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De novo biosynthesis of *trans*-cinnamic acid derivatives in *Saccharomyces cerevisiae*

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Abstract The production of natural aroma compounds is an expanding field within the branch of white biotechnology. Three aromatic compounds of interest are cinnamaldehyde, the typical cinnamon aroma that has applications in agriculture and medical sciences, as well as cinnamyl alcohol and hydrocinnamyl alcohol, which have applications in the cosmetic industry. Current production methods, which rely on extraction from plant materials or chemical synthesis, are associated with drawbacks regarding scalability, production time, and environmental impact. These considerations make the development of a sustainable microbial-based production highly desirable. Through steps of rational metabolic engineering, we engineered the yeast *Saccharomyces cerevisiae* as a microbial host to produce *trans*-cinnamic acid derivatives cinnamaldehyde, cinnamyl alcohol, and hydrocinnamyl alcohol, from externally added *trans*-cinnamic acid or de novo from glucose as a carbon source. We show that the desired products can be de novo synthesized in *S. cerevisiae* via the heterologous overexpression of the genes encoding phenylalanine ammonia lyase 2 from *Arabidopsis thaliana* (*AtPAL2*), aryl carboxylic acid reductase (acar) from *Nocardia* sp., and phosphopantetheinyl transferase (*entD*) from *Escherichia coli*, together with endogenous alcohol dehydrogenases. This study provides a proof of concept and a strain that can be further optimized for production of high-value aromatic compounds.

Keywords *trans*-cinnamic acid · Bioconversion · Cinnamaldehyde · Cinnamyl alcohol · Hydrocinnamyl alcohol

Manuela Gottardi and Jan Dines Knudsen contributed equally to the work.

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Introduction

In recent years, an increasing interest has developed towards production of aromatic compounds in microbial hosts, involving industrial as well as academic research (Hansen et al. 2009; Kim et al. 2013). Among other products, cinnamaldehyde ((*2E*)-3-phenylprop-2-enal; cinALD), cinnamyl alcohol ((*2E*)-3-phenylprop-2-en-1-ol; cinOH), and hydrocinnamyl alcohol (3-phenylpropan-1-ol; HcinOH) are widely used aroma compounds, with applications in the food and cosmetic industry, but also as nematicide (Bang et al. 2016), anti-inflammatory (Hanci et al. 2016), and antimicrobial agents (Utchariyakiat et al. 2016). cinALD is found in the bark of plants belonging to the *Cinnamomum* species (Singh et al. 2007) and it has the characteristic odor and taste of cinnamon. cinOH and HcinOH, which can be purified from the leaves of

Cinnamomum species, are usually chemically synthesized from cinALD. They have a sweet-spicy odor and are mainly applied in perfumery and personal care products. Current production methods rely on extracting the pure compounds from plants or on chemical synthesis (Richmond 1947). Both approaches have drawbacks such as scalability, production time, and environmental impact. Industrial biotechnology offers a promising alternative as it allows for the bio-based production of natural aromatic compounds from renewable resources. Plant secondary metabolites have been successfully produced through metabolic engineering of microorganisms such as bacteria or yeasts. Among them, we find flavonoids for medical applications (Koopman et al. 2012), hydroxycinnamic and cinnamic acids, used as precursors for a variety of products for flavoring; plastic and medical applications (Vargas-Tah and Gosset 2015); vanillin, one of the most used flavoring compounds (Hansen et al. 2009); and resveratrol with beneficial functions for human health (Becker et al. 2003; Li et al. 2015; Wang et al. 2011). The yeast *Saccharomyces cerevisiae* presents well-established advantages as production host when compared to other microorganisms; it is robust and stable under harsh industrial conditions, resistant to phages, and able to ferment sugars at low pH (Liu 2011; Weber et al. 2010).

To date, biotechnological production of cinALD was reported only in metabolically engineered *Escherichia coli* (Bang et al. 2016). The production was achieved by heterologous expression of phenylalanine ammonia lyase (EC 4.3.1.24), 4-coumarate:CoA ligase (EC 6.2.1.12), and cinnamoyl-CoA reductase (EC 1.2.1.44). In this pathway, two enzymatic steps are necessary to convert *trans*-cinnamic acid (tCA) to cinALD, via the biosynthesis of cinnamoyl-CoA.

In this work, we present a novel heterologous pathway expressed in yeast for the production of the aroma compounds cinALD, cinOH, and HcinOH starting from tCA or glucose as substrates, which is different from that previously described in *E. coli* (Bang et al. 2016) in that tCA is directly converted to cinALD. The novel synthetic pathway requires the overexpression of three genes, encoding phenylalanine ammonia lyase, aryl carboxylic acid reductase (EC 1.2.1.30), and a phosphopantetheinyl transferase (EC 2.7.8.7). The conversion of cinALD to cinOH can be catalyzed by endogenous alcohol dehydrogenases of *S. cerevisiae* (Larroy et al. 2002) (Fig. 1). Finally, due to the aromatic nature of the intermediates, we investigated the toxic effect that tCA and its derivatives might have on yeast cells (Ramos et al. 2002). Although further optimization is needed, we were able to de novo synthesize maximum titers of cinALD, cinOH, and HcinOH of 0.3, 27.8, and 113.1 mg/L, respectively.

Methods

Strains and plasmids

S. cerevisiae strains and plasmids used in the study are listed in Table 1. *E. coli* DH10β (Gibco BRL, Gaithersburg, MD) cells were used for subcloning. CEN.PK113-7D-derived cells bearing plasmids (MGY1, MGY2, MGY3, MGY4) were always freshly transformed and streaked on YPD plates (20 g/L peptone, 10 g/L yeast extract, 20 g/L D-glucose, and 20 g/L agar) with appropriate antibiotic markers.

Plasmid and strain construction

The codon-optimized DNA sequence ($AtPAL2^{opt}$, GeneBank accession number KY203339) encoding for *At*Pal2 protein (GeneBank accession number NP_190894.1) was generated according to Wiedemann and Boles (2008) and obtained from GENEWIZ (New Jersey, USA). A PCR product of $AtPAL2^{opt}$ with 5′ overhangs homologous to a linearized vector backbone (pRS72N) was generated; primers are listed in Table 2. $AtPAL2^{opt}$ was cloned into the backbone plasmid by transforming (Gietz and Schiestl 2007) both fragments into yeast and exploiting the yeast's native homologous recombination system to assemble the entire plasmid. It resulted in the multicopy plasmid pRS72N_MGV9, with $AtPAL2^{opt}$ sequence under the control of the truncated *HXT7* promoter, ensuring a strong constitutive expression (Hauf et al. 2000). Yeast transformations were performed and cells were streaked out on selective YPD medium containing antibiotics G418 (200 mg/L) or G418 and clonNAT/Nourseothricin (100 mg/L), added to select for *kanMX4* or *natNT2* markers, respectively. Electrocompetent cells of *E. coli* DH10β were used for subcloning, according to previously described methods (Dower et al. 1988). *E. coli* transformants were selected on lysogeny broth (LB) agar plates (Sambrook and Russel 2001) supplemented with 100 mg/L ampicillin.

Strain cultivations

Precultures for high cell density bioconversion were propagated in 2-L Erlenmeyer flasks containing 400 mL of medium. Precultures for toxicity assays were grown in 100 mL of media in 500-mL flasks. All the remaining cultivations were performed in a volume of 50 mL in 300-mL Erlenmeyer flasks. All cultivation experiments were performed at 30 °C in a rotary shaker at 180 rpm. Synthetic minimal medium (Verduyn et al. 1992) containing 0.1 M phosphate buffer at pH 6.4 and 50 g/L D-Glucose was used for the high cell density bioconversion experiments and toxicity assays, or 20 g/L D-Glucose, for

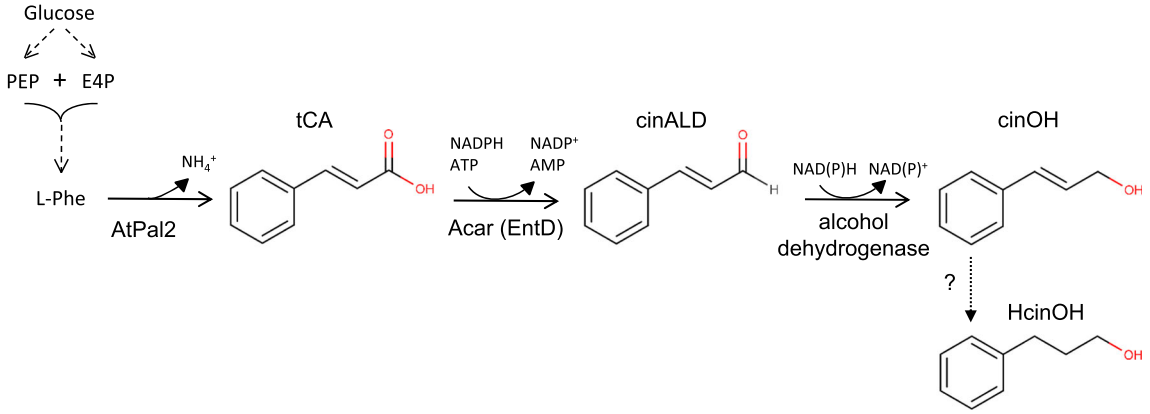

Fig. 1 Representation of the heterologous biosynthetic pathway yielding cinnamaldehyde and cinnamyl alcohol in *S. cerevisiae*. Phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) are condensed through the shikimate pathway towards phenylalanine (L-Phe). The first heterologous reaction is catalyzed by phenylalanine ammonia lyase 2 from *Arabidopsis thaliana* (AtPal2), converting L-Phe to *trans*-cinnamic acid (tCA). The second heterologous enzymatic step, reduction of tCA to cinnamaldehyde (cinALD), is catalyzed by the aryl carboxylic acid reductase (Acar) from *Nocardia* sp. For the activation of Acar, a phosphopantetheinyl transferase (EntD) from *Escherichia coli* is additionally overexpressed. cinALD can be reduced to cinnamyl alcohol (cinOH) by alcohol dehydrogenases natively expressed in *S. cerevisiae*. *Dashed arrows* indicate multiple catalytic steps, and the involvement of unknown enzymes in the reduction of cinOH to hydrocinnamyl alcohol (HcinOH) is depicted by *dotted arrows*

de novo biosynthesis experiments and all precultures. Medium was appropriately supplemented with antibiotics (200 mg/L G418, 100 mg/L clonNAT) for the selection of transformants and further maintenance of the plasmid(s).

For high cell density bioconversion experiments, tCA at concentrations of 400, 200, or 100 mg/L and cinALD or cinOH at concentrations of 200 mg/L were freshly added to the culture; for toxicity assays, tCA was added at concentrations of 25, 50, 100, and 200 mg/L, whereas cinALD, cinOH, and HcinOH were added at equimolar concentrations to the tCA concentrations, being 0.17, 0.34, 0.68, and 1.35 mM, respectively.

The cells for high cell density bioconversions were harvested from precultures at the late exponential phase, whereas precultures for toxicity assays and de novo biosynthesis experiments were harvested in the exponential phase. Cells harvested from precultures were washed in sterile water and inoculated at an optical density (λ = 600 nm) of 10 for high cell density bioconversions and of 0.2 for toxicity assays and for de novo biosynthesis cultivations.

High cell density bioconversions and toxicity assays were performed in biological duplicates and de novo biosynthesis experiments in biological triplicates. During high cell density bioconversion experiments with tCA, samples were taken at 0, 1, 2, 3, 4, 6, 8, 10, and 24 h of cultivation. During high cell density bioconversion experiments with tCA, cinALD, or cinOH, samples were taken at 0, 2, 5, and 24 h of cultivation. During de novo biosynthesis experiments, samples were taken at 0, 2, 4, 6, 8, 10, 24, and 26 h of cultivation. At each time

Table 1 Strains and plasmid used in the present study

Plasmid	Relevant features	Reference
pRS41K	*CEN6, ARS4, kanMX, Amp*^{r}	(Taxis and Knop 2006)
pRS41K_*optACAR_optEntD*	*CEN6*, *ARS4, kanMX*, *Amp*^{r}; *HXT7* shortened promoter, *CYC1* terminator, *acar*^{opt}; 1000 bp *PGK1* promoter sequence, *entD*^{opt}, +433 bp downstream sequence of *ZWF1* terminator sequence	(Bruder and Boles 2016)
pRS72N	2 μ,*TDH3* promoter, *natNT2, Amp*^{r}	(Farwick et al. 2014)
pRS72N_MGV9	*2* μ, *TDH3* promoter, *natNT2, Amp*^{r}; *HXT7* shortened promoter, *CYC1* terminator, *AtPAL2*^{opt}	This work
Strain		
CEN.PK113-7D	*MATα, MAL2-8c SUC2*	EUROSCARF
MGY1	*MATα, MAL2-8c SUC2,* pRS41K	This work
MGY2	*MATα, MAL2-8c SUC2,* pRS41K_*optACAR_optEntD*	This work
MGY3	*MATα, MAL2-8c SUC2,* pRS72N_MGV9,	This work
MGY4	*MATα, MAL2-8c SUC2,* pRS72N_MGV9, pRS41K_*optACAR_optEntD*	This work

Table 2 Primers used in this study

Primer name	Sequence 5′–3′	Application
MGP36.optPAL2.opHXT7.fw	CACAAAAACAAAAAGTTTTTTTAATTTTAATC AAAAAATGGACCAAATTGAAGCTATG	Cloning of pRS72N_MGV9
MGP37.optPAL2.otCYC1.rev	GCGTGAATGTAAGCGTGACATAACTAATTACA TGACTCGAGTTAACAAATTGGAATTGGAGC	Cloning of pRS72N_MGV9

point, the cell density and principal extracellular metabolites were analyzed.

Evaporation assays

The same cultivation conditions of high cell density bioconversions were used to evaluate evaporation of cinALD and cinOH. Three hundred-milliliter Erlenmeyer flasks with 50 ml of synthetic minimal medium (Verduyn et al. 1992) containing 0.1 M phosphate buffer at pH 6.4 and 50 g/L D-Glucose were used. Eighty-nine milligrams per liter of cinALD or 200 mg/L cinOH was added to the media before starting the experiments. Flasks were incubated in a rotary shaker at 180 rpm and 30 °C for 24 h. Samples for HPLC analysis were taken at 0, 2, 5, and 24 h of incubation. The experiment was performed in biological triplicates.

Growth and metabolite analyses

Samples for monitoring cell growth were analyzed directly after collection, while samples for metabolite analysis were stored at −20 °C until analyzed. Cell growth was followed by measuring the optical density at 600 nm (OD_{600nm}) using a spectrophotometer (Ultrospec 2100 pro spectrophotometer, GE Healthcare, USA).

For real-time measurements of cell growth in toxicity assays, the Cell Growth Quantifier (CGQ, Aquila Biosystems) was used (Bruder et al. 2016). Thereby, the intensity of backscattered light was measured every 20 s over a period of 18 or 24 h. Samples for OD_{600nm} measurement and metabolite concentrations via HPLC were collected at 0 and 24 h after the inoculation.

Culture supernatants for HPLC analysis were obtained by centrifugation, treated with 5-sulfosalycilic acid to a final concentration of 5% (*w*/*v*) for the analysis of glucose, ethanol, acetate, and glycerol or used directly for the analysis of aromatic metabolites. Glucose, ethanol, acetate, and glycerol were separated via HPLC (ThermoScientific) equipped with a HyperREZ XP Carbohydrate H+ column (300 × 7.7 mm, 8 μm; ThermoScientific) and a refractive index detector (Thermo Shodex RI-101, Shoko Scientific Co., Kanagawa, Japan). The mobile phase was 5 mM H_2SO_4, and temperature and flow rate were kept constant at 60 °C and 0.6 mL/min, respectively. Aromatic metabolites, tCA, cinALD, and cinOH, were separated using an HPLC (Dionex) equipped with an Agilent Zorbax SB-C8 column (4.6 × 150mm, 3.5 μm), kept at constant temperature of 40 °C. The eluent was composed of 20% (*v*/*v*) acetonitrile in water, 10 mM KH_2PO_4, pH 2.5, at a flow rate of 1 mL/min. These conditions allowed elution and detection of tCA, cinALD, cinOH, and HcinOH, by a UV detector (Dionex UltiMate 3000 Variable Wavelength Detector) at 258 nm for tCA and cinOH, at 220 nm for cinALD and at 210 nm for HcinOH. To identify unknown compounds, GC-MS (Agilent) analysis of culture supernatants was performed. The gas chromatograph was equipped with capillary column Agilent HP-Innowax (25 m × 0.20 mm × 0.2 μm), using helium at a flow rate of 0.6 mL/min as carrier gas. The temperatures of injector, column, and detector were initially set at 250, 50, and 230 °C, respectively. The oven temperature was increased from 50 to 250 °C with a ramp of 10 °C/min.

Data analysis and graphing were carried out using the software Prism 5 (GraphPad, USA).

Results

Construction of a yeast strain expressing $acar^{opt}$ and $entD^{opt}$ for bioconversion of tCA

Strains for high cell density bioconversions and de novo biosynthesis experiments were generated by yeast transformation (Gietz and Schiestl 2007). Centromeric plasmid pRS41K (empty vector control) or pRS41K_*optACAR_optEntD* encoding aryl carboxylic acid reductase (Acar*)* from *Nocardia* sp. and phosphopantetheinyl transferase (EntD) from *E. coli* was used to transform the *S. cerevisiae* laboratory strain CEN.PK113-7D, generating the strains MGY1 (control) and MGY2, respectively. EntD is required to activate Acar, which was previously shown to convert benzoic acid into benzaldehyde in yeast (Bruder and Boles 2016). Growth on agar plates supplemented with 200 mg/L G418 allowed for selection of transformants maintaining the plasmid. MGY1 and MGY2 were subsequently used for high cell density bioconversions of tCA, cinALD, or cinOH.

Toxicity of *trans*-cinnamic acid is alleviated by its consumption

Due to a $\log P_{o/w}$ partition coefficient of 2.1, tCA can act as a toxic agent on microbial cells. Such toxic effect is believed to be exerted as small hydrophobic molecules can disrupt membrane integrity and lead to the release of ions. This has been described in Gram-negative bacteria (Ramos et al. 2002). Moreover, tCA might act as a weak organic acid causing cellular stress (Martani et al. 2013). Therefore, we evaluated the toxicity of tCA on a wild-type *S. cerevisiae* (MGY1) and a *S. cerevisiae* strain expressing the aryl carboxylic acid reductase (MGY2), aiming to assess if the ability of Acar to metabolize the substrate could mitigate the toxicity of tCA. The cells were inoculated from agar plates into liquid media and precultivated until late exponential phase in order to increase their robustness (Steels et al. 1994). The cells were then harvested and re-inoculated at OD_{600nm} 0.2 in minimal synthetic media supplemented with different concentrations of tCA: 0, 25, 50, 100, and 200 mg/L. The growth was measured by the CGQ every 20 s (Fig. 2) and by a spectrophotometer (OD_{600nm}; Table 3), after 24 h, showing consistent trends. Strain MGY1 showed a decreasing growth performance at increasing concentrations of tCA, whereas in strain MGY2, the expression of Acar and EntD appeared to confer a growth advantage with respect to tCA toxicity. Indeed, until a concentration of 50 mg/L tCA, the growth profile of strain MGY2 was comparable to the setup without tCA. Moreover, the final cell density did not differ significantly from the control condition (Fig. 2). Cultivations of MGY2 cells with 100 and 200 mg/L reached lower cell densities than the tCA-free control, but higher if compared to the strain MGY1 under the same conditions (Fig. 2, Table 3, Fig. S1).

Metabolite analyses showed almost complete exhaustion of tCA after 24 h in MGY2 cultivations. Nevertheless, no cinALD was detected in the supernatant of the cultures. Instead, we detected three additional peaks in the HPLC analysis of MGY2 supernatants, which were not present in the analyses of the supernatants of MGY1 control cultures. Based on the elution time, one of them could be identified as cinOH, the direct reduction product of cinALD. A second peak could later be assigned to HcinOH by GC/MS analysis, while the identity of the compound eluting in the third peak could not be clarified. MGY1 was not able to consume significant amount of tCA.

The toxicity of the expected and identified products derived from tCA was assayed as well. The same experimental conditions of the tCA toxicity assay were applied to assess the toxicity of cinALD, cinOH, and HcinOH. cinALD, cinOH, and HcinOH were tested at equal molar concentrations of tCA, being 0.17, 0.34, 0.68, and 1.35 mM. Figure 3 and Fig. S2 show that cinALD exerts a high toxicity from a concentration of 0.68 mM, while the effect of cinOH and HcinOH is mild even at the highest concentration of 1.35 mM.

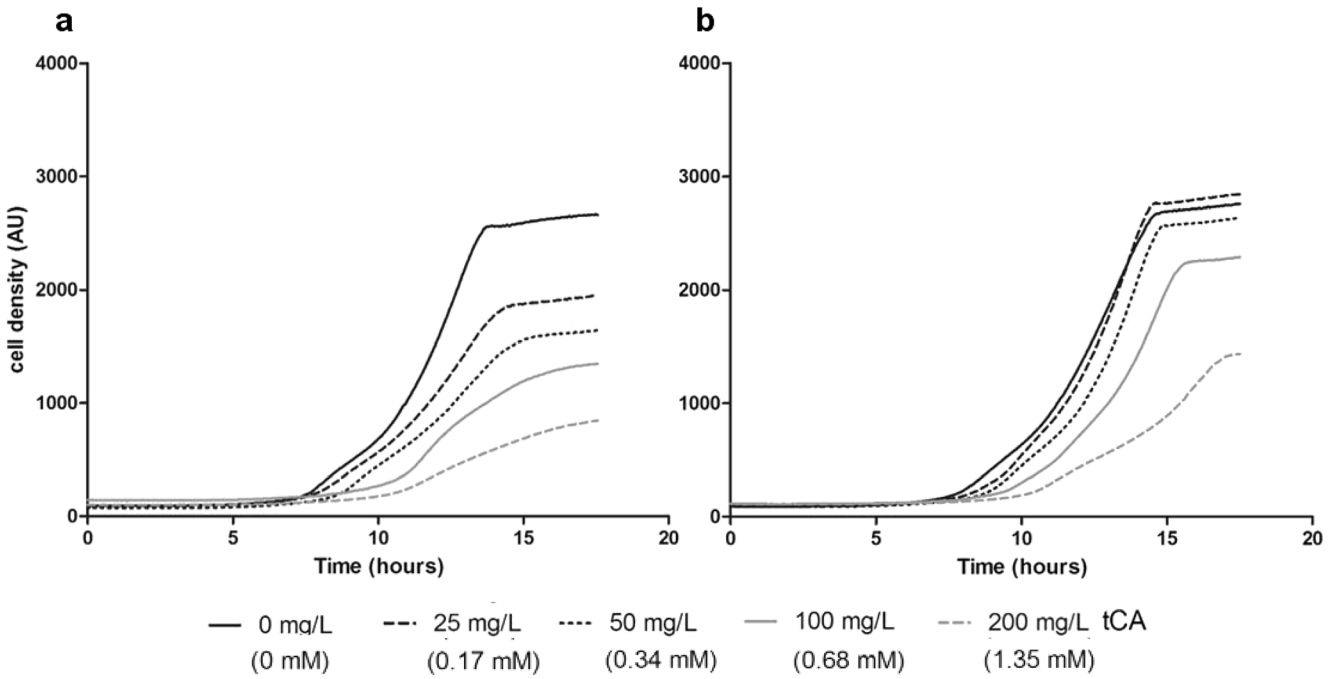

Fig. 2 Growth-based assay of tCA toxicity. Growth curves of the control strain MGY1 (**a**) and MGY2 (**b**), expressing $acar^{opt}$ and $entD^{opt}$, are shown in the presence of different concentrations of tCA added to the media: 0 (*black line*), 25 (*black dashed line*), 50 (*black dotted line*), 100 (*gray line*), and 200 mg/L(*gray dashed line*). In situ cell density, expressed in arbitrary units, AU, was measured every 20 s over 18 h of flask cultivation by the Cell Growth Quantifier system. Results are shown as mean values of duplicate experiments. Standard deviation values do not exceed 15% of the mean value. The *error bars* are omitted for clarity (graphs with error bars are shown in the supplementary material, Fig. S1)

Table 3 Optical cell density, OD_{600nm}, measurements of toxicity assays. Strains MGY1 (empty vector control) and MGY2 (expressing $acar^{opt}$) were assayed after 24 h of cultivation in synthetic minimal media, 50 g/L D-glucose, with different concentrations of tCA

	Optical cell density, 600 nm	
tCA (mg/L)	MGY1	MGY2
0	17.15 ± 0.05	16.9 ± 0.6
25	13.25 ± 0.45	17.4 ± 0.7
50	10.35 ± 0.55	16.75 ± 0.05
100	8.25 ± 0.65	14.95 ± 0.25
200	5.95 ± 0.25	10.85 ± 0.15

High cell density bioconversion of tCA

Since no cinALD was detected in the culture supernatant after 24 hours of cultivation in the previous experiment, we performed high cell density bioconversions with a starting OD_{600nm} of 10, which also allowed to further increase the initial concentration of tCA (as the severity of toxic effects also depends on the initial cell density). tCA was added to the media in concentrations of 0, 100, 200, or 400 mg/L. The metabolite analyses (Fig. 4) revealed that, in all applied conditions, the strain MGY2 completely consumed tCA, producing cinALD at concentrations ranging from 0.1 to 2.4 mg/L (data not shown) and cinOH to a maximum measured concentration of 112 mg/L, after 3 h in the setup with 200 mg/L tCA. In all the three experimental setups, the molar conversion yield of tCA to cinOH in the first 2 h ranged between 85 and 100%, meaning that cinOH was the main initial product of the conversion. However, the amount of cinOH gradually decreased over time, with a concomitant increase of the concentration of HcinOH (Fig. 4) and of the unknown byproduct (since the latter is not quantifiable, the peak areas are plotted over time and shown in Supplementary Fig. S3). In all bioconversions with strain MGY2, HcinOH is the major terminal product that accounts for about 60% of the converted tCA (Fig. 4).

In order to assess whether cinALD and cinOH are consumed by the yeast's native metabolism or if the consumption relates to the presence of Acar and EntD, further bioconversions with strains MGY1 and MGY2 were performed, whereby tCA, cinALD, or cinOH was added as substrates. Figure 5 shows an overlay of HPLC chromatograms measured after 5 h of cultivation. In the absence of Acar (MGY1), a small proportion of cinALD and cinOH appears to be converted back to tCA, possibly by spontaneous, oxygen-dependent, or enzymatic oxidation. When Acar is overexpressed, no peak corresponding to tCA is detected. Apart from this difference, no significant changes in peak patterns are observed between MGY1 and MGY2 samples, indicating that cinALD and cinOH are consumed by the endogenous metabolism, independently of the presence of Acar and EntD.

Evaporation assays of cinALD and cinOH were also performed to clarify if evaporation could cause an underestimation of the metabolites. HPLC analyses revealed that after 24 h of incubation under the same cultivation conditions of the bioconversion experiments, cinALD and cinOH were present at 97.6 ± 6.4 and 99.5 ± 3.2% of the initial added amounts, respectively (data are expressed as percent mean values of triplicate experiments with standard deviation).

De novo biosynthesis of cinALD, cinOH, and HcinOH

After having assessed toxicity of tCA and functionality of Acar in *S. cerevisiae* by adding tCA as substrate, the possibility of producing cinALD, cinOH, and HcinOH de novo from glucose was investigated. The plasmid pRS72N_MGV9 bearing $AtPAL2^{opt}$ was transformed into the strains MGY1 and MGY2, generating the strains MGY3 and MGY4, respectively. One hundred milligrams per liter of Nourseothricin together with 200 mg/L G418 allowed for the selection of transformants bearing both plasmids. The enzyme Pal2 from *Arabidopsis thaliana* was chosen among four other *A. thaliana* phenylalanine ammonia lyase proteins due to its superior kinetic parameters towards phenylalanine. It has the lowest k_m towards phenylalanine and very low activity towards tyrosine. The k_{cat}/k_m value for phenylalanine is 51.200 M^{-1} s^{-1} and for tyrosine only 40 ± 12 M^{-1} s^{-1} (Cochrane et al. 2004; McKenna and Nielsen 2011). Figure 6 shows the HPLC metabolite analyses of the cultivations of strains MGY3 and MGY4. The additional expression of $AtPAL2^{opt}$ gene in the strain MGY3 allowed the de novo biosynthesis of tCA from glucose to a final and maximum concentration of 28.6 mg/L after 26 h of cultivation (Fig. 6a). It is worth noticing that when glucose was almost completely consumed (10-h time point), tCA reached a concentration of 18.2 mg/L and its titer continued to increase in the following 10 h. The co-expression of $AtPAL2^{opt}$, $acar^{opt}$, and $entD^{opt}$ genes in the strain MGY4 resulted in the production of tCA, cinALD, cinOH, and HcinOH. Additionally, the unknown compound as observed in bioconversion experiments described above was also detected (Supplementary Information, Fig. S3). The maximum titer of cinOH (27.8 mg/L) was reached after 10 h of cultivation, when the residual glucose concentration was 1.9 g/L, and diminished till 3.5 mg/L at 24 h. Because of their conversion into cinOH, tCA and cinALD did not accumulate over time and their maximum titers were measured after 8 h of cultivation, being 2.1 and 0.3 mg/L, respectively. HcinOH, differently, accumulated overtime and it resulted in a stable titer of 113.1 mg/L after 26 h of cultivation (Fig. 6). Thus, the product profile of the de novo synthesis is consistent with that obtained with externally added tCA.

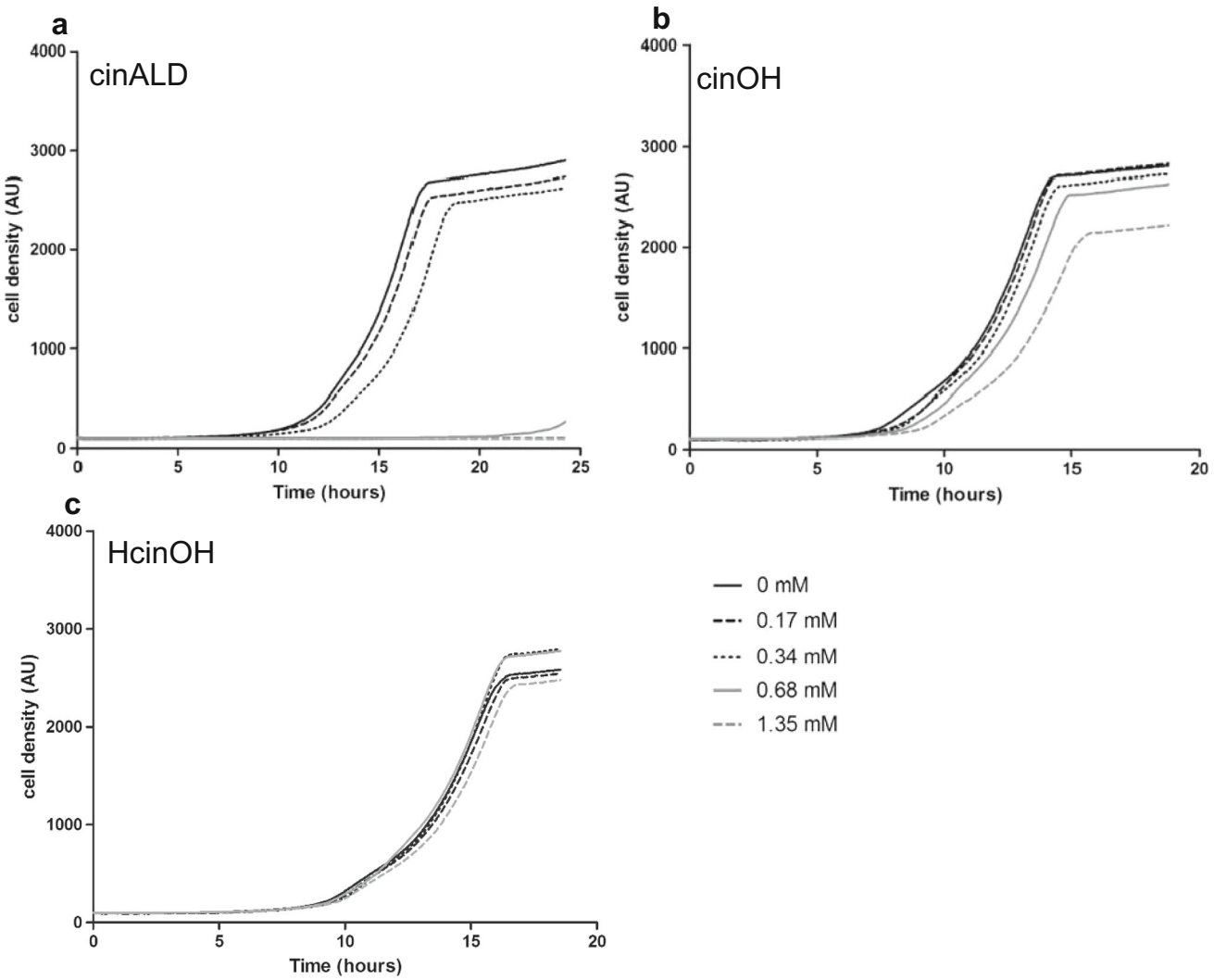

Fig. 3 Growth-based assay of cinALD, cinOH, and HcinOH toxicity. Growth curves of the strain MGY2, expressing *acar*^{opt} and *entD*^{opt}, are shown in the presence of different concentrations of cinALD (**a**), cinOH (**b**), or HcinOH (**c**) added to the media: 0 (*black line*), 0.17 (*black dashed line*), 0.34 (*black dotted line*), 0.68 (*gray line*), and 1.35 mM (*gray dashed line*). In situ cell density, expressed in arbitrary units, AU, was measured every 20 s over 18–24 h of flask cultivation by the Cell Growth Quantifier system. Results are shown as mean values of duplicate experiments. Standard deviation values do not exceed 15% of the mean value. The error bars are omitted for clarity (graphs with error bars are shown in the supplementary material, Fig. S2)

Discussion

In this work, we present the establishment of a novel biosynthetic pathway yielding the industrially important compounds cinALD, cinOH, and HcinOH in the yeast *S. cerevisiae* as production host. The toxicity of intermediates in a pathway can hamper the production of the user-specified end products, and we therefore investigated whether tCA, cinALD, cinOH, and HcinOH affect the performance of the *S. cerevisiae* cell factory. As shown in Fig. 2 and Table 3, the toxicity of tCA on yeast cells affects their growth, suggesting that production of tCA derivatives is likely to pose further challenges beyond establishing a functional heterologous pathway. The expression of the Acar enzyme apparently reduced the toxicity of tCA (Fig. 2), by reducing this organic acid and therefore lowering its titer. Interestingly, the toxicity of cinALD is even higher than that of tCA. During 24 h of cultivation (Fig. 3a), cinALD up to 0.34 mM did not significantly hamper cell growth, but from 0.68 mM, the growth is almost completely abolished. It is possible to observe that, at the end of the cultivation, in the setup with 0.68 mM cinALD, cells are starting to grow, probably due to the fact that cinALD is finally slowly being converted. cinOH (Fig. 3b) has a milder effect than cinALD and tCA, whereas HcinOH has no effect on growth (Fig. 3c). Thus, further reduction of cinALD to cinOH and HcinOH (Figs. 4, 5, and 6) can be explained by a cellular detoxification mechanism.

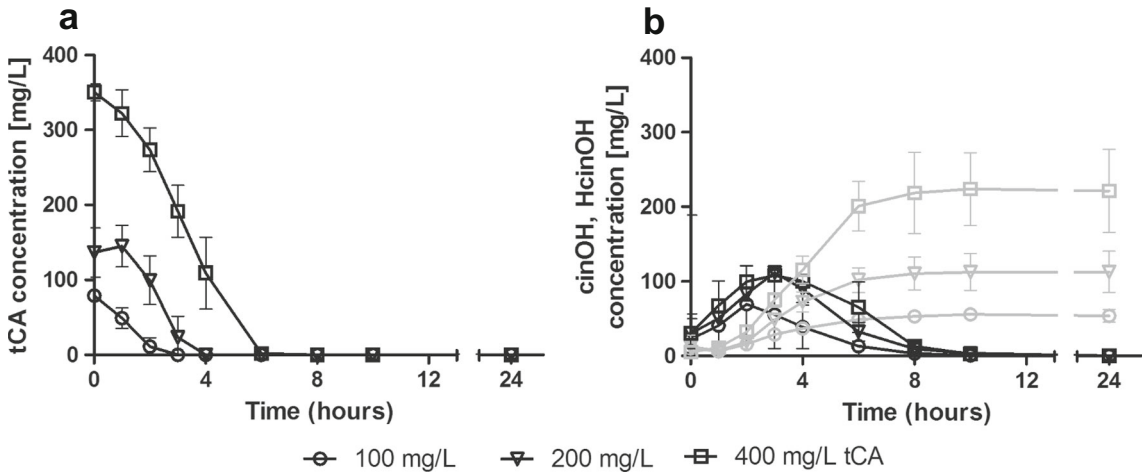

Fig. 4 Metabolite analysis of bioconversion experiments with added tCA. **a** tCA consumption in the *acar*^{opt} expressing strain, MGY2, and **b** the production of cinOH (*black line*) and hydrocinnamyl alcohol (HcinOH, *gray line*). tCA was added at a concentration of 400 (*open square*), 200 (*open downward triangle*), and 100 mg/L (*open circle*). Samples were taken at 0, 1, 2, 3, 4, 6, 8, 10, and 24 h of cultivation. Results are shown as mean values of biological duplicates, with standard deviation as *error bar*

The high cell density bioconversion experiments proved the activity of Acar towards tCA in *S. cerevisiae* and allowed for the production of cinALD, cinOH, and HcinOH. Only small concentrations of cinALD were detected, due to its rapid conversion to cinOH. As previously described, the conversion of cinALD to cinOH could be catalyzed by alcohol dehydrogenases such as Adh6 (Larroy et al. 2002). Therefore, to further investigate the metabolism of cinALD, an alcohol dehydrogenase deletion strain should be constructed and evaluated. Furthermore, cinALD might be condensed with acetaldehyde by pyruvate decarboxylase to yield 5-phenylpent-4-ene-2,3-diol (Miyakoshi et al. 2016). Although the unidentified compound detected during the HPLC analysis can also be formed from cinOH (Fig. 5), it is possible that a part of the alcohol is oxidized back to cinALD, which can undergo this reaction.

Even though a significant amount of cinOH was produced both in bioconversion experiments (Fig. 4) and de novo (Fig. 6), its titer was not stable, which is explained by the formation of byproducts—HcinOH and the unidentified compound. The endogenous double-bond reductase Tsc13 might be

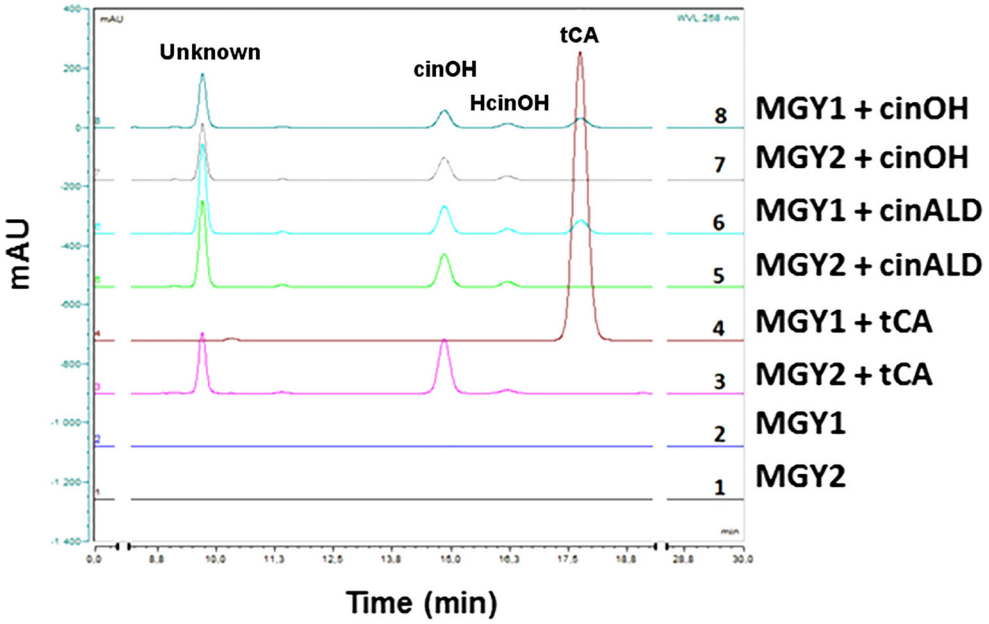

Fig. 5 Comparison of HPLC chromatograms of high cell density bioconversions with added tCA, cinALD, or cinOH. Two hundred milligrams per liter of tCA, cinALD, or cinOH was added to the cultures of MGY1 or MGY2 as indicated on the right (chromatograms 3–8) or omitted in negative controls (chromatograms 1 and 2). After 5 h of high cell density bioconversion experiments, culture supernatants were analyzed by HPLC and the chromatograms were overlaid. A peak eluting at 9.7 min corresponds to a yet unidentified compound (*Unknown*), while cinOH elutes at 14.8 min followed by HcinOH at 16.2 min and tCA at 17.7 min

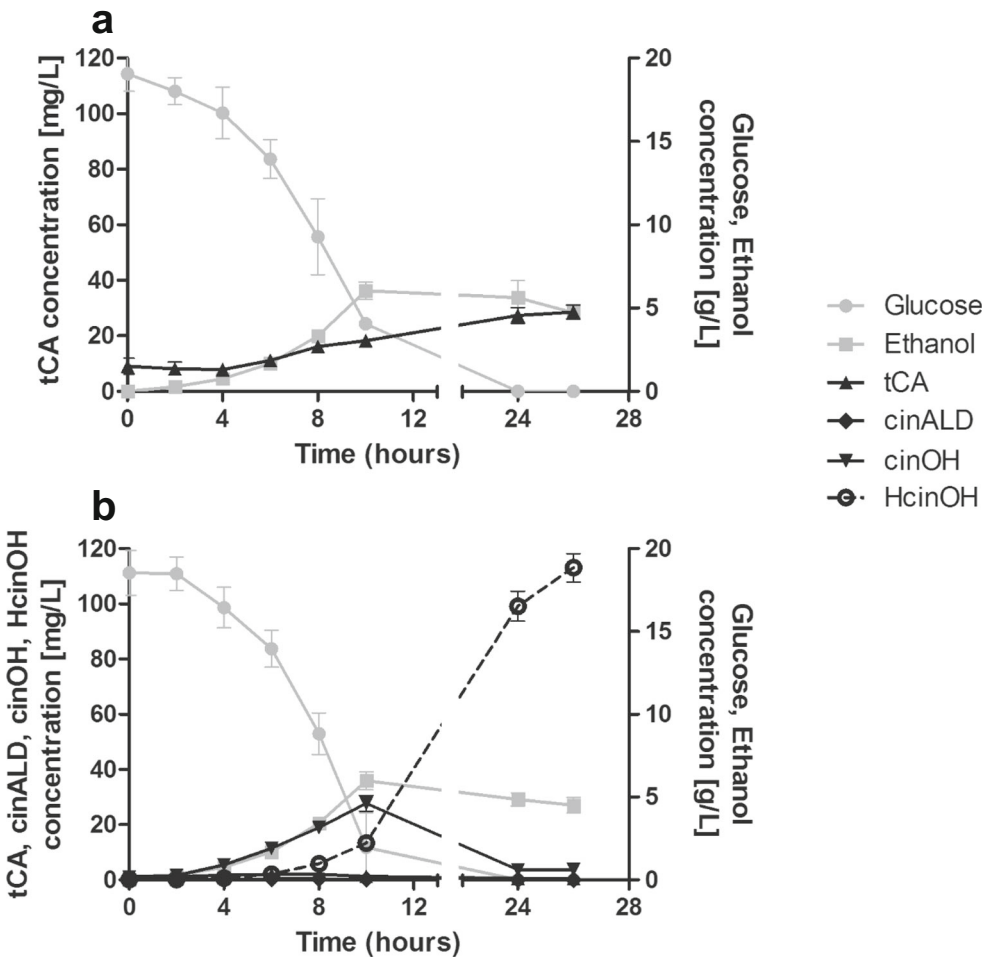

Fig. 6 De novo synthesis of tCA, cinALDcinOH, and HcinOH. **a** tCA biosynthesis in the strain MGY3. **b** tCA, cinALD, cinOH, and HcinOH production in the strain MGY4. In both graphs, glucose and ethanol concentrations refer to the right Y axis. Samples were taken at 0, 2, 4, 6, 8, 10, 24, and 26 h of cultivation. Results are shown as mean values of biological triplicate experiments with standard deviation as *error bars*

involved in the reduction of cinOH to HcinOH (Lehka et al. 2017). The production of HcinOH was unexpected, but can be regarded as desirable from a biotechnological point of view as HcinOH finds applications in the cosmetic industry, amplifying the range of valuable products that can be produced by the presented heterologous pathway. If cinOH or HcinOH are terminal compounds to be produced, this intrinsic property might be an additional benefit of using *S. cerevisiae* instead of *E. coli*, which does not convert cinALD to alcohols (Bang et al. 2016).

In a separate experiment, the evaporation of cinALD and cinOH was also assayed but proved not to be of concern, as cinALD and cinOH did not significantly evaporate over a time of 24 h.

As the bioconversion experiments proved that it is possible to convert tCA to cinALD, cinOH and HcinOH in vivo in *S. cerevisiae* cells, *AtPAL2*^{opt}, the link of the L-Phe biosynthesis pathway to the heterologous pathway, was expressed (McKenna et al. 2014). The de novo pathway was proved to be functional, which provides the basis for future strain engineering by optimizing the flux through the shikimate pathway as previously described (Luttik et al. 2008; Rodriguez et al. 2015). To specifically increase the production of tCA derivatives, the carbon flux towards L-Phe production should be increased (Gold et al. 2015; Koopman et al. 2012). In our conditions with no upstream pathway engineering, the strain expressing only *atPal2*^{opt} (MGY3) produced up to 28.6 mg/L tCA at 24 h and the strain expressing Acar, MGY4, 27.8 mg/L cinOH, after 8 h of cultivation and 113.1 mg/L of HcinOH after 26 h of cultivation.

Moving towards an industrial application, for sustainable and profitable production of tCA derivatives, several strain rational engineering steps or evolutionary approaches should be undertaken. Among other, upstream pathway engineering (Gold et al. 2015; Koopman et al. 2012) and identification of enzymes involved in the conversion of the desired end products need to be addressed. Moreover, if cinALD is to be produced as the terminal compound, the production strains will need to be engineered to tolerate higher concentrations of the aldehyde than the laboratory strain (see Fig. 3a).

It will be also of great interest to introduce the pathway towards tCA derivatives into *S. cerevisiae* strains which are able to utilize alternative carbon sources such as lignocellulosic hydrolysates or industrial wastes.

To summarize, with this work, we show the first proof of concept for the production of cinALD, cinOH, and HcinOH in *S. cerevisiae*.

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Authors' contribution MG performed the experimental work, analyzed the data, and wrote the manuscript. MO and EB initiated the work on tCA production. MG, JDK, and PB designed the experiments. JDK and PB helped in data analysis. LP helped in the identification of unknown compounds. JDK, PB, MO, and EB helped in drafting the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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