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Current Opinion in Biotechnology

Current state of aromatics production using yeast: achievements and challenges

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Aromatics find a range of applications in the chemical, food, cosmetic and pharmaceutical industries. While production of aromatics on the current market heavily relies on petroleum-derived chemical processes or direct extraction from plants, there is an increasing demand for establishing new renewable and sustainable sources of aromatics. To this end, microbial cell factories-mediated bioproduction using abundant feedstocks comprises a highly promising alternative to aromatics production. In this review, we provide the recent development of *de novo* biosynthesis of aromatics derived from the shikimate pathway in yeasts, including the model *Saccharomyces cerevisiae* as well as other non-conventional species. Moreover, we discuss how evolved metabolic engineering tools and strategies contribute to the construction and optimization of aromatics cell factories.

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Introduction

By rebuilding and optimizing the cellular metabolism, metabolic engineering enables the enhanced biosynthesis of native metabolites or heterologous new products using genetically modified organisms [1]. Such efforts comprise an attractive alternative for chemical production, which is generally obtained by non-sustainable processes, including plant extraction using various solvents and chemical synthesis. In the last decade, various cellular platforms, ranging from prokaryotic to mammalian cells, have been tested for their capacity to biosynthesize chemicals, with several demonstrating promise for producing a wide array of bulk chemicals, value-added specialty chemicals and pharmaceutical proteins [2–4]. Commercially viable processes using this cell-mediated approach have subsequently been established for chemical building blocks 1,3-propanediol and 1,4-butanediol, flavor enhancer L-glutamic acid and anti-malarial drug artemisinic acid [5]. Importantly, the community is constantly exploring and expanding the potential production space using available metabolic engineering strategies. This includes the recently established budding yeast strains that were engineered to produce elevated levels of oleoylethanolamide [6], a phospholipid derivative with promising pharmacological applications, as well as the major cannabinoids that have medicinal properties [7].

Aromatics, or aromatic compounds, which typically contain conjugated cyclic six-carbon structures, are also traditionally recognized as chemicals of value, due to their ability to stimulate the olfactory senses enabling their use as a fragrance. Being a vast group of chemical substances, aromatics have various applications in the chemical, food, cosmetic and pharmaceutical industries [8]. Most aromatics on the market are produced by petroleum-derived chemical synthesis or by isolating natural compounds produced from plants [9]. As a result, there is an increasing demand for new sources of aromatics to be established which includes more renewable and sustainable processes. In this context, microbe-mediated bioproduction using abundant carbon feedstocks constitutes a highly promising alternative, and numerous efforts have been made on developing cell platforms and processes that are dedicated to aromatics production. Here, we review the recent advances in research aimed at the *de novo* biosynthesis of aromatics in yeasts, including the model *Saccharomyces cerevisiae* as well as other non-conventional species (Table 1). In addition, emerging difficulties in strain construction and optimization as well as potential countermeasures are presented and discussed.

Production of aromatics in yeast

The shikimate pathway, which connects the central carbon metabolism to the biosynthesis of aromatic amino acids (AAA, L-phenylalanine, L-tyrosine, and L-tryptophan), is widespread across bacteria, archaea, fungi, algae and plants. While detailed study and characterization of enzymes and genetic regulations involved in this key metabolic route have been well presented and reviewed elsewhere [10], our first focus will be on the metabolic engineering efforts recently performed to create yeast platforms capable of

Table 1

Selection of reconstructed *de novo* biosynthesis of aromatics in yeast systems

Category	Aromatics	Host	Titer and yield[a]	Fermentation type	Reference
Phenol derivatives	*cis*, *cis*-Muconic acid	*S. cerevisiae*	1.2 g/L (Glu); 33 mg/g (Glu)	Shake-flask	[17]
		S. cerevisiae	2.0 g/L (Glu); 13.4 mg/g (Glu)	Fed-batch bioreactor	[18]
		S. cerevisiae	2.1 g/L (Glu); 12.9 mg/g (Glu)	Fed-batch bioreactor	[13]
		S. cerevisiae	2.7 g/L (CCM and PCA, Glu); 51.6 mg/g (CCM and PCA, Glu)	Shake-flask	[15]
		S. cerevisiae	1.2 g/L (Glu and Suc) and 5.1 g/L (Glu and Sur with amino acids supplementation); 58 mg/g (Glu and Sur with amino acids supplementation)	Fed-batch bioreactor	[19•]
	Vanillin	*S. cerevisiae*	45 mg/L (Glu)	Shake-flask	[20]
		S. pombe	65 mg/L (Glu)	Shake-flask	[20]
		S. pombe	>100 mg/L (vanillin β-D-glucoside, Glu)	Shake-flask	[20]
	2-Phenylethanol	*S. cerevisiae*	1.6 g/L (Glu); 76.6 mg/g (Glu)	Batch bioreactor	[23]
		P. pastoris	1.2 g/L (Glu); 58.5 mg/g (Glu)	Shake-flask	[24]
Phenylpropanoids	*p*-Coumaric acid	*S. cerevisiae*	12.5 g/L (Glu); 139.6 mg/g (Glu)	Fed-batch bioreactor	[25•]
		S. cerevisiae	242 mg/L (Xyl); 9.7 mg/g (Xyl)	Shake-flask	[26]
Flavonoids	Naringenin	*S. cerevisiae*	90 mg/L (Glu); 4.5 mg/g (Glu)	Shake-flask	[27]
		Y. lipolytica	898 mg/L (Glu)	Fed-batch bioreactor	[28]
	Kaempferol	*S. cerevisiae*	26.6 mg/L (Glu)	Shake-flask	[29]
	Quercetin	*S. cerevisiae*	20.4 mg/L (Glu)	Shake-flask	[29]
	Fisetin	*S. cerevisiae*	2.3 mg/L (Glu)	Shake-flask	[29]
	8-Prenylnaringenin	*S. cerevisiae*	0.12 mg/L (Glu)	Shake-flask	[30]
	Eriodictyol	*Y. lipolytica*	134.2 mg/L (Glu)	Shake-flask	[31]
	Taxifolin	*Y. lipolytica*	110.5 mg/L (Glu)	Shake-flask	[31]
Alkaloids	Noscapine	*S. cerevisiae*	2.2 mg/L (Glu with Gly supplementation)	Shake-flask	[35•]
	8-Hydroxygeraniol	*S. cerevisiae*	227 mg/L (Glu)	Fed-batch bioreactor	[36]
	Nepetalactol	*S. cerevisiae*	5.9 mg/L (Glu)	Shake-flask	[36]
	Pseudotropine	*S. cerevisiae*	0.08 mg/L (Glu)	Shake-flask	[37]
	Tropine	*S. cerevisiae*	0.13 mg/L (Glu)	Shake-flask	[37]
		S. cerevisiae	6 mg/L (Glu with amino acids and Gly supplementation)	Shake-flask	[38]
	Cinnamoyltropine	*S. cerevisiae*	6 mg/L (Glu with amino acids and Gly supplementation)	Shake-flask	[38]

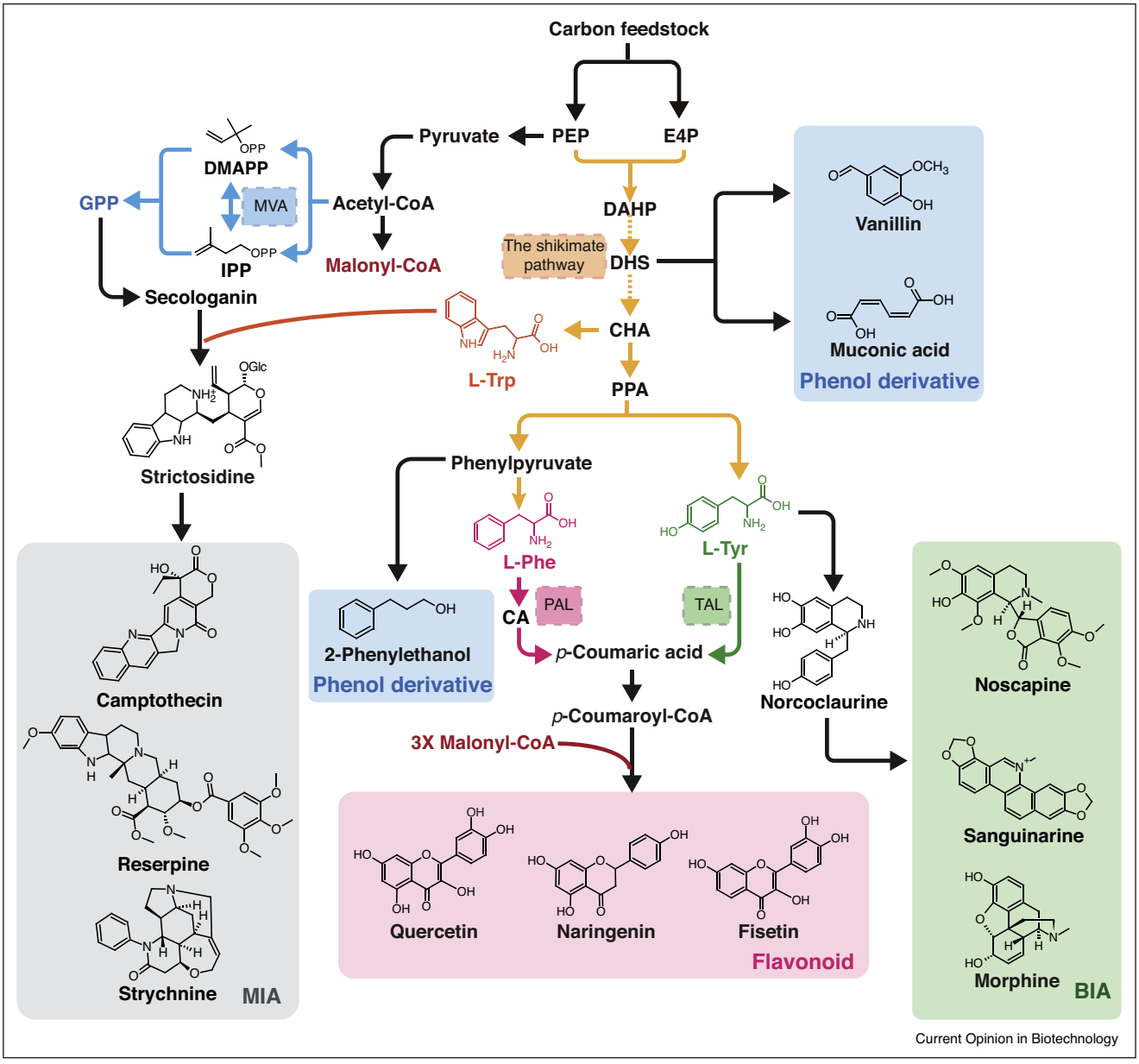
[a] Carbon sources used for yeast cultivations, Glu glucose, Suc sucrose, Xyl xylose, Gly, glycerol.

de novo biosynthesizing various aromatic chemicals derived from this pathway (Figure 1). Here, we focus on several groups of aromatic chemicals, including phenol derivatives, flavonoids, and alkaloids.

cis,cis-Muconic acid and Vanillin

cis,cis-Muconic acid (CCM) biosynthesis is of high importance to establish a potential biotechnological process for generating the precursor for adipic acid, which is widely used for the production of plastics and which is also mainly produced by fossil-based and environmentally unfriendly processes. While bacterial hosts, such as *Escherichia coli* has been engineered to produce a high level of CCM [11], the yeast *S. cerevisiae* is often considered as an attractive CCM production host as its fermentations can be performed at a low pH, which may facilitate downstream separation. However, only a trace 1.56 mg/L of CCM was produced by the first proof-of-concept of *S. cerevisiae* strain, in which three bacterial enzymes 3-dehydroshikimate (DHS) dehydratase, protocatechuic acid (PCA) decarboxylase, and catechol dioxygenase were expressed to convert endogenous DHS to the formation of heterologous CCM [12]. Since this initial work, the yield and titer of CCM have been substantially improved [13–15], more importantly, it becomes increasingly evident that yeast CCM production mainly suffers from two factors: (i) low performance of the CCM-forming enzymes and (ii) metabolic imbalance between the shikimate pathway and the heterologous branch. Specifically, the activity of PCA decarboxylase responsible for PCA decarboxylation was demonstrated to limit yield coming from the CCM pathway [16], thus resulting in remarkable buildup of pathway intermediates. Using an alternative approach, Bruckner *et al.* selected a new PCA decarboxylase derived from the yeast *Arxula adeninivorans* with the resultant strain

Figure 1



Schematic illustration of aromatics biosynthesis. Structures of building blocks, key intermediates and representative compounds of phenol derivatives (blue panel), flavonoids (magenta panel), benzylisoquinoline alkaloids (BIA, green panel), and monoterpene indole alkaloids (MIA, grey panel) are shown. PAL phenylalanine ammonia-lyase, TAL tyrosine ammonia-lyase. PEP phosphoenolpyruvate, E4P erythrose-4-phosphate, DAHP 3-deoxy-D-arabino-2-heptulosonic acid 7-phosphate, DHS 3-dehydroshikimate, CHA chorismic acid, PPA prephenate, L-Phe L-phenylalanine, L-Tyr L-tyrosine, L-Trp L-tryptophan, MVA, the mevalonate pathway, DMAPP dimethylallyl pyrophosphate, IPP isopentenyl pyrophosphate, GPP geranyl diphosphate.

generating up to 1.2 g/L CCM and a yield of 31 mg/g glucose in a shake-flask batch culture [17]. Moreover, a prokaryotic transcriptional regulator-mediated synthetic selection system, which enables the coupling of the abundance of CCM to cell growth, was also recently developed to rapidly identify CCM high-producing yeast strains [18]. Here, an identified variant out of a large strain library was able to generate approximately 2 g/L CCM under bioreactor conditions. In *S. cerevisiae*, DHS production is undertaken by the unique pentafunctional enzyme Aro1, which determines the carbon flux distribution towards the native AAA biosynthesis and heterologous CCM pathway. Hence, effective modulation of Aro1 activity should also render more DHS for CCM

production. To this end, a C-terminal degron tag was introduced to destabilize Aro1 protein, which resulted in remarkable accumulation of the precursor PCA [19•]. Further combination of functional FMN prenyltransferase-coding *PAD1*, which is responsible for activating PCA decarboxylase activity, enabled nearly stoichiometric conversion (95%) of PCA to downstream products. Indeed, under the fed-batch fermentation conditions, the optimized strain produced 5.1 g/L CCM with a yield of 58 mg/g sucrose (supplemented with amino acids), representing the highest titer and yield obtained for CCM biosynthesis in yeast to date [19•].

The DHS node has also been exploited as an entry point for the heterologous biosynthesis of vanillin, currently one of the most widely used flavoring compounds on the market. With the introduction of only a small number of exogenous enzymes, including a fungal dehydratase, a bacterial aromatic carboxylic acid reductase, and a human *O*-methyltransferase, Hansen *et al.* demonstrated the capacity for vanillin biosynthesis in both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* with titers of 45 and 65 mg/L, respectively [20]. Growth inhibition assay showed that vanillin was toxic to *S. cerevisiae* at a concentration less than 0.5 g/L, while vanillin β-D-glucoside (VG) was non-toxic even at 25 g/L. By further expressing a plant glycosyltransferase in *S. pombe*, which converts vanillin to VG, and this enabled an increase in production of VG at titers exceeding 100 mg/L [20]. Furthermore, a recent study by Strucko *et al.* indicated that the laboratory strain S288c produced up to 10-fold more VG compared with that of the industrially relevant CEN.PK strain [21]. Thus, selection of yeast strain backgrounds should also be carefully considered in establishing a cell factory for vanillin or VG production.

2-Phenylethanol

As well as vanillin, the aromatic compound 2-phenylethanol (2-PE) is also widely used in the food and cosmetic industries due to the appeal of its rose-like scent. *S. cerevisiae* possesses the endogenous capacity to generate 2-PE by the means of the Ehrlich pathway, which has been characterized as the degradation pathway of branched-chain and aromatic amino acids as well as methionine [22]. For *de novo* 2-PE biosynthesis, the phenylpyruvate node from the shikimate pathway represents the key branch point. Very recently, Hassing *et al.* reported an engineered yeast strain capable of producing 1.6 g/L of 2-PE with a yield of 76.6 mg/g glucose, representing the highest yield for *de novo* produced 2-PE in *S. cerevisiae* [23]. Specifically, the expression of pyruvate kinase variants exhibiting reduced catalytic activity was demonstrated to improve phosphoenolpyruvate (PEP) supply while down-regulation of L-Tyrosine (L-Tyr) pathway showed to eliminate the formation of byproduct *p*-hydroxy-phenylethanol, with the latter significantly increasing 2-PE production in *S. cerevisiae*. Furthermore, by overexpressing key genes involved in the Ehrlich pathway *ARO10* and *ADH6*, in combination with de-regulating the upstream shikimate pathway, Kong *et al.* also demonstrated the first *de novo* 2-PE production using glucose in methylotrophic yeast *Pichia pastoris* at a level of 1.2 g/L [24].

p-Coumaric acid and (iso)flavonoids

Unlike the aromatics so far discussed, most of which have been produced mainly due to their aromas, flavonoid production has been researched due to their various favorable human health-related properties, such as antioxidant, anti-inflammatory, antiallergic, and anti-oncogenic. These compounds are a diverse group of phenylpropanoid-derived plant secondary metabolites with, in most plants, L-Phenylalanine (L-Phe) serving as the building block for phenylpropanoid biosynthesis, with another AAA, L-Tyr, taking this role in only certain flowering plants (partial monocots). During flavonoid synthesis, *para*-coumaric acid (*p*-HCA) occurs as the obligatory metabolic intermediate fueling the downstream (iso)flavonoids pathways. In *S. cerevisiae*, *p*-HCA production can be enabled by the introduction of specific plant or bacterial biosynthetic genes with recent systematic metabolic efforts resulting in a maximum *p*-HCA titer of 12.5 g/L with a yield of 139.6 mg/g glucose [25•]. The best producing strain harbors four lines of distinct genetic manipulation: (i) the combination of both L-Phe and L-Tyr-derived *p*-HCA forming routes, (ii) the inclusion of inducible promoters to control gene transcription, (iii) the implementation of a phosphoketalose-based strategy to enhance the supply of the limiting AAA precursor erythrose 4-phosphate (E4P), and (iv) further orchestrating of the carbon flux between glycolysis and the AAA biosynthesis pathway through promoter engineering of key gene branchpoints connecting these two pathways. In addition, the production of *p*-HCA using xylose, the second most abundant sugar after glucose, has also been developed in *S. cerevisiae* as well. Based on starting with an evolved xylose utilizing strain, a bacterial tyrosine ammonia-lyase (*TAL*) route was introduced together with overexpression of several important AAA biosynthetic genes, resulting in the production of 242 mg/L *p*-HCA in shake flask cultivation [26]. This engineered strain provides a platform for further improving the xylose catabolism to generate diverse phenylpropanoid chemicals.

Starting with *p*-HCA and another precursor malonyl-CoA, a multiple-step process catalyzes the formation of naringenin, a key intermediate to the biosynthesis of dihydroflavonols, flavonols, isoflavones and anthocyanins. In *S. cerevisiae*, a modular engineering strategy was applied to improve naringenin biosynthesis using tyrosine as the precursor. Through stepwise evaluation of the heterologous naringenin pathway (module 1) as well as the supply of native precursors malonyl-CoA (module 2) and L-Tyr (module 3), the final strain produced up to 90 mg/L of naringenin in shake flask fermentations,

a 20-fold increase compared with the parental strain [27]. However, no positive effect so far was observed with respect to tuning the biosynthesis of malonyl-CoA, which has been proposed to act as a limiting factor. Interestingly, high-level *de novo* production of naringenin in the oleaginous yeast *Yarrowia lipolytica*, well-known for its superior malonyl-CoA-derived fatty acid (FA) biosynthesis, was recently established through import of a heterologous pathway in a mutant background [28]. Specifically, the overexpression of *PEX10*, encoding a peroxisomal membrane protein required for peroxisome biogenesis, led to greater supply of acetyl-CoA by β-oxidation-mediated FA degradation. Subsequent building of enhanced activities of malonyl-CoA-producing acetyl-CoA carboxylase and mutated 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, in combination with more copies of naringenin biosynthetic genes, brought about an impressive 124.1 mg/L naringenin in test-tube fermentation [28]. This capacity was significantly amplified to generate 898 mg/L naringenin under optimized fed-batch bioreactor, the highest titer reported to date in a microbial host.

Growing efforts have also been made to extend the flavonoid biosynthetic pathway in order to provide an alternative source of more commercially valuable aromatics. For example, the reconstruction of long biosynthetic pathways, consisting of up to eight plant genes, resulted in production of 26.6 mg/L kaempferol and 20.4 mg/L quercetin, respectively [29]. Moreover, while in combination with additional chalcone reductase from the legume plant, the *de novo* biosynthesis of isoflavonoid fisetin to a titer of 2.3 mg/L was established in budding yeast for the first time [29]. By introducing other modifying enzymes responsible for prenylation or hydroxylation, *de novo* production of the potent phytoestrogen 8-prenylnaringenin and eriodictyol, and taxifolin were obtained in hosts *S. cerevisiae* [30] or *Y. lipolytica* [31], respectively.

Alkaloids

Alkaloids, comprising a wide array of naturally occurring secondary compounds, are popularly defined by structurally containing a heterocyclic nitrogen derived from amino acids. While the beneficial effects of alkaloids have long been exploited by traditional medicine worldwide, two subfamilies of alkaloids having particularly medical application, these are benzylisoquinoline alkaloids (BIA, including morphinan alkaloids and noscapine) from opium poppy and monoterpenoid indole alkaloids (MIA, including vincristine and vinblastine) found in the Madagascar periwinkle [32]. Among the known BIA and MIA biosynthetic pathways found to exist in plants, membrane-bound cytochrome P450 (CYP) enzymes, responsible for hydroxylation, constitute a remarkable portion of catalytic enzymes. Thus, the model eukaryotic microorganism *S. cerevisiae*, whose native endomembrane system should support appropriate function of CYPs, has been preferably chosen as the host for the latter's heterologous expression. Indeed, pioneering work has showcased the feasibility of producing representative BIA opioids [33] as well as key MIA intermediate strictosidine [34], respectively, in this yeast. With the availability of biosynthetic genes identified from *Papaver somniferum*, Li *et al.* demonstrated the *de novo* production of noscapine, a promising anticancer agent, in *S. cerevisiae* [35•]. This reconstructed biosynthetic pathway is composed of dozens of catalytic enzymes from bacteria, mammals, and especially several plant endoplasmic reticulum (ER)-targeted enzymes. Optimization of both heterologous and host endogenous metabolic pathways, and fermentation conditions resulted in the production of 2.2 mg/L noscapine, representing a greater than 18 000-fold improvement compared with the initial titers. One of the two basic precursors involved in MIA biosynthesis is geraniol, derived from the native mevalonate (MVA) pathway. By compartmentalizing the geraniol biosynthetic pathway in the mitochondria, in order to create dedicated geranyl pyrophosphate (GPP) pool that could not be consumed by the cytosolic competing pathway, a sixfold increase in geraniol production was achieved in budding yeast [36]. Further integration of plant geraniol hydroxylase, geraniol oxidoreductase, and iridoid synthase, also led to the substantial generation of downstream MIA precursors 8-hydroxygeraniol at a titer of 227 mg/L in fed-batch fermentation and 5.9 mg/L nepetalactol under shake flask conditions, the highest *de novo* nepetalactol production reported so far [36].

The sustainable production of other alkaloids, such as tropane alkaloids (TAs), which play a role in treating neurological disorders, also draws the attention of the metabolic engineering community. TAs comprise a family of more than 200 specialized metabolites, which are primarily produced in *Solanaceous* (nightshade) species. For the biosynthesis of TAs, tropine represents an important intermediate, which can be further combined with other molecules, such as L-Phe-derived phenyllactyl-CoA, to produce downstream TAs. With known plant TAs biosynthetic information and the newly characterized enzyme from *Anisodus acutangulus*, Ping *et al.* successfully reconstructed two six-step biosynthetic pathways leading to the production of 0.13 mg/L tropine and 0.08 mg/L pseudotropine, key intermediates in TAs biosynthesis, in *S. cerevisiae* from simple feedstocks in shake flask cultures [37]. Interestingly, another research group independently presented a set of TAs-producing yeast strains almost at the same time, which not only exhibited improved performance on tropine biosynthesis at a titer of 6 mg/L, but was also able to produce cinnamoyltropine, a non-canonical TA [38]. All these outcomes collectively provide a platform for further characterization of TAs-related plant enzymes as well

as motivate efforts towards generating medicinal TAs using industrial fermentation methods.

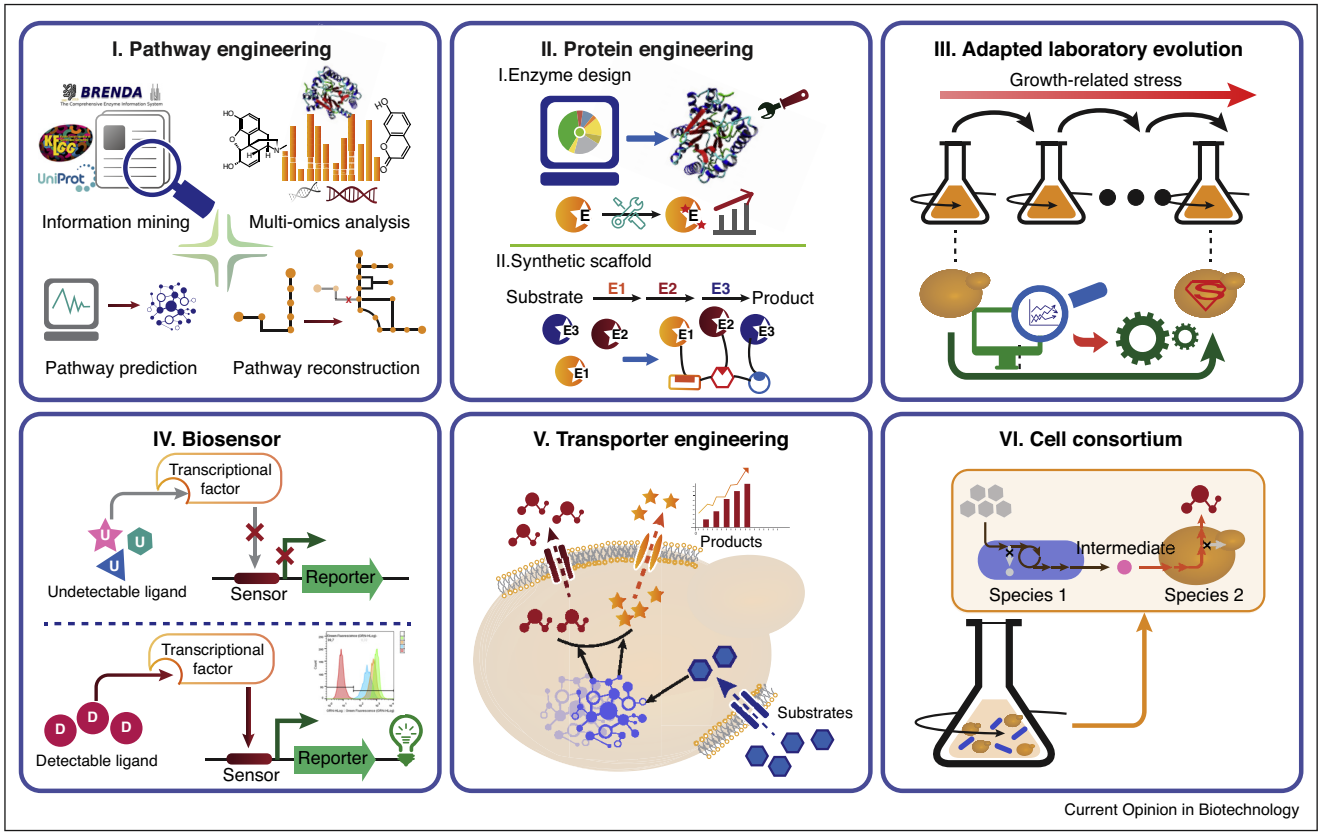
Challenges and perspectives

So far, the aforementioned efforts and outcomes have clearly demonstrated the feasibility of converting yeast hosts to cell factories for the production of various aromatics, which can be achieved through manipulating native yeast metabolism or by grafting heterologous biosynthetic pathways onto the cell's endogenous metabolic network. Despite their promising potential however, current performance of most generated strains is not at a level where fermentation-based production represents an economically viable alternative to traditional methods in industry. Multiple factors challenge the improvement of cell growth, product titer, rate of production, and yield (TRY). These include cellular toxicity of end-products, low activity and stability of catalytic enzymes, and metabolic imbalances at the levels of the biosynthetic pathway and across the cellular network. In addition, a lack of key fundamental information on the interactivity across and regulation within the metabolic network also handicaps this process, leading to a lag in the time taken to discover and elucidate the contribution of potential biosynthetic pathways in aromatics' production. Incomplete knowledge in these areas subsequently represents a major barrier in the construction of yeast cell factories for new or complex aromatics. However, rapid evolution of metabolic engineering principles is likely to create new reliable solutions for existing problems currently hindering the industrial bioproduction of aromatic chemicals. In the following sections, we discuss the development and application of such enabling strategies and tools (Figure 2).

Omics-driven elucidation of missing biosynthetic pathways

The identification of important biosynthetic enzymes in native producers, makes up the foundation for constructing microbe-based production platforms. This is aided by detailed and reliable omics data, such as genomics, transcriptomics, proteomics and metabolomics. Moreover,

Figure 2



Metabolic engineering strategies and tools for improving the phenotypes of engineered cell factories. (I) Mining omics data for discovering novel or missing genes of aromatic biosynthetic pathways. (II) Protein engineering is applied for improving the catalytic properties of enzymes. (III) Growth-based adaptive laboratory evolution is used to increase chemical tolerance and product yield. (IV) Biosensor engineering is designed for optimizing synthetic pathways. (V) Application of membrane transporters provides additional driving force for high-level chemical production. (VI) Cell consortium is devised to alleviate metabolic burden by means of an artificial microbial community.

with the increasing availability of cost-effective, high-throughput DNA sequencing technologies, the disclosure of new genomes and transcriptomes has begun in the last decade to be conducted at an unprecedented level. A great example of this is the One Thousand Plant Transcriptomes Initiative, which recently released the vegetative transcriptomes of 1124 species that cover diverse plants across green plants, glaucophytes and red algae [39]. Coincidentally, in 2018, the 10KP (10 000 Plants) Genome Sequencing Project was launched with the objective of completing the sequencing and characterizing of representative genomes from major clades of embryophytes, green algae, and protists within the next five years [40]. This increasing access to high-quality plant genomes and the acceleration in progress related to functional genomics approaches is effectively reshaping our understanding of genetic foundation underlying plant aromatic biosynthesis. For example, Dastmalchi *et al.* reported the screening and characterization of a pathogenesis-related 10 protein, namely neopinone isomerase (NISO), which was found to be able to catalyze the isomerization of neopinone to codeinone, a presumed non-catalytic conversion in morphine biosynthesis [41]. The addition of NISO to previously established opiate-producing *S. cerevisiae* strains markedly enhanced the biosynthesis of codeine and morphine, by reducing the accumulation of structural isomers neopine and neomorphine. Another prominent example relates to representative MIA vincristine and vinblastine, which are clinically applied to the treatment of lung and brain cancers. By combing *in silicon* analysis of annotated genes in the transcriptome from the native producing plant *Catharanthus roseus* alongside extensive chemical investigations, Caputi *et al.* were able to complete the biosynthetic pathway for vindoline and catharanthine, key intermediates in the vinblastine biosynthesis, with the discovery of two enzymes precondylocarpine acetate synthase (PAS) and dihydroprecondylocarpine synthase (DPAS) [42•].

Enhancing enzyme activity via protein engineering

Low metabolic activities accompanied by unsatisfactory chemical titers and yields have been widely observed for non-optimized heterologous aromatics biosynthesis in yeast hosts. Two potential reasons may in part contribute to this phenomenon: (i) being secondary metabolism in most cases, the enzymes related to aromatics biosynthesis have been evolutionarily shaped to be much less efficient than those involved in central carbon and energy metabolism [43], and (ii) the absence of an appropriate environment in yeast that could enable the full functionality of expressed enzymes, that is their usage at 100% capacity [33]. A substantial improvement in the biochemical kinetics of selected enzymes is therefore of great importance for expediting the establishment of cell factories for aromatic compound production. To enable this improvement, several protein engineering methods have been applied. These include site-directed mutation and random mutagenesis, which are commonly used to optimize the *in vitro* activity of individual enzymes for bioengineering. The ancestral sequence reconstruction (ASR)-mediated strategy has also recently emerged as an effective approach for directed evolution of enzymes [44•]. This involved inferring the ancient protein sequences using evolutionarily related extant ones, enabling ASR to identify key amino acid residues that readily influence substrate and/or reaction specificity, thus promoting the isolation of new enzyme mutants which may exhibit beneficial properties by medium-throughput or even low-throughput screening. Besides, reconstructed ancestors are believed to possess higher structural stability and enzymatic evolvability compared to the contemporary counterparts, thus comprising appealing starting points for delineating the protein engineering space. Recent application of ASR has indeed revealed that this approach could substantially improve the biological activity and stability of coagulation factor VIII [45] or generate highly functional thermostable enzymes [46].

Alternatively, the metabolic activity of biosynthetic enzymes or pathways can be optimized *in vivo*. This is by the means of metabolite-responsive biosensors [47], which transform changes in chemical production to readily quantifiable factors, such as a fluorescence signal. By repurposing the TtgR regulatory protein to a resveratrol-responsive biosensor in *E. coli*, Xiong *et al.* demonstrated the rapid and efficient selection of resveratrol hyper-producers and identification of *Arabidopsis thaliana* *p*-coumarate:CoA ligase (4CL) variants displaying improved catalytic properties relative to wild type for the production of this aromatic compound [48]. Furthermore, the best 4CL mutant was found to be applicable for increasing production of naringenin. Similarly, in *S. cerevisiae*, the development and characterization of a naringenin-induced biosensor afforded the efficient optimization of a naringenin associated biosynthetic pathway [49], in which a strain library featured by the combinatorial assembly of biosynthetic genes and promoters has been screened. On the other hand in addition, intensive investigations towards characterizing the outstanding variety of plant secondary metabolism, have shown it increasingly evident that metabolons, weakly bound and ordered protein complexes composed by sequential biosynthetic enzymes, play a crucial role in maximizing the channeling of metabolic intermediates and reducing undesired metabolic cross-talk [50,51]. Hence, mimicking this protein interaction in yeast hosts, either by simple peptide linkers [52] or synthetic protein scaffolds [53], should significantly reduce the buildup of intermediates or byproducts thus fully releasing the biosynthesis potential of aromatics originating from plants.

Host engineering towards improved phenotypes

The biosynthesis and accumulation of heterologous chemicals frequently exert dramatic cytotoxicity,

potentially decreasing the cellular fitness and metabolic robustness of engineered cell factories. For example, a few aromatics, such as 2-PE [23] and vanillin [20], are known to be inhibitory to cell proliferation of yeast hosts even at levels less than gram-per-liter. However, considering the anti-microbial and anti-oxidative properties of flavonoids, it may come as no surprise that cytotoxicity might occur when pursuing their high-level production in yeast strains. Accordingly, multiple lines of strategy, including adaptive laboratory evolution (ALE) and transporter engineering, have been devised to alleviate the issue of product toxicity, concomitant with improving the chemical production titer and yield.

By leveraging a key mechanism in evolution natural selection, ALE offers an emerging but increasingly important approach to rapidly generating novel microbial phenotypes [54]. This strategy, wherein phenotype of interest is coupled to cell growth, also capitalizes on recent developments in multi-omics techniques and genome engineering to help identify underlying genetic mechanisms in the final evolved strains that contain improved phenotypes. With the establishment of this powerful fitness selection system, through which adaptive mutant strains are enriched, ALE can be applied to improve microbial hosts performance in four ways: (i) substrate utilization, (ii) increasing tolerance, (iii) growth rate optimization, and (iv) increasing product biosynthesis. For example, the tolerance mechanisms adopted in *S. cerevisiae* to tackle cellular stress imposed by inhibiting concentrations of dicarboxylic acids, including adipic acid, were recently investigated by Pereira *et al.* principally using ALE experiments [55]. Detailed genome sequences analysis indicated that yeast membrane transporters play a key role in affording strains evolved tolerance, with the overexpression of identified multidrug transporter-coding gene *QDR3* in particular showing to be beneficial to *cis*, *cis*-muconic acid biosynthesis in an engineered *S. cerevisiae* host. Interestingly, membrane transporter was also genetically associated with *p*-HCA tolerance in the soil bacterium *Pseudomonas putida* KT2440, an isolate exhibiting native tolerance to various types of stress [56]. These findings imply that engineering membrane transporters may provide additional driving force for high-level chemical production using microbial systems, in particular for those that tend to accumulate intracellularly, such as methylated flavonoids [57] which have also been engineered for their production in *S. cerevisiae*. In addition, the rational selection and application of transporters from different organisms, have been employed for both enhancing the biosynthesis of fatty alcohol [58] and for the efficient transport of dicarboxylic acids [59] in *S. cerevisiae*, represents a promising alternative strategy to enhance chemical producing capability.

Metabolic burden, resulting from the competition between chemical production pathways and native metabolism for building blocks (including chemical precursors, energy molecules, and reducing equivalents), exists as one of the major challenges during current recombinant strain construction and optimization. The cell consortium is one such approach devised to alleviate metabolic burden in a single host by dividing the labor among several, by means of an artificial microbial community. This innovative approach has shown to drastically improve metabolic efficiency compared to the use of monoculture, with Jones *et al.* recently providing one of the first reports using this approach to enable substantial *de novo* production of the anthocyanin molecule calistephin [60•], a previously recalcitrant metabolic engineering target, by building and optimizing a synthetic, 4-strain *E. coli* polyculture that collectively performed the function of 15 unique heterologous or modified enzymes from diverse plants and other microbes. Although as yet unreported, it is anticipated that similar coculture approach, using artificial microbial communities to divide the chemical production workload, should be applicable for engineering aromatics production in yeast, especially for those derived from lengthy biosynthetic pathways, on the premise that the efficient transport of intermediates could be solved.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Quanli Liu: Conceptualization, Writing - original draft. **Yi Liu:** Conceptualization, Writing - original draft. **Yun Chen:** Data curation, Writing - review & editing. **Jens Nielsen:** Supervision, Writing - review & editing, Funding acquisition.

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