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Minireview

MINIREVIEW

Pathway engineering for the production of heterologous aromatic chemicals and their derivatives in *Saccharomyces cerevisiae*: bioconversion from glucose

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One sentence summary: The aromatic amino acid pathway of *Saccharomyces cerevisiae* can be engineered for sustainable production of valuable chemicals

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ABSTRACT

Saccharomyces cerevisiae has been extensively engineered for optimising its performance as a microbial cell factory to produce valuable aromatic compounds and their derivatives as bulk and fine chemicals. The production of heterologous aromatic molecules in yeast is achieved via engineering of the aromatic amino acid biosynthetic pathway. This pathway is connected to two pathways of the central carbon metabolism, and is highly regulated at the gene and protein level. These characteristics impose several challenges for tailoring it, and various modifications need to be applied in order to redirect the carbon flux towards the production of the desired compounds. This minireview addresses the metabolic engineering approaches targeting the central carbon metabolism, the shikimate pathway and the tyrosine and phenylalanine biosynthetic pathway of *S. cerevisiae* for biosynthesis of aromatic chemicals and their derivatives from glucose.

Keywords: *Saccharomyces cerevisiae*; aromatic amino acid pathway; phenylalanine and tyrosine branch; Shikimic acid pathway; metabolic engineering; biotechnology

INTRODUCTION

The majority of aromatic compounds and their derivatives are chemically synthesised from petroleum-derived sources (Richmond 1947; Wu, Koylinski and Bozik 1981; Castellan, Bart and Cavallaro 1991; Mimura and Saito 1999). However, such procedures are not sustainable and leave environmental footprints, as they are very energy intensive processes and cause the release of greenhouse gasses. The production of fine chemicals from plants, such as vanillin and flavonoids, relies on plant life cycles, climate conditions, and is susceptible to plant diseases. Moreover, the final yield is usually low and high purity is difficult to obtain. These disadvantages can be avoided by using biotechnological procedures. Biotechnology is focusing on the development of engineered microorganisms as cell factories able to convert biomass into the desired chemicals (Hong and Nielsen 2012; Borodina and Nielsen 2014; Thompson, Machas and Nielsen 2015). The yeast *Saccharomyces cerevisiae* is a well-established model organism and has several advantages as a cell factory, due to its robustness and high tolerance to stress in fermentative processes (Gibson *et al.* 2007). Therefore, *S. cerevisiae* has been engineered to produce different classes of valuable compounds (Hong and Nielsen 2012).

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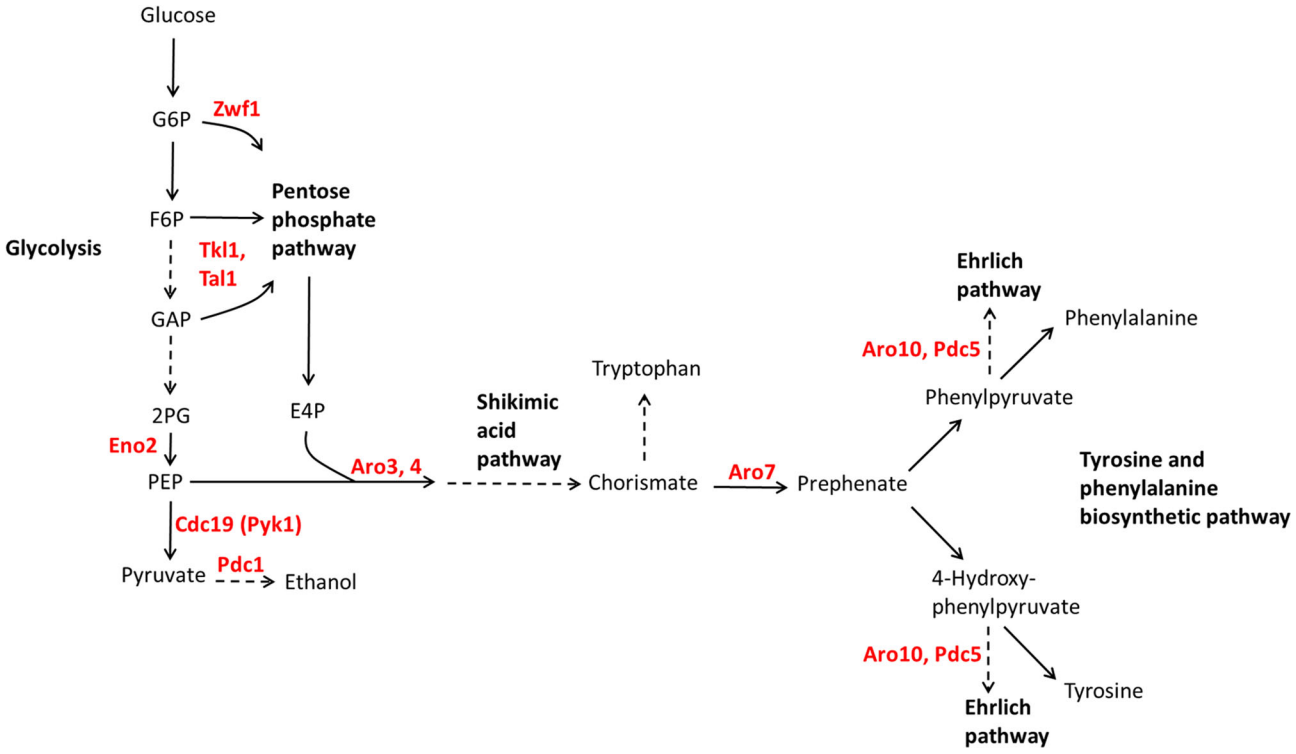

Figure 1. Biosynthesis of aromatic amino acids in *S. cerevisiae*. The precursors of the shikimic acid pathway, PEP and E4P are generated by glycolysis and the pentose phosphate pathway, respectively. From the end product of the shikimic acid pathway, chorismate, the tryptophan and the phenylalanine/tyrosine biosynthetic branches diverge. Enzymes which are important targets for metabolic engineering strategies are highlighted in red. For simplification, multiple enzymatic steps are depicted as dashed lines when appropriate. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glycerin-aldehyde-3-phosphate; 2PG, 2-phosphoglycerate; Eno2, Enolase isoenzyme 2; Zwf1, glucose-6-phosphate dehydrogenase; Tkl1, transketolase isoenzyme 1; Tal1, transaldolase isoenzyme 1; Cdc19 (Pyk1), pyruvate kinase isoenzyme 1; Pdc1, pyruvate decarboxylase isoenzyme 1; Aro3, 4, DAHP synthases; Aro7, chorismate mutase; Aro10, aromatic carboxylic acid decarboxylase; E4P, erythrose-4-phosphate;; Pdc5, Pyruvate decarboxylase isoenzyme 5.

In *S. cerevisiae*, aromatic compounds are synthesised via the aromatic amino acid biosynthetic pathway (AAP) (Braus 1991). This highly regulated pathway is a central node of yeast metabolism and feeds several other pathways (i.e. quinone, folate and Ehrlich pathways). Its core constitutes of the shikimate pathway, from which two main pathways for amino acid production branch out: the tryptophan, and the tyrosine and phenylalanine branch (Fig. 1).

The focus of this minireview is on metabolic engineering approaches for the bioconversion of glucose to industrially attractive compounds derived from the shikimate and the tyrosine and phenylalanine biosynthetic pathways in *S. cerevisiae* (Table 1).

METABOLIC ENGINEERING OF *SACCHAROMYCES CEREVISIAE* TYROSINE AND PHENYLALANINE BIOSYNTHETIC PATHWAY

Increasing precursor supply for the aromatic amino acid pathway

The enzyme 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase catalyses the initiating step into the AAP. It facilitates the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), thereby releasing phosphate. PEP is an intermediate of the lower part of glycolysis, whereas E4P is derived from the pentose phosphate pathway (PPP) (Fig. 1). However, on glucose under aerobic conditions, the available carbon flux to E4P is significantly lower than the flux to PEP (Vaseghi *et al.* 1999). Therefore, balancing the ratio of the two substrates and increasing their availability seems to be a promising step towards increasing the flux into the AAP.

Using flux balance analysis, Curran *et al.* (2013) could show that optimizing the availability of E4P requires a shift in the PPP. Normally, flux enters into the PPP through the glucose-6-phosphate dehydrogenase encoded by *ZWF1*, reducing the maximum theoretical carbon yield due to the release of CO_2 in the 6-phosphogluconate dehydrogenase reaction. E4P is then produced by transaldolase in the non-oxidative part of the PPP, which is the limiting step of the pathway (Becker and Boles 2003). Moreover, in the next step, transketolase rather consumes E4P and converts it together with xylulose-5-phosphate (X5P) to produce fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P). Therefore, in order to optimise synthesis of E4P, *ZWF1* was deleted and *TKL1*-encoding transketolase was overexpressed to reverse flux from the glycolytic intermediates F6P and G3P to E4P and X5P (Fig. 1). This resulted in an up to 7-fold increase of the flux into the AAP (Deaner and Alper 2017). Another approach to improve the flux towards the AAP is the overexpression of transaldolase (Tal1) and enolase 2 (Eno2) (Mao *et al.* 2017). Tal1 favours the conversion of sedoheptulose-7-phosphate (S7P) and G3P to E4P and F6P, whilst Eno2 converts 2-phosphoglycerate to PEP.

On the other hand, most of the other precursor, PEP, is converted by pyruvate kinase into pyruvate and then further metabolised to ethanol by pyruvate decarboxylases and alcohol dehydrogenases, due to the Crabtree effect in *S. cerevisiae*. A study conducted with ^{13}C labelling showed that only 1% of

Table 1. List of heterologous compounds produced from glucose via the engineering of the shikimate pathway and the tyrosine-phenylalanine aromatic amino acid pathway in *Saccharomyces cerevisiae*.

Product	Endogenous precursors	Application field	Reference
Shikimic acid pathway			
Vanillin	3-DHS	Flavour and fragrance	Hansen *et al.* (2009); Brochado *et al.* (2010)
cis, cis-muconic acid	3-DHS	Plastic	Weber *et al.* (2012); Curran *et al.* (2013); Skjoedt *et al.* (2016); Suastegui *et al.* (2016 b); Leavitt *et al.* (2017)
pHBA	Chorismate	Plastic	Krömer *et al.* (2013)
Benzylisoquinoline alkaloids			
(S)-Reticuline	Tyrosine, 4-HPAA	Medical	DeLoache *et al.* (2015); Trenchard *et al.* (2015)
Flavonoids, stilbenoids and dihydrochalcones			
Naringenin	Tyrosine, Phenylalanine	Medical	Jiang, Wood and Morgan (2005); Koopman *et al.* (2012); Lehka *et al.* (2017)
Resveratrol	Tyrosine, Phenylalanine	Medical, food	Li *et al.* (2015, 2016)
Phloretin	Tyrosine	Medical, food	Eichenberger *et al.* (2017)
Pinocembrin DHC	Phenylalanine	Medical, food	Eichenberger *et al.* (2017)
Trans-Cinnamic acid derivatives			
Styrene	Phenylalanine	Plastic	McKenna *et al.* (2014b)
Cinnamaldehyde	Phenylalanine	Flavour and fragrance, medical, agricultural	Gottardi *et al.* (2017)
Cinnamyl alcohol	Phenylalanine	Flavour and fragrance	Gottardi *et al.* (2017)
Hydrocinnamyl alcohol	Phenylalanine	Flavour and fragrance	Gottardi *et al.* (2017)

pHBA, p-hydroxybenzoic acid; 3-DHS, 3-dehydroshikimate; 4-HPAA, 4-hydroxyphenylacetaldehyde.

PEP is consumed via the entry into the AAP (Blank, Kuepfer and Sauer 2005), and even in a strain engineered for an optimised initial AAP pathway, <8% of the flux into PEP was channelled into the AAP (Suastegui *et al.* 2016a). Therefore, a potential strategy to increase the availability of PEP seems to lower the activity of pyruvate kinase or pyruvate decarboxylase. However, by using a mutant version of pyruvate kinase exhibiting impaired activity and resulting in a corresponding increase in intracellular PEP concentrations, no increase of carbon flux into the AAP was observed (Gold *et al.* 2015). In contrast, deletion of *PDC1* encoding the main isoform of pyruvate decarboxylases reduced pyruvate decarboxylase activity by about 30% and resulted in a 2-fold improvement of flux into the AAP (Brochado *et al.* 2010). Nevertheless, pyruvate kinase and pyruvate decarboxylase are also important enzymes for the fitness of the cells; elimination or reduction of their activities lead to severely reduced cell growth and productivities. One possible solution to counteract these obstacles could be to reduce sugar uptake activity, e.g. by (over-) expression of mutant forms of Mth1, a transcriptional regulator of hexose transporters (Oud *et al.* 2012).

Shikimate pathway engineering, and production of vanillin, *cis,cis*-muconic acid and p-hydroxybenzoic acid

The shikimic acid pathway directly provides precursors for heterologous products, such as vanillin and muconic acid. Furthermore, some of the seven reaction steps of the pathway are targets for improvement of downstream pathways and product yields. These aspects will be discussed in this section.

The first enzymatic step of the pathway is catalysed by DAHP synthase, which condenses E4P and PEP into DAHP. *ARO3* and *ARO4* encode for two DAHP synthase isoforms in yeast. The DAHP synthase reaction represents an important target in metabolic engineering approaches. The two protein isoforms Aro3 and Aro4 catalysing this reaction are feedback inhibitable by phenylalanine and tyrosine, respectively (Braus 1991). Studies on a cavity in the structure of Aro4 demonstrated that modifications of specific residues in this cavity lead to a relief in feedback inhibition of Aro4 (Hartmann, Schneider and Pfeil 2003). The mutation K229L in Aro4 caused the protein to be phenylalanine and tyrosine insensitive, but did not affect the overall activity of the protein. In combination with the deletion of *ARO3*, overexpression of $Aro4^{K229L}$ increased the flux through the aromatic amino acid pathway by 4- to 5-fold (Luttik *et al.* 2008). Consequently, this mutant was used successfully to increase the yield of various aromatic products (Curran *et al.* 2013; McKenna *et al.* 2014b; Li *et al.* 2015; Rodriguez *et al.* 2015; Suastegui *et al.* 2016b) (Figs 1 and 2). Likewise, overexpression of the tyrosine-insensitive mutant $Aro4^{G226S}$ improved the production of the flavonoid naringenin (Koopman *et al.* 2012), even though its activity is still repressed by phenylalanine (Hartmann, Schneider and Pfeil 2003). The tyrosine-insensitive mutant $Aro4^{Q166K}$ was used for the production of tyrosine-derived opioids (Galanie *et al.* 2015).

The following enzymatic steps in the pathway are exerted by the pentafunctional enzyme Arom, encoded by *ARO1*. Arom converts DAHP into 5-enolpyruvylshikimate-3-phosphate (EPSP) through five reactions, which include the cyclisation of the molecule, generating 3-dehydroquinate (3-DHQ), and introduction of the first double bond of the aromatic ring, forming the intermediate 3-dehydroshikimate (3-DHS) (Fig. 2). 3-DHS is converted to shikimate in an NADPH-dependent reaction. Subsequently, shikimate-3-phosphate is formed from shikimate and ATP. In the last reaction step, EPSP is produced from shikimate-3-phosphate and PEP. Due to its five catalytic domains (C-, E-, D-, B- and A-domain), Arom is considered as a natural scaffold for substrate channelling (Duncan, Edwards and Coggims 1987). Expression of monofunctional homologues of Arom from *Escherichia coli* revealed that the kinase reaction, converting shikimate to shikimate-3-phosphate, is a flux controlling step in

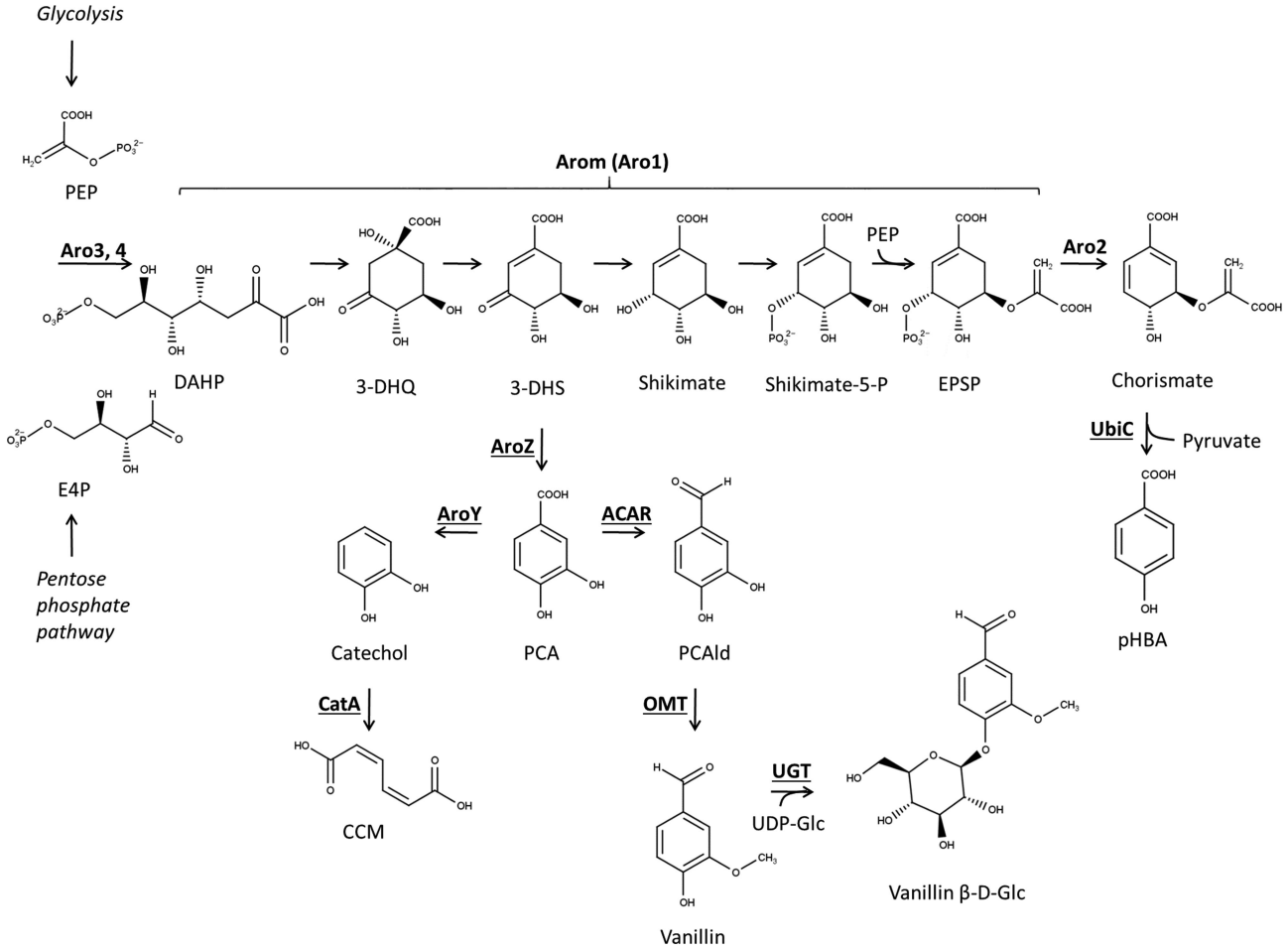

Figure 2. Shikimic acid pathway intermediates and heterologous products derived from it. Enzyme names are depicted in bold, heterologous enzymes are underlined. PEP, phosphoenolpyruvate; Aro3, 4, DAHP synthase isoenzymes; E4P, erythrose-4-phosphate; Arom (Aro1), aromatic pentafunctional enzyme; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; 3-DHQ, 3-dehydroquinate; 3-DHS, 3-dehydroshikimate; Shikimate-5-P, shikimate-5-phosphate; EPSP, 5-enolpyruvylshikimate-3-phosphate; Aro2, chorismate synthase; AroZ, 3-DHS dehydratase; PCA, protocatechuic acid; AroY, protocatechuic acid decarboxylase; CatA, catechol 1, 2-dioxygenase; CCM, *cis, cis*-muconic acid; ACAR, aromatic carboxylic acid reductase; PCAld, protocatechuic aldehyde; OMT, O-methyl transferase; UGT, UDP glycosyltransferase; UDP-Glc, UDP-glucose; Vanillin β-D-Glc, Vanillin β-D-glucoside; UbiC, chorismate pyruvate lyase; pHBA, *para*-hydroxybenzoic acid.

the sequence of these reactions (Rodriguez *et al*. 2015) that can be overcome by expression of the shikimate kinase AroL from *E. coli*.

Two important heterologous products that we encounter in the shikimic acid pathway are the aroma compound vanillin and the precursor of the bulk chemical adipic acid, *cis, cis*-muconic acid (CCM), which are derived from 3-DHS. The production of vanillin from glucose in yeast was first described by Hansen *et al*. (2009), who conducted a parallel study on *Schizosaccharomyces pombe* and *S. cerevisiae*. To produce vanillin in *S. cerevisiae*, three heterologous enzymatic steps were introduced into the strain. The first step is a dehydration reaction, converting 3-DHS to protocatechuic acid (PCA), catalysed by a 3-DHS dehydratase from the mold *Podospora pauciseta*. Then PCA is converted into protocatechuic aldehyde by an aryl carboxylic acid reductase (ACAR) from the bacterial *Nocardia* genus in an ATP and NADPH-dependent reaction (Fig. 2). As ACAR needs to be activated by phospopantetheinylation, a *Corynebacterium glutamicum* phosphopantetheine transferase was co-expressed. Finally, protocatechuic aldehyde is methylated by an S-adenosyl-methionine-dependent O-methyl-transferase from *Homo sapiens*, thereby generating vanillin. The main obstacles limiting the production of high yields are the generation of the unwanted byproduct vanillyl alcohol and product toxicity. To avoid the direct reduction of vanillin to vanillyl alcohol, *S. cerevisiae* strains deleted of potential reductases were tested. Adh6 was found to be the one most active on vanillin, therefore it was deleted in the final strain. With only these engineering steps, *S. cerevisiae* was able to produce 45 mg L^{-1} of vanillin.

As vanillin and other phenolic compounds are present in lignocellulosic hydrolysates used as biomass for the production of second-generation biofuels, their toxicity has been largely studied (Adeboye, Bettiga and Olsson 2014; Nguyen *et al*. 2014; Shen *et al*. 2014). The nature of functional groups on the aromatic ring of these compounds contributes to their toxicity, whereby aldehydes and ketones are more toxic than acids, and acids are more toxic than alcohols (Almeida *et al*. 2007). Moreover, properties and position of additional substituents play an important role (Adeboye, Bettiga and Olsson 2014). Phenolic compounds affect the integrity of biological membranes (Almeida *et al*. 2007) and can cause oxidative stress (Nguyen *et al*. 2014). The diffusion of weak aromatic acids across the plasma membrane in their undissociated form and subsequent dissociation in the less acidic cytosol leads to acidification of the cytosol.

Several yeast strains found to be more tolerant to vanillin were able to convert vanillin to other byproducts (Shen *et al*.

2014; Zheng *et al.* 2017). Therefore, such strains would not be of advantage for the production of vanillin. However, the conversion of vanillin to the less toxic product vanillin β-D-glucoside by introduction of an UDP-dependent glycosyltransferase (UGT) has proven to be a successful strategy to decrease the problem of product toxicity (Hansen *et al.* 2009). Interestingly, a strain with high ergosterol content and thus with altered membrane properties was found to be more resistant to vanillin (Endo, Nakamura and Shima 2009). Evolutionary engineering approaches might be useful to improve the resistance of intracellular targets against aromatic compounds.

In a genome-scale modelling approach, pyruvate decarboxylase 1 (*PDC1*) and glutamate dehydrogenase 1 (*GDH1*) were identified as promising targets for deletion (Brochado *et al.* 2010) to improve the product yield of the pathway. Since Gdh1 is an NADPH-dependent enzyme, its deletion leads to an increased NADPH-pool, thereby favouring the reduction of PCA to protocatechuic aldehyde. The reduced nitrogen assimilation rate associated with its deletion was compensated by overexpression of the NADH-dependent glutamate dehydrogenase Gdh2. Deletion of *PDC1* led to a more respiratory metabolism, consequently providing more ATP for the ACAR reaction and increasing the availability of PEP for the entrance into the shikimic acid pathway, as outlined in the previous section (Brochado *et al.* 2010).

Another heterologous chemical compound derived from 3-DHS is CCM, a precursor of the bulk chemicals adipic acid and terephtalic acid. To achieve CCM production, three heterologous enzymes have to be expressed in *S. cerevisiae*. The pathway was first implemented in yeast using a 3-DHS dehydratase (AroZ) from *P. anserina*, a PCA decarboxylase (AroY) consisting of subunits B, C and D from *Klebsiella pneumoniae* and catechol-1, 2-dioxygenase (CatA) from *Acinetobacter calcoaceticus* (Weber *et al.* 2012). Together with these heterologous enzymes, the pentafunctional enzyme Arom was overexpressed in a mutated form. Here, a deletion of the E-domain encoding the dehydratase function required for conversion of 3-DHS into shikimate was introduced to provide more substrate for the conversion of 3-DHS into PCA (Fig. 2). In a recent study, the point mutation D1409A was introduced in the E-domain, with the same effect, but presumably less disturbing for the overall structure and activity of the enzyme (Suastegui *et al.* 2016b). Even though modifications in the general carbon metabolism and introduction of feedback-resistant variants of DAHP synthase enabled the production of higher titers (Curran *et al.* 2013; Skjoedt *et al.* 2016; Suastegui *et al.* 2016b), the decarboxylation step still remains a bottleneck, leading to secretion of high amounts of PCA into the medium. An improvement in the activity of AroY was achieved by growing the cells under oxygen-limited conditions, as the enzyme is oxygen sensitive (Suastegui *et al.* 2016b). Recently, it was shown that the activity of AroY isomeric subunit C (AroY-C^{iso}), the subunit of AroY with the actual decarboxylase function, depends on the presence of the cofactor prenylated flavin mononucleotide (prFMN), which can be generated by AroY subunit B (Weber *et al.* 2017). Since the synthesis of the cofactor is performed more efficiently by the endogenous yeast enzyme Pad1 (Weber *et al.* 2017), its coexpression will allow for a better cofactor supply and an improved enzymatic activity in the pathway.

An innovative approach to facilitate the use of high-throughput screenings to identify engineered strains with higher performance is described in the study of Skjoedt *et al.* (2016). A biosensor for CCM was developed based on the prokaryotic LysR-type transcriptional regulator BenM in *S. cerevisiae*. Here, the operator BenO was introduced in a modified *CYC1* promoter in front of the reporter GFP, allowing for expression of the reporter when BenM binds CCM and alters its position on the operator. In another recent approach, an aromatic amino-acid-inducible biosensor based on *ARO9* promoter elements was used to identify beneficial mutations improving the flux into the AAP (Leavitt *et al.* 2017), thereby creating a strain for improved CCM production.

The seventh and last step of the shikimate pathway is catalysed by a chorismate synthase, encoded by *ARO2*. Aro2, which requires the cofactor flavin mononucleotide (FMN), acts by removing a phosphate and introducing the second double bond of the aromatic ring, converting EPSP into chorismate (Jones, Reusser and Braus 1991; Quevillon-Cheruel *et al.* 2004) (Figs 1 and 2). In an approach to identify bottlenecks in the AAP, Aro2 was overexpressed and caused a 2-fold improvement in the levels of *p*-coumaric acid, an aromatic compound that can be derived from tyrosine (Mao *et al.* 2017).

From chorismate, *p*-hydroxybenzoic acid (pHBA) has been produced (Krömer *et al.* 2013). pHBA, a compound used in liquid crystal polymers, is synthesised as a part of the ubiquinone biosynthesis pathway. For generation of pHBA, a route directly from chorismate and another route from tyrosine via coumarate seem to exist in *S. cerevisiae* (Meganathan 2001). But neither the genes encoding the enzymes involved in the tyrosine route nor for the conversion of chorismate into PHBA have been identified in *S. cerevisiae* so far. Therefore, the *ubiC* gene from *E. coli* was expressed (Krömer *et al.* 2013) (Fig. 2). To avoid a drain of chorismate into the aromatic amino acid biosynthesis pathways, chorismate mutase encoded by *ARO7* was deleted. With this strategy, a yield of 6 mg pHBA g^{-1} glucose could be obtained.

Tyrosine and phenylalanine branch and Ehrlich pathway engineering

Further downstream, from chorismate towards the tyrosine and phenylalanine branch, a common enzymatic step catalysed by Aro7 converts chorismate into prephenate (Figs 1 and 3). Aro7 is the third feedback regulatable enzyme of the aromatic amino acid biosynthetic pathway. It is inhibited by tyrosine, and its activity is enhanced by tryptophan (Braus 1991). This regulation is necessary for the cell in order to regulate the metabolic flux between the tryptophan and the tyrosine-phenylalanine branch. Luttik *et al.* (2008) showed that the mutation G141S in Aro7 relieves the feedback regulation. Therefore, overexpression of $ARO7^{G141S}$ in a strain already engineered with an overexpression of $ARO4^{K229L}$ improved the titers of the intermediates of tyrosine and phenylalanine pathway as well as the Ehrlich pathway intermediates, when compared to the equally engineered strain overexpressing the wild-type isoform of *ARO7* (Luttik *et al.* 2008). Not only the mutated isoform $Aro7^{G141S}$ was found to be of advantage for the production of downstream aromatics, but also another feedback-resistant isoform $Aro7^{T226I}$ (Schmidheini *et al.* 1989; Trenchard *et al.* 2015) improved the production of tyrosine-derived (*S*)-reticuline.

The next reaction step is the conversion of prephenate to phenylpyruvate (PP), precursor of phenylalanine, or to hydroxyphenylpyruvate (4-HPP), precursor of tyrosine. Tyr1 catalyses the reaction to 4-HPP, and it was recently published that its overexpression in combination with upper pathway modifications increased the production of tyrosine-derived *p*-coumaric acid (Mao *et al.* 2017).

Another common engineering step to redirect the flux towards tyrosine or phenylalanine abolishing competing pathways is the deletion of the aromatic decarboxylase-encoding

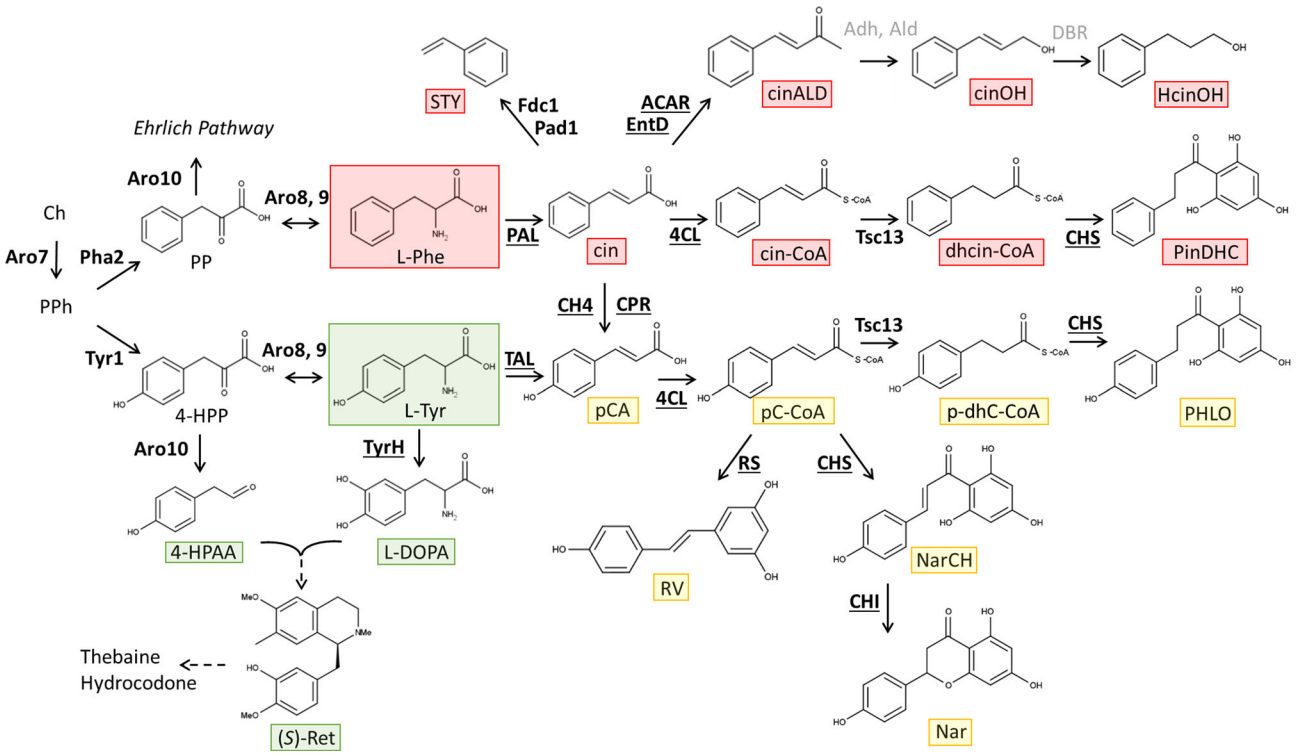

Figure 3. Overview of the aromatic compounds produced from chorismate towards the tyrosine and phenylalanine biosynthetic branch. Dashed arrows indicate multiple enzymatic steps. Enzymes are shown in bold, underlined enzymes have a heterologous source; grey coloured enzyme names represent hypothetical reactions. Ch, chorismate; PPh, prephenate; Aro7, chorismate mutase; Pha2, prephenate dehydratase; Aro10, aromatic carboxylic acid decarboxylase; PP, phenylpyruvate; Aro8, 9, phenylalanine and tyrosine transaminases; L-Phe, phenylalanine; PAL, phenylalanine ammonia lyase; cin, *trans*-cinnamic acid; Fdc1, ferulic acid decarboxylase 1; Pad1, phenylacrylic acid decarboxylase 1; STY, styrene; C4H, cinnamate 4-hydroxylase; CPR1, cytochrome P450 reductase; ACAR, aryl carboxylic acid reductase (*Nocardia* genus); EntD, phosphopantetheinyl transferase (*E. coli*); 4CL, 4-coumaric acid-CoA ligase; cinALD, cinnamaldehyde; Adh, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; cinOH, cinnamyl alcohol; DBR, double bound reductase; HcinOH, hydrocinnamyl alcohol; cin-CoA, cinnamoyl-CoA; Tsc13, double bond reductase; dhcin-CoA, dihydrocinnamoyl-CoA; CHS, chalcone synthase; PinDHC, pinocembrin dihydrochalcone; Tyr1, prephenate dehydrogenase; 4-HPP, 4-hydroxyphenylpyruvate; L-Tyr, tyrosine; TAL, tyrosine ammonia lyase; pCA, *p*-coumaric acid; pC-CoA, *p*-coumaroyl-CoA; p-dhC-CoA, *p*-dihydrocoumaroyl-CoA; PHLO, phloretin; 4-HPAA: 4-hydroxyphenylacetaldehyde; TyrH, mammalian tyrosine hydroxylase/CYP76AD1 (*Beta vulgaris*); L-DOPA, L-3, 4-dihydroxyphenylalanine; (*S*)-Ret, (S)-reticuline; RS, resveratrol synthase; RV, resveratrol; NarCH, naringenin chalcone; CHI, chalcone isomerase; Nar, naringenin.

gene *ARO10*. Aro10 catalyses the entrance reaction into the catabolism of amino acids, the Ehrlich pathway. Together with Aro10, Pdc5 and Pdc6 were found to have activity towards PP (Romagnoli *et al.* 2012), the direct precursor of phenylalanine. By deletion of *ARO10*, *PDC5* and *PDC6*, the titer of the Ehrlich pathway intermediate phenylethanol decreased 22-fold in a strain producing the flavonoid naringenin, whose production will be later discussed (Koopman *et al.* 2012). On the other hand, the overexpression of *ARO10* allowed for the accumulation of Ehrlich pathway intermediates, such as 4-hydroxyphenylacetaldehyde (4-HPAA), whose overproduction was necessary for the biosynthesis of the already mentioned (S)-reticuline (Trenchard *et al.* 2015). It is clear that at this stage, the Ehrlich pathway plays an important role in the accumulation of the desired intermediates, allowing researchers to define the preferred route by decreasing or increasing the flux towards the Ehrlich pathway (Figs 1 and 3).

Tyrosine biosynthesis branch and production of benzylisoquinoline alkaloids

Moving towards the production of tyrosine, a large and medically relevant class of compounds was produced in *S. cerevisiae*. Benzylisoquinoline alkaloids (BIAs) are derived from L-3, 4-dihydroxyphenylalanine (L-DOPA) and 4-HPAA, and include opioids such as barberine, codeine and morphine (Fig. 3). First, a crucial part of the BIAs pathway was reconstructed in yeast, until the biosynthesis of (S)-reticuline. Two different groups independently recreated the entrance into the pathway. This was possible thanks to the identification of highly active isoforms of tyrosine hydroxylase (TyrH), able to convert tyrosine to L-DOPA. DeLoache *et al.* (2015) took advantage of an enzyme-coupled biosensor that converts L-DOPA into a yellow fluorescent pigment, betaxanthin. Using this system, they could identify a cytochrome P450 enzyme from *Beta vulgaris* with high tyrosine hydroxylase activity. This enzyme was further modified by PCR mutagenesis, leading to higher concentrations of L-DOPA. In another approach, Trenchard *et al.* (2015) focused on mammalian tyrosine hydroxylases, which are considered to have better substrate specificity for tyrosine. Nevertheless, mammalian tyrosine hydroxylases require an electron carrier cosubstrate which is not present in microbial hosts. Therefore, four additional genes had to be expressed in yeast in order to allow for production and recycling of the electron carrier cosubstrate. Once the production of L-DOPA was achieved, the pathway was extended towards (S)-reticuline production. L-DOPA was converted into dopamine by expression of a DOPA decarboxylase from *Pseudomonas putida*. The second precursor necessary for the entry into the pathway is 4-HPAA, whose production was improved by the overexpression of Aro10, as mentioned earlier, and the transaminase Aro9, involved in the catabolism of tyrosine (Fig. 3) (Iraqui *et al.* 1999; Trenchard *et al.* 2015). Then, a norcoclaurine synthase from *Papaverum somniferum* (DeLoache *et al.* 2015) or

from *Coptis japonica* (Trenchard *et al.* 2015) was expressed in order to condensate 4-HPAA and dopamine. From here on, a previously described pathway for the production of (*S*)-reticuline was implemented (Hawkins and Smolke 2008; DeLoache *et al.* 2015; Trenchard *et al.* 2015). Galanie *et al.* (2015) extended the production of (*S*)-reticuline by six catalytic steps, being able to produce a wide range of BIAs *de novo* in yeast, including thebaine and hydrocodone.

Tyrosine and phenylalanine towards p-coumaric acid, and production of flavonoids, stilbenoids and dihydrochalcones

Other classes of relevant compounds produced from tyrosine and phenylalanine are flavonoids, stilbenoids and dihydrochalcones (DHCs). The importance of flavonoids, stilbenoids and DHCs mainly concerns their antioxidant activities (Trantas, Panopoulos and Ververidis 2009; Koopman, *et al.* 2012; Eichenberger *et al.* 2017). These compounds are derived from the phenylpropanoid pathway of plants, where *p*-coumaric acid and *trans*-cinnamic acid are the entry metabolites into the pathway.

The formation of *p*-coumaric acid in *S. cerevisiae* can be accomplished via a single heterologous enzymatic step from tyrosine or via two enzymatic steps from phenylalanine (Fig. 3). Expression of a tyrosine ammonia lyase (TAL) allows for the conversion of L-tyrosine to *p*-coumaric acid. Phenylalanine on the other hand can be converted by a phenylalanine ammonia lyase (PAL) to *trans*-cinnamic acid, which can be further converted to *p*-coumaric acid by a cinnamate-4-hydroxylase (C4H). As C4H is a cytochrome P450 enzyme, a cytochrome P450 reductase (CPR) is required for its activity (Werck-Reichhart and Feyereisen 2000). Trantas, Panopoulos and Ververidis (2009) showed that even though *S. cerevisiae* carries a CPR gene, it is beneficial for C4H activity to coexpress a plant CPR (Fig. 3).

Most TAL or PAL enzymes that have been characterised also exhibit low activity towards phenylalanine or tyrosine, respectively (Cochrane, Davin and Lewis 2004; Vannelli *et al.* 2007; Jendresen *et al.* 2015). Jendresen *et al.* (2015) aimed to find specific TALs and expressed a broad variety of genes in *S. cerevisiae*, chosen by synteny information. They compared the enzymes to previously characterised TALs and analysed their specificity towards tyrosine. The best performing TALs with high specificity towards tyrosine were derived from *Flavobacterium johnsoniae* (*Fj*TAL) and *Herpetosiphon aurantiacus* (*Ha*TAL1), which produced higher *p*-coumaric acid titers than the previously characterised specific TALs from *Rhodobacter sphaeroides* (*Rs*TAL) (Watts, Lee and Schmidt-Dannert 2004; Huang and Xue 2006) and *Saccharothrix espanaensis* (*Se*Sam8) (Berner *et al.* 2006). However, focusing solely on final *p*-coumaric acid yields and not on specificity towards tyrosine, the previously characterised TAL/PAL enzyme from *Phanerochaete chrysosporium* (*Pc*XAL) (Xue *et al.* 2007) outperformed the newly characterised enzymes (Jendresen *et al.* 2015).

So far, only few PAL variants have been tested in *S. cerevisiae* (Jiang, Wood and Morgan 2005; Koopman *et al.* 2012; Jendresen *et al.* 2015; Li *et al.* 2016). Most commonly PALs from *Arabidopsis thaliana* (AtPal1 or AtPal2) (Cochrane, Davin and Lewis 2004) are used for the conversion of phenylalanine to *trans*-cinnamic acid in *S. cerevisiae* (Koopman *et al.* 2012; Li *et al.* 2016; Gottardi *et al.* 2017). Other attempts expressing PALs from *Rhodosporidium toruloides* (Jiang, Wood and Morgan 2005) or *Physcomitrella patens* subsp. *patens* (Jendresen *et al.* 2015) in *S. cerevisiae* resulted in rather low titers of *p*-coumaric acid.

For the production of the flavonoid naringenin from *p*-coumaric acid, two additional enzymes need to be introduced into *S. cerevisiae*, a 4-coumaric acid-CoA ligase (4CL) and a chalcone synthase (CHS). Naringenin chalcone can be converted to naringenin spontaneously or enzymatically by introduction of a chalcone isomerase (CHI) (Fig. 3) (Jiang, Wood and Morgan 2005; Trantas, Panopoulos and Ververidis 2009; Koopman *et al.* 2012). Jiang, Wood and Morgan (2005) were the first to report the successful integration of the plant pathway in *S. cerevisiae*, enabling naringenin production in YPD medium. Koopman *et al.* (2012) were able to produce naringenin in *S. cerevisiae* in minimal medium from glucose. Their attempt included the expression of six naringenin biosynthesis genes of *A. thaliana*. In plants, the enzymes involved in flavonoid production have been previously hypothesised to form protein complexes, which could allow for substrate channelling. Therefore, the choice of the heterologous genes for naringenin production was based on a gene expression correlation analysis. *At*Pal1, *At*C4H (codon optimised), *At*CPR1 (codon optimised), *At*4CL3, *At*CHS1 and *At*CHI1 were chosen based on the analysis. They could further optimise the strain by introducing feedback-resistant Aro4^{G226S}, deleting *ARO10*, *PDC5* and *PDC6* and introducing three copies of codon optimised *At*CHS3. Moreover, in order to use the pool of tyrosine for naringenin production as well, a codon-optimised TAL from *Rhodobacter capsulatus* (*Rc*TAL1) was expressed additionally. With this final optimisation, naringenin titers of 200 μM (54 mg/L) were reached (Koopman *et al.* 2012).

Lehka *et al.* (2017) recently published another approach to further optimise flavonoid production in *S. cerevisiae*. The authors found out that Tsc13, an essential endogenous double-bond reductase involved in fatty acid synthesis, is responsible for the conversion of *p*-coumaroyl-CoA to *p*-dihydrocoumaroyl-CoA (Fig. 3). This conversion leads to carbon loss from the heterologous flavonoid production and to accumulation of the unwanted side product phloretic acid. As the deletion of *TSC13* is lethal, *TSC13* was exchanged for a homologous plant gene, and thereby the production of phloretic acid could be abolished completely, reaching higher *p*-coumaric acid and naringenin titers (Lehka *et al.* 2017).

So far, naringenin is the only flavonoid which was produced in minimal medium from glucose in *S. cerevisiae*. However, it was previously shown that introducing an isoflavone synthase or a flavanone 3-hydroxylase and a flavonol synthase enables *S. cerevisiae* to convert naringenin to different flavonoids such genistein, kaempferol and quercetin (Trantas, Panopoulos and Ververidis 2009). Moreover, Rodriguez *et al.* (2015) aimed to construct a strain yielding higher *p*-coumaric acid concentrations from glucose, allowing further modifications to engineer flavonoid or stilbenoid producing strains. They chose to avoid the cytochrome P450 enzymatic step and used only the pool of tyrosine for *p*-coumaric acid production introducing *Fj*TAL. They deleted downstream enzymes encoded by *ARO10*, *PDC5* and *PDC6* and expressed *ARO4*^{K229L} and *ARO7*^{G141S}. Additionally, overexpressing the isoform of the shikimate kinase from *E. coli* AroL, Rodriguez *et al.* (2015) reached a final titer of 1.9 g L^{-1} on feed-in-time medium, which is higher than the previously published *p*-coumaric acid production in yeast (Vannelli *et al.* 2007)

Similar to flavonoids, resveratrol is a valuable fine chemical of medical interest, which is used as food supplement and in cosmetics. Resveratrol can be produced from *p*-coumaric acid by introducing two additional heterologous enzymes (Fig. 3). As for

the production of flavonoids, a 4-coumaric acid-CoA ligase is required to produce 4-coumaryl-CoA. Additionally, a heterologous resveratrol synthase (RS) is needed to produce the antioxidant in *S. cerevisiae*. Li *et al.* (2015) were the first to produce resveratrol directly from glucose. In this work, three heterologous enzymes, namely *Ha*TAL, *At*4CL1 and a RS from *Vitis vinifera* (*VvVST1*) were expressed in *S. cerevisiae*. The aromatic precursor production was optimised by overexpression of feedback-resistant Aro4^{K229L} and Aro7^{G141S}. Additionally, the synthesis of the precursor malonyl-CoA was improved by overexpressing a deregulated Acc1 (Acc^{1S659A,S1157A}). Such modifications in addition to multiple genome integration of *Ha*TAL, *At*4CL1 and *VvVST1* allowed the production of 236 mg L^{-1} of resveratrol (Li *et al.* 2015).

A follow-up strategy by the same group (Li *et al.* 2016) aimed to use the pool of phenylalanine for the production of resveratrol. *AtPAL2*, *At*C4H, *At*4CL2 and *VvVST1* were introduced in a strain harbouring the same upstream pathway modifications described in their previous approach. Resveratrol production was increased by enhancing P450 activity, via the expression of cytochrome P450 reductases *AtATR2* from *A. thaliana* and the endogenous *CYB5*, and by integrating two copies of *AtPAL2*, *At*C4H, *At*4CL2 and *VvVST1* into the genome. Additionally, the deletion of aromatic decarboxylase *ARO10* and expression of a non-regulated version of acetyl-CoA synthase of *Salmonella enterica* (*Se*ACS^{L641P}) allowed a final resveratrol titer of 273 mg L^{-1}.

As the low stability of resveratrol limits its bioactivity, Li *et al.* (2016) also aimed to further convert resveratrol to other stilbenoid antioxidants of higher stability. They subsequently introduced two different resveratrol O-methyltransferases previously shown to convert resveratrol to pinostilbene (*Sorghum bicolor*, *Sb*ROMT) and pterostilbene (*Vitis vinifera*, *Vv*ROMT). Expression of *Sb*ROMT and *Vv*ROMT in the resveratrol producing strain enabled production of 1.4 mg L^{-1} pinostilbene and 5.5 mg L^{-1} pterostilbene, respectively (Li *et al.* 2016).

Another class of secondary plant metabolites produced from p-coumaric acid are the DHCs. DHCs are of commercial interest as they exhibit antioxidant and antidiabetic properties and can be used as food additives due to their sweetening properties. Eichenberger *et al.* (2017) recently engineered *S. cerevisiae* for the *de novo* production of a wide range of DHCs. They enabled *p*-coumaroyl-CoA formation by introducing *AtPAL2*, a C4H from *Ammi majus* (*Am*C4H), *At*4CL2 and overexpressing *CPR1* from *S. cerevisiae*. Furthermore, they overexpressed different double-bond reductases and analysed whether they enabled formation of *p*-dihydrocoumaroyl-CoA, which can be further converted to the DHC phloretin by a CHS. Most effective was the previously mentioned Tsc13 from *S. cerevisiae*. The side activity of Tsc13 for p-coumaroyl-CoA, which leads to carbon loss from the flavonoid pathway, was used here to enable production of DHCs (Eichenberger *et al.* 2017; Lehka *et al.* 2017). The comparison of eight different CHS enzymes showed that the CHS from *Hypericum androsaemum* (*Ha*CHS) produced the highest titer of phloretin (43 mg L^{-1}). They found the CHS from *Hordeum vulgare* (*Hv*CHS2) to exhibit unexpected specificity for *p*-dihydrocoumaroyl-CoA as a substrate, circumventing the production of naringenin from p-coumaryl-CoA. When additionally introducing different UGTs with known specificities for DHCs (Lim *et al.* 2004; Willits *et al.* 2004; Jugdé *et al.* 2008; Brazier-Hicks *et al.* 2009; Gosch *et al.* 2010), they enabled production of trilobatin, the sweetener naringenin DHC, the antioxidant nothofagin and the antidiabetic phlorizin. Introduction of a cytochrome P450 enzyme (CH3H from *Cosmos sulphureus*) (Hutabarat *et al.* 2016) and a cytochrome P450 reductase (*At*ATR2) in addition to the phloretin pathway enabled *de novo* production of 3-hydroxyphloretin.

Phenylalanine biosynthesis branch, and production of pinocembrin DHC, styrene and cinnamyl alcohol

Focusing now solely on the phenylalanine branch, another series of valuable fine and bulk chemicals was produced via metabolic engineering in yeast. These compounds, namely pinocembrin DHC, styrene, cinnamaldehyde, cinnamyl alcohol and hydrocinnamyl alcohol find applications in the medical field, like pinocembrin DHC and cinnamaldehyde; in the cosmetic industry, like cinnamaldehyde, cinnamyl alcohol and hydrocinnamyl alcohol; and in the plastic production field, like styrene.

A shared entry step for the biosynthesis of these compounds exists. It is catalysed by PALs, converting phenylalanine to *trans*-cinnamic acid. This step was achieved by several groups, as already mentioned in this minireview (Koopman *et al.* 2012; McKenna *et al.* 2014b; Gottardi *et al.* 2017).

Pinocembrin DHC is part of the previously described DHCs. The set of reactions that allowed for the production of phloretin from *p*-coumaric acid was implemented from *trans*-cinnamic acid on (Eichenberger *et al.* 2017). The overexpression of *AtPAL2*, *At*4CL2 and *Ha*CHS allowed the production of 1.47 mg L^{-1} of pinocembrin DHC, and the additional overexpression of *TSC13* increased the titers up to 2.6 mg L^{-1}. This work showed that *At*4CL2, *Ha*CHS and Tsc13 can also act on *trans*-cinnamic acid, missing the 4-hydroxy group, however, with a decreased activity.

Another compound produced from *trans*-cinnamic acid is styrene. It is a valuable bulk chemical typically produced via chemical synthesis from ethyl-benzene, an aromatic compound derived from petroleum. Its market represents more than $28 billion in the USA. The *de novo* production of styrene in *S. cerevisiae* was achieved by the combination of a classical evolutionary approach with rational engineering (McKenna *et al.* 2014b). In fact, *S. cerevisiae* strain BY4741 was evolved to overproduce phenylalanine by using ethylmethanesulphonate as mutagen, coupled with selection on plates supplemented with the phenylalanine analog m-fluoro-DL-phenylalanine as antimetabolite. Combined with the overexpression of *AtPAL2* from *A. thaliana*, the evolved strain allowed for the production of 18 mg L^{-1} of styrene, compared to 5 mg L^{-1} in the wild-type strain. It was also established that the endogenous enzyme Fdc1 of *S. cerevisiae* is necessary to decarboxylate *trans*-cinnamic acid to styrene. Further studies demonstrated that the co-expression of endogenous functional Pad1 is required for this enzymatic step by providing the cofactor prenylated FMN (Richard, Viljanen and Penttila 2015; Weber *et al.* 2017). In the same work, McKenna *et al.* (2014b) further rationally engineered the evolved strain by deleting *ARO10* and overexpressing *ARO4*^{K229L}, leading to a *de novo* production of 29 mg L^{-1} of styrene after 48 h of cultivation. An interesting aspect of the work is the mRNA analysis of the phenylalanine overproducing strain. mRNAs of the main genes involved in the aromatic amino acid pathway were quantified, showing increased amounts of *ARO7*, *ARO4*, *ARO3* and especially *ARO1*, *ARO2* and *ARO8* mRNAs. Such results might be of interest in order to design further rational engineering approaches on the aromatic amino acid biosynthetic pathway.

Despite being volatile, styrene is toxic to yeast cells. In another work, McKenna *et al.* (2014a) investigated different approaches to extract styrene from the media. Such *in situ* extraction strategies might not only reduce toxicity problems, but also improve production, due to the shifted chemical equilibrium of the reaction.

Finally, a proof of concept for the production of cinnamaldehyde, cinnamyl alcohol and hydrocinnamyl alcohol was recently published (Gottardi *et al.* 2017). These compounds find applications mainly in the cosmetic and perfumery industry, and cinnamaldehyde in the medical and agricultural field as well (Bang *et al.* 2016; Gottardi *et al.* 2017). Together with *AtPAL2*, an ACAR from *Nocardia* and a phosphopantetheinyl transferase (*entD*) from *E. coli* were expressed in *S. cerevisiae* in their codon-optimised versions (Wiedemann and Boles 2008). With no further upstream engineering, it was possible to biosynthesise up to 0.3, 27.8 and 113.1 mg L^{-1} of cinnamaldehyde, cinnamyl alcohol and hydrocinnamyl alcohol, respectively. More work will be necessary to unravel the metabolic fate of cinnamaldehyde and cinnamyl alcohol in order to accumulate them in the culture supernatant (Gottardi *et al.* 2017), as cinnamaldehyde and cinnamyl alcohol are consumed by the native metabolism of yeast. Furthermore, using growth assays, the toxicity of intermediates and final products was evaluated in this work, suggesting that further improvement of the strain will be necessary to obtain higher titers of the toxic products. In fact, cinnamaldehyde, the most toxic compound among the investigated ones, is rapidly converted to cinnamyl alcohol in order to face its toxicity (Gottardi *et al.* 2017). Other strategies, like *in situ* removal bioprocesses, could be helpful in order to avoid stress induced by aromatic compounds (Lomascolo *et al.* 2001; Beekwilder *et al.* 2014; McKenna *et al.* 2014a).

CONCLUSIONS AND OUTLOOK

This minireview provides an overview of the metabolic engineering approaches applied to produce heterologous aromatic chemicals derived from the tyrosine and phenylalanine biosynthesis pathway in *Saccharomyces cerevisiae*. We focused on the *de novo* synthesis from glucose, with the future possibility to develop yeast strains able to synthesise competitive amounts of desired products from a sustainable carbon source.

As it is possible to witness, several obstacles hinder the biosynthesis of aromatic compounds in *S. cerevisiae* at high yields. Not only establishing novel pathways in yeast but also increasing precursor pools and limiting the toxicity of the desired compounds are challenges that need to be faced. Moreover, the yeast aromatic amino acid pathway is strictly regulated at gene expression and protein levels. Therefore, redirecting the carbon flux towards the desired metabolic branch requires a fine and balanced tuning.

In order to overcome the mentioned obstacles, both rational engineering and evolutionary approaches have been undertaken, and the synergy of them should be used to improve the yields of the compound of interest (McKenna *et al.* 2014b). Combining the described approaches in one strain will surely improve the production of the final desired compound, keeping into account possible limitations such as redox balance. Additionally, development of high-throughput screening systems such as the biosensor tool developed by Leavitt *et al.* (2017) for improved aromatic amino acid production, as well as better screening systems for highly active heterologous enzymes in yeast (DeLoache *et al.* 2015) will accelerate and improve the research in this field.

Despite the many challenges, academic as well as industrial partners are centring their efforts to develop better strains and new metabolic pathways, to help shifting the production of aromatic fine and bulk chemicals from a petroleum-based to a sustainable green production.

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