses for aromatic compounds forecast considerable increase in national and worldwide demands in all major commercial segments, from food and nutraceuticals to drugs and pain management medications. This emphasizes the strong need for quick development of microbial hosts to gradually reduce the utilization of non-renewable resources as the main feedstocks. This goal can be accomplished by developing efficient (1) high-throughput screening methods, (2) co-culture systems, and (3) genetic tools for engineering non-conventional yeast.

The development of high-throughput screening methods allows exploration of large combinatorial spaces (strain libraries) for selection of improved phenotypes. Such methods can be tied with a growth response [16] or an optical or electrochemical response [6, 34]. One example of a biosensor to improve production of aromatic secondary metabolites was developed recently [15]. It was based on screening a library of L-tyr hydroxylase mutants from *Beta vulgaris* (*CYP74AD1*) with enhanced activity. By coupling this enzyme with the L-DOPA dioxygenase from *Mirabilis jalapa*, L-DOPA was converted to a yellow fluorescent pigment betaxanthin. An identified mutant had a 2.8-fold improvement in activity, which translated into a 7.4-fold improvement in dopamine titer. This new mutant enzyme can be used to streamline the production of many valuable BIAs.

Co-culturing systems have garnered attention in recent years as an alternative to efficiently produce biorenewable chemicals [77]. One advantage of such systems is the partitioning of large metabolic pathways into two or more microbial hosts, to reduce the metabolic burden imposed on a monoculture system, as well as to facilitate modular optimization. In the realm of aromatic compounds, Jones et al. [41] implemented an *E. coli* co-culturing process to improve the production of flavonoids. By dividing the pathway into two strains (malonyl-CoA-requiring upstream and NADPH-requiring downstream), and optimizing culturing conditions through computational modeling, they obtained a system with 65 % improved titer in flavan-3-ols. Such an approach could also be implemented in yeast cultures with the advantage of a more efficient expression of cytochrome P450 enzymes.

Finally, although *S. cerevisiae* has been greatly domesticated and serves as a model for quick and robust engineering, a major disadvantage stems from being Crabtree positive yeast. This entails the elevated production of ethanol, rather than biomass precursors, under aerobic respiration in high glucose concentrations. As a result, lower titers for value-added compounds, such as aromatic chemicals, are observed. Although research has been devoted to diminish this effect in *S. cerevisiae* [54], a potential alternative solution is to engineer Crabtree negative non-conventional yeasts. Such yeasts include, but are not limited to *Yarrowia lipolytica* and *Schefferomyces stipitis*. Special attention should be directed to the latter species, since it possesses a highly efficient xylose utilization pathway. As a result, it is expected to carry a higher carbon flux into the PPP, which could ultimately lead to an enhanced production of aromatic compounds due to an elevated availability of the precursor E4P.

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