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Biosynthesis of *cis,cis*-Muconic Acid and Its Aromatic Precursors, Catechol and Protocatechuic Acid, from Renewable Feedstocks by *Saccharomyces cerevisiae*

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Adipic acid is a high-value compound used primarily as a precursor for the synthesis of nylon, coatings, and plastics. Today it is produced mainly in chemical processes from petrochemicals like benzene. Because of the strong environmental impact of the production processes and the dependence on fossil resources, biotechnological production processes would provide an interesting alternative. Here we describe the first engineered *Saccharomyces cerevisiae* strain expressing a heterologous biosynthetic pathway converting the intermediate 3-dehydroshikimate of the aromatic amino acid biosynthesis pathway via protocatechuic acid and catechol into *cis,cis*-muconic acid, which can be chemically dehydrogenated to adipic acid. The pathway consists of three heterologous microbial enzymes, 3-dehydroshikimate dehydratase, protocatechuic acid decarboxylase composed of three different subunits, and catechol 1,2-dioxygenase. For each heterologous reaction step, we analyzed several potential candidates for their expression and activity in yeast to compose a functional *cis,cis*-muconic acid synthesis pathway. Carbon flow into the heterologous pathway was optimized by increasing the flux through selected steps of the common aromatic amino acid biosynthesis pathway and by blocking the conversion of 3-dehydroshikimate into shikimate. The recombinant yeast cells finally produced about 1.56 mg/liter *cis,cis*-muconic acid.

Adipic acid is a chemical precursor used for the production of nylon, lubricants, coatings, plastics, and plasticizers. It belongs to the top 50 bulk chemicals, with an annual production of about 2×10^9 kg. Currently, it is produced mainly by a two-stage process which starts with the oxidation of benzene-derived cyclohexane resulting in a cyclohexanol-cyclohexanone mixture that is further oxidized by nitric acid (47). This chemical process requires a high energy input and further leads to the production of large amounts of the greenhouse gas N_2O (46). Moreover, the chemical intermediates are toxic compounds and are partially linked to carcinogenesis (16). Thus, because of the heavy environmental impact and the dependence on finite fossil resources, there is high interest in alternative biotechnological production processes. Currently, no commercial biotechnological process exists. One possible biochemical route is via *cis,cis*-muconic acid (CCM), which can be hydrogenated to adipic acid (38). Several bacteria are known to convert aromatic compounds into CCM (37, 53). The first synthetic route using renewable resources like glucose has been established in *Escherichia coli* (10) (Fig. 1). This nonnatural route is based on the expression of three heterologous genes that encode a 3-dehydroshikimate (3-DHS) dehydratase (AroZ), a protocatechuic acid (PCA) decarboxylase (AroY) from *Klebsiella pneumoniae*, and a catechol 1,2-dioxygenase (CatA) from *Acinetobacter calcoaceticus*. These enzymes are found in various microorganisms normally enabling the use of aromatic compounds as carbon sources (10, 21). The enzymes convert 3-DHS, an intermediate of the common aromatic amino acid biosynthesis pathway, to CCM via PCA and catechol. This production pathway has been further optimized for improved carbon flow into the aromatic amino acid pathway by increasing the levels of the basal metabolic intermediates phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), which serve as the initial substrates for aromatic amino acid biosynthesis, and by blocking of aromatic amino acid synthesis at the level of 3-DHS by deleting the 3-DHS dehydrogenase gene *aroE* (11, 16, 49). Moreover, a feedback-resistant mutant form of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase was overexpressed. The final recombinant *E. coli* strain produced 36.8 g/liter of CCM, with a yield of 22% (mol/mol) within 48 h of culturing under fed-batch fermentor conditions (38).

Even though the optimized *E. coli* strain already produces considerable amounts of CCM, it does not allow a cost-competitive industrial production process. This is due to the intrinsic ability of *E. coli* to grow only under neutral-pH conditions. Purification of CCM from the fermentation broth in its undissociated form is done at low pH values (26). Thus, in the case of *E. coli*, substantial acidification of the broth after fermentation and a subsequent recycling step with a high salt load would be required. These steps are very cost-intensive and would make the downstream process uneconomical. In contrast, *Saccharomyces cerevisiae* fermentations can be performed at low pH values. Moreover, *S. cerevisiae* has further beneficial properties for industrial production processes like high robustness, high resistance to toxic inhibitors and fermentation products, resistance to microbial contamination, and a high level of public acceptance (32, 35, 50).

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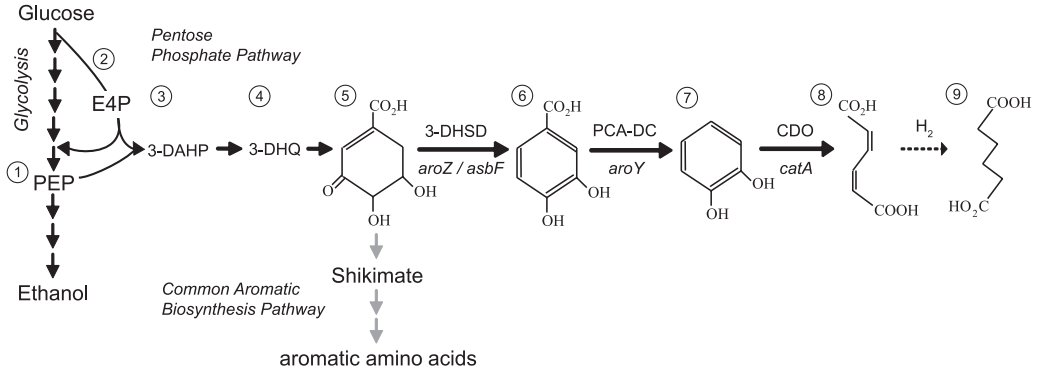

FIG 1 Schematic representation of the *de novo* CCM biosynthesis pathway. The central intermediates of the pathway shown are PEP (step 1), E4P (step 2), DAHP (step 3), 3-dehydroquinate (3-DHQ; step 4), 3-DHS (step 5), PCA (step 6), catechol (step 7), CCM (step 8), and adipic acid (step 9). Adipic acid can be produced by chemical hydrogenation (dashed arrow). Bold arrows indicate heterologous enzymatic reactions: 3-DHSD, 3-DHS dehydratase; PCA-DC, PCA decarboxylase; CDO, catechol 1,2-dioxygenase. Undesired yeast metabolic reactions are indicated by gray arrows.

In this work, we established a heterologous CCM production pathway in *S. cerevisiae* by the expression of three enzymes. We further optimized this pathway, resulting in the production of 1.56 mg/liter CCM.

MATERIALS AND METHODS

Strains and media. The yeast and bacterial strains used in this work are listed in Table 1. The bacterial strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *S. cerevisiae* strains were grown at 30°C with shaking at 180 rpm in synthetic complete medium (6.7 g/liter Difco yeast nitrogen base without amino acids) supplemented with amino acids as described previously (54) and with 20 g/liter D-glucose as a carbon source (SCD medium), adjusted to pH 6.3. For maintenance of plasmids, selective SCD medium lacked the auxotrophy markers and/or contained 200 mg/liter G418 or 200 mg/liter hygromycin, respectively.

Metabolite analysis. For metabolite analysis, the yeast cells were removed from samples by centrifugation. Proteins were precipitated by the addition of sulfosalicylic acid to a final concentration of 5% and metabolites were measured by high-performance liquid chromatography (HPLC). The metabolites were separated by HPLC (Dionex) using a Nucleogel Sugar 810 H exchange column (Macherey-Nagel GmbH & Co., Düren, Germany). The column was eluted with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min and a temperature of 65°C. The detection of glucose, glycerol, acetic acid, and ethanol was done by means of a Shodex RI-101 refractive-index detector. A UV detector was used for the detection of PCA (220 nm), catechol (220 nm), and CCM (250 nm). For data evaluation, the Chromeleon software (version 6.50) was used.

Plasmid and strain construction. The plasmids and primers used in this study are listed in Tables 2 and 3. Molecular techniques were performed according to previously published procedures (51). Bacterial genomic DNA was prepared for PCR amplification as described in reference 2. Alternatively, PCRs were performed using broken cells as templates. Codon-optimized gene versions for increased protein expression in *S. cerevisiae* were obtained from DNA2.0, including the use of a DNA2.0 optimization algorithm. Yeast transformations and reisolation of plasmid DNA from yeast cells were carried out as described previously (1, 17). Genes were cloned by homologous recombination. The coding regions of the respective genes were amplified by PCR from genomic DNA or plasmids by using specific primer pairs with 5′ extensions overlapping vector sequences. PCR fragments were cotransformed into yeast cells together with a linearized vector. All vector-carried genes were under the control of strong promoters (Table 2). Plasmids were amplified in *E. coli* strain DH5α (Gibco BRL, Gaithersburg, MD). *E. coli* transformations were performed via electroporation according to the methods of Dower et al. (9). *E. coli* was grown on Luria-Bertani medium with 40 μg/ml ampicillin for plasmid selection. Strain CEN.PK2-1C$^{\Delta aroE}$ was constructed by using the *cre-loxP-kanMX4-loxP* system (19). The *loxP-kanMX-loxP* deletion cassette was PCR amplified from plasmid pUG6 by using primers with 5′ extensions homologous to the integration site, allowing homologous recombination. The deletion cassette was transformed into CEN.PK2-1C, which was selected for Geneticin (G418) resistance. Positive clones were transformed with plasmid pSH47, which encodes the Cre recombinase. Expression of the enzyme was induced by shifting the transformed cells to galactose-containing medium. The deletion of the *aroE* domain and the removal of the deletion cassette were verified by PCR analysis.

Fermentations and feeding experiments. Cultures of yeast strains (50 ml) were grown in 300-ml shake flasks (Erlenmeyer flasks) at 30°C in a rotary shaker (180 rpm). Precultures were grown into the exponential phase in selective SCD medium. Cells were washed with sterile water and inoculated to an optical density at 600 nm (OD_{600}) of 1.0 in the same medium. Growth and fermentation experiments were performed up to

TABLE 1 Strains used in this study

Species or strain	Genotype	Accession no.	Source
Burkholderia xenovorans		DSMZ-17367	DSMZ[a]
Acinetobacter sp.		DSM-586	DSMZ
Bacillus thuringiensis		DSM-6074	DSMZ
S. cerevisiae strains			
CEN.PK2-1C	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*		EUROSCARF, Frankfurt, Germany
CEN.PK2-1C$^{\Delta aroE}$	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2 ARO1$^{\Delta 1359\text{-}1588}$::loxP*		This work

[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

TABLE 2 Plasmids used in this study

Plasmid	Regulatory elements, markers, genes	GenBank accession no.	Reference
p423-HXT7	*HXT7* promoter, *CYC1* terminator, *HIS3*		20
p425-HXT7	*HXT7* promoter, *CYC1* terminator, *LEU2*		20
p426-HXT7	*HXT7* promoter, *CYC1* terminator, *URA3*		20
pUG6	*loxP-kanMX4-loxP, URA3*		19
pRS42K	*TEF* promoter, *TEF* terminator, *kanMX*		45
pRS42H	*TEF* promoter, *CYC1* terminator, Hyg^r		45
pRS42K-HXT7	*HXT7* promoter, *CYC1* terminator, *kanMX*		This work
pRS42H-HXT7	*HXT7* promoter, *FBA1* terminator, Hyg^r		This work
pRS42H-HXT7-FBA1	*HXT7* promoter, *FBA1* terminator		This work
pSH47	*GAL1* promoter, *cre* recombinase, *CYC* terminator, *URA3*		19
p426-HXT7-aroE	*HXT7* promoter, *aroE* from *E. coli*, *CYC1* terminator, *URA3*	YP001732122	This work
p425-HXT7-Bx-catA	*HXT7* promoter, *catA* from *B. xenovorans*, *CYC1* terminator, *LEU2*	YP558911	This work
p425-HXT7-Ar-catA^{opt}	*HXT7* promoter, *catA* from *A. radioresistens* codon optimized (DNA2.0), *CYC1* terminator, *LEU2*	AF380158	This work
p423-HXT7-Bt-aroZ	*HXT7* promoter, *asbF* from *B. thuringiensis*, *CYC1* terminator, *HIS3*	NC014171	This work
p423-HXT7-Bt-aroZ^{opt}	*HXT7* promoter, *asbF* from *B. thuringiensis* codon-optimized (DNA2.0), *CYC1* terminator, *HIS3*	NC014171	This work
p423-HXT7-As-aroZ	*HXT7* promoter, *aroZ* from *Acinetobacter* sp., *CYC1* terminator, *HIS3*	Q43922	This work
p423-HXT7-Pa-aroZ^{opt}	*HXT7* promoter, *3DSD* from *P. anserina* codon-optimized (DNA2.0), *CYC1* terminator, *HIS3*	CAD60599	This work
pRS42H-HXT7-ARO1$^{\Delta aroE}$	*HXT7* promoter, *ARO1* from *S. cerevisiae* lacking the *aroE* encoding domain (amino acids 1359-1588), *FBA1* terminator, Hyg^r	YDR127W	This work
pRS42K-Kp-aroY^{opt}	*HXT7* promoter, *aroY* subunit *B*, *CYC1* terminator; *PGK1* promoter, *aroY* subunit C, *PGK1* terminator; *TPI1* promoter, *aroY* subunit D, *TAL1* terminator, *kanMX*; all subunits from *K. pneumoniae* codon optimized (DNA2.0); the expression cassette can be integrated into the *PYK2* locus	AAY57854, AAY57855, AAY57856	This work
pRS42K-Kp-aroY$^{iso.opt}$	*HXT7* promoter, *aroY* subunit B, *CYC1* terminator; *PGK1* promoter, *aroY* subunit C isoform, *PGK1* terminator; *TPI1* promoter, *aroY* subunit D, *TAL1* terminator, *kanMX*; all subunits from *K. pneumoniae* codon optimized (DNA2.0)	AAY57854, AB479384, AAY57856	This work
pRS42K-Sh-aroY	*HXT7* promoter, *aroY* subunit B, *CYC1* terminator; *PGK1* promoter, *aroY* subunit C, *PGK1* terminator; *TPI1* promoter, *aroY* subunit D, *TAL1* terminator, *kanMX*; all subunits from *S. hydroxybenzoicus*	AAY67850 AAD50377 AAY67851	This work

three times by using the same precultures, with the given standard deviations. Samples for OD_{600} measurement and metabolite analyses were taken at different time points. Anaerobic/oxygen-limited fermentations (100 ml) were done using 100-ml shake flasks (Erlenmeyer flasks) sealed with an airlock and incubation at 30°C. The cell suspension was mixed by using a magnetic stirrer. The oxygen was initially removed by sparging with nitrogen gas. Samples were taken with a syringe using a separate output connection. Since the oxygen level could not be controlled completely, these fermentations are seen as semianaerobic, oxygen-limited conditions. To analyze the rate of turnover of catechol or PCA in yeast cultures, each compound was supplied at a final concentration of 5 or 3.4 mM, respectively.

RESULTS

A heterologous biosynthetic route to CCM in yeast. In *S. cerevisiae*, no natural biosynthetic route to CCM exists. Therefore, we aimed to establish a pathway similar to that previously described for *E. coli* by expression of the heterologous enzymes 3-DHS dehydratase (AroZ), PCA decarboxylase (AroY), and catechol-1,2-dioxygenase (CatA). These enzymes should convert the intermediate 3-DHS of the common aromatic amino acid biosynthesis pathway (4) via PCA and catechol to CCM (Fig. 1). We first set out to identify suitable enzymes for functional expression in yeast. Therefore, for each enzyme activity, we expressed various potential candidate genes in *S. cerevisiae* and analyzed their activities individually in bioconversion and biotransformation experiments. Finally, we combined the best-performing enzymes into an optimized biosynthetic pathway.

Identification of suitable 3-DHS dehydratases and optimization of the biosynthetic route to 3-DHS. In the first heterologous reaction, 3-DHS is dehydrated to PCA. Several 3-DHS dehydratases (AroZ) acting in various cellular pathways have been identified in different microbial systems (14, 15, 18, 22, 28, 30, 38, 39, 40). To identify a suitable candidate for the new biosynthetic route in yeast, we chose three different genes, *aroZ* of the aromatic degradation pathway from *Acinetobacter* sp. (*As-aroZ*), *aroZ* of *Podospora anserina* (*Pa-aroZ*) (40), and *asbF* of the petrobactin biosynthesis pathway from *Bacillus thuringiensis* (*Bt-aroZ*) (15, 39). The corresponding protein sequences exhibit a very low overall homology of about 17% (data not shown). The coding sequences of *As-aroZ* and *Bt-aroZ* were amplified by PCR from genomic microbial DNA and cloned into high-copy-number yeast vector p423-HXT7. To further optimize gene expression, we additionally cloned a codon-optimized variant of the gene from *B. thuringiensis* (*Bt-aroZ*^{opt} [opt, optimized]). The gene from *P. anserina* (*Pa-aroZ*^{opt}) was expressed only in a codon-optimized version. To examine the performance of the different 3-DHS dehydratases, the respective plasmids were transformed into the CEN.PK2-1C yeast strain, the transformants were grown in selective SCD medium, and the production of PCA was assessed by

TABLE 3 Primers used in this study

Primer	5′–3′ sequence	Target[a]
Pyk2_f	CGCACCTATATCTGCGTGTTG	Plasmid pRS42K-Sh-aroY
pHXT7_r	TTTTTGATTAAAATTAAAAAAACTTTTTGT	Plasmid pRS42K-Sh-aroY
DecBsh_f	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGAGGCTAGTAATTGGAATTTC	Plasmid pRS42K-Sh-aroY
DecBsh_r	CGTGAATGTAAGCGTGACATAACTAATTACATGACTCGAGCTAATCATCTTTTGTCCCTTCC CAAGCC	Plasmid pRS42K-Sh-aroY
Tcyc_f	CTCGAGTCATGTAATTAGTTATG	Plasmid pRS42K-Sh-aroY
pPGK1_r	TGTTTTATATTTGTTGTAAAAAGTAG	Plasmid pRS42K-Sh-aroY
DecCsh_f	AAGGAAGTAATTATCTACTTTTTACAACAAATATAAAACAATGGCTAAAGTATACAAAGATTT	Plasmid pRS42K-Sh-aroY
DecCsh_r	AAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATTTATCTGTTTTGA TTTTTTAATAATTC	Plasmid pRS42K-Sh-aroY
tPGK_f	ATTGAATTGAATTGAAATCGATAGAT	Plasmid pRS42K-Sh-aroY
pTpI1_r	TTTTAGTTTATGTATGTGTTTTTTG	Plasmid pRS42K-Sh-aroY
DecDsh_f	TTAAATCTATAACTACAAAAAACACATACATAAACTAAAAATGAAATGTCATAGATGTGGCTC	Plasmid pRS42K-Sh-aroY
DecDsh_r	ATAAGGACATGGCCTAAATTAATATTTCCGAGATACTTCCCTATTTTTTCAA GGGTGGTATAGG	Plasmid pRS42K-Sh-aroY
tTal_f	GGAAGTATCTCGGAAATATTAAT	Plasmid pRS42K-Sh-aroY
tTal_r	ATATTAAGGGTTGTCGACCTGCAGCGTACGAAGTTTAAACGCTAGCCCTAGGGGCGCGCC GACGTTGATTTAAGGTGGTTCC	Plasmid pRS42K-Sh-aroY
DecCklp_f	AAGGAAGTAATTATCTACTTTTTACAACAAATATAAAACAATGACCGCTCCAATTCAAGAC	Plasmid pRS42K-Kp-aroY^{opt}
DecCklp_r	AAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATTACTTAGCAGAACCTTGGTTC	Plasmid pRS42K-Kp-aroY^{opt}
DecBklpopt_f	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGAAACTGATAATCGGGATGAC	Plasmid pRS42K-Kp-aroY$^{iso.opt}$
DecBklpopt_r	CGTGAATGTAAGCGTGACATAACTAATTACATGACTCGAGTTATTCGATTTCCTGAGCGA ATTGTTCAG	Plasmid pRS42K-Kp-aroY$^{iso.opt}$
DecCklpopt_f	AAGGAAGTAATTATCTACTTTTTACAACAAATATAAAACAATGGCCTTTGACGACCTTAGA	Plasmid pRS42K-Kp-aroY$^{iso.opt}$
DecCklpopt_r	AAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATTATTTACGATTGGCTAACATAGC	Plasmid pRS42K-Kp-aroY$^{iso.opt}$
DecDklpopt_f	TTAAATCTATAACTACAAAAAACACATACATAAACTAAAAATGATATGCCCAAGATGTGCC	Plasmid pRS42K-Kp-aroY$^{iso.opt}$
DecDklpopt_r	ATAAGGACATGGCCTAAATTAATATTTCCGAGATACTTCCTTATCTCTTATCTTCAG GTAAAAG	Plasmid pRS42K-Kp-aroY$^{iso.opt}$
catA_A.rad.opt_f	AAAAAGTTTTTTTAATTTTAATCAAAAAGTTAACATGCATATGACTGCAGCAAATGTTAAGA	Plasmid p425-HXT7-Ar-catA^{opt}
catA_A.rad.opt_r	GAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGACTTAGGCTTGCAATCT TGGTCTATC	Plasmid p425-HXT7-Ar-catA^{opt}
AroZ_B.th._opt_f	AAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAGTTAACATGAAGTACTCATTA TGCACTATC	Plasmid p423-HXT7-Bt-aroZ^{opt}
AroZ_B.th._opt_r	TATTAGTCAGTACTTTCATTTCAGCCTTCAAGATATCCTTTTAAGATGTGACAACT TCAAGATTTC	Plasmid p423-HXT7-Bt-aroZ^{opt}
AroZ_P.ans._opt_f	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGCCAAGTAAACTGGCTATTAC	Plasmid p423-HXT7-Pa-aroZ^{opt}
AroZ_P.ans._opt_r	ATTCTAATAGGTTGTTGAGTTGCCTGTTGTTGGGTAGGAGTTAAAGTGCGGCAGA TAGAGATAAC	Plasmid p423-HXT7-Pa-aroZ^{opt}
S1_ARO11359-1588_F	GCACAATTGGTGAAAGAAAAACTTTTGGACGGAAACAAGTGATTCGTACGCTGCAGGTCGAC	Strain CEN.PK2-1C$^{\Delta aroE}$
S2_ARO11359-1588_R	AAGCATTGTAAAATATAAAAAAGGATAGATATATTATTGTGCATAGGCCACTAGTGGATCTG	Strain CEN.PK2-1C$^{\Delta aroE}$
A1_ARO11359-1588_F	CTGTTTGTTGTTGGAAAGCCA	Strain CEN.PK2-1C$^{\Delta aroE}$
A4_ARO11359-1588_R	AACTTGTAATTATCTAACTGTTGC	Strain CEN.PK2-1C$^{\Delta aroE}$
CATA_F	AACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGAACAGGCAAGCTATCGAC	Plasmid p425-HXT7-Bx-catA
CATA_R	GAATGTAAGCGTGACATAACTAATTACATGACTCGAGTCAGGCTTCAGCGCGCAAA	Plasmid p425-HXT7-Bx-catA
aro1ΔE_ÜbrexS1	AACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGGTGCAGTTAGCCAAAGTC	Plasmid pRS42H-HXT7-ARO1$^{\Delta aroE}$
aro1ΔE_ÜbrexS2	GAATGTAAGCGTGACATAACTAATTACATGACTCGAGCTACTTGTTTCCGTCCAAAAGTTTT	Plasmid pRS42H-HXT7-ARO1$^{\Delta aroE}$
DHSase B.t_S1	AACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGAAATATTCACTATGTACCATT	Plasmid p423-HXT7-Bt-aroZ
DHSase B.t._S2	GAATGTAAGCGTGACATAACTAATTACATGACTCGAGTTAAGAAGTTACTACTTCTAAATTTC	Plasmid p423-HXT7-Bt-aroZ

[a] Plasmid or strain for the construction of which the primer was used.

HPLC analysis of the supernatants of batch cultures at different time points. However, only very low PCA levels (<7 mg/liter) could be detected even after cultivation for up to 120 h (Fig. 2 and data not shown). We could not detect higher PCA concentrations when the yeast cells were included in the PCA extraction process, indicating that PCA did not accumulate within the yeast cells.

As low production of PCA might be explained by the competition of the 3-DHS dehydratases with the 3-DHS dehydrogenase activity of the common aromatic amino acid biosynthesis pathway, we blocked the production of phenylalanine, tryptophan, and tyrosine at the level of 3-DHS by the elimination of 3-DHS dehydrogenase activity. In yeast, this reaction is mediated by the pentafunctional enzyme Arom (encoded by *ARO1*) (12, 13), which mediates the stepwise conversion of 3-DAHP to 5-enolpyruvyl-shikimate-3-phosphate via 3-DHS. Therefore, it was necessary to block only the 3-DHS dehydrogenase activity without affecting the dehydroquinate synthase and dehydroquinate dehydratase activities, which convert 3-DAHP to 3-DHS. The dehydrogenase domain of Arom, which mediates the conversion of 3-DHS to shikimate, has already been identified in a previous work by sequence alignment with the respective *aroE*-encoded enzyme from *E. coli* and is the last domain of yeast Arom (amino acids 1359 to 1588) (Fig. 3A) (12). The coding region of the whole dehydrogenase domain was replaced with a stop codon in wild-type strain CEN.PK2-1C by homologous recombination using the *cre-loxP-kanMX* system (Fig. 3A). After removal of the *kanMX* cassette, a single *loxP* site was left behind the truncated gene before the terminator region, resulting in strain CEN.PK2-1C$^{\Delta aroE}$. As expected, the strain turned out to be auxotrophic for aromatic amino acids (Fig. 3B).

To confirm that the other enzymatic activities of the truncated Arom enzyme, especially the dehydroquinate synthase and dehydroquinate dehydratase activities, were not affected, the aromatic amino acid auxotrophy of the mutant strain should be complemented by overexpression of the *aroE* gene from *E. coli* (Fig. 3B). In *E. coli*, the individual enzyme activities of Arom are encoded by

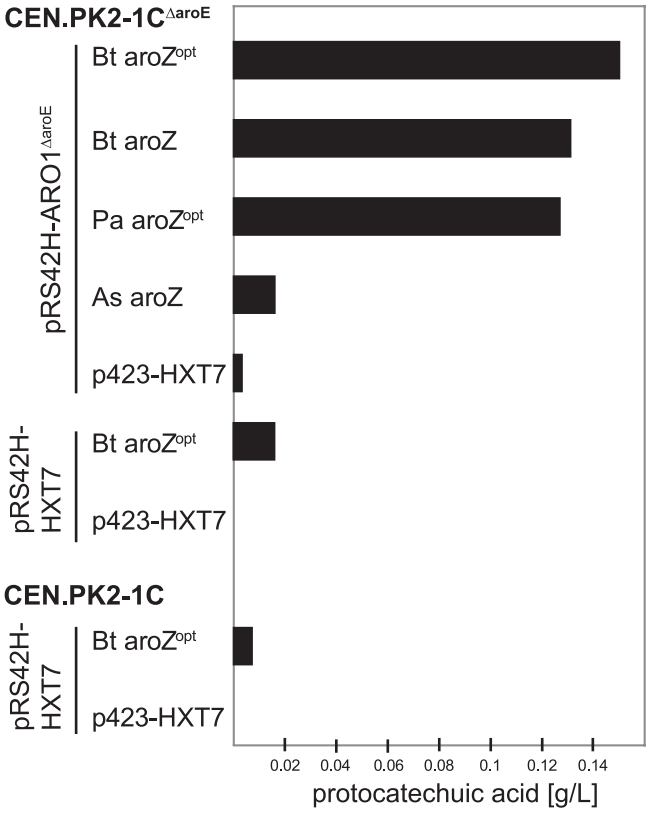

FIG 2 Analysis of PCA production with different 3-DHS dehydratases. To analyze the activities of the different 3-DHS dehydratases, the respective expression vectors and the empty control vector (p423-HXT7) were cotransformed with pRS42H-HXT7-ARO1$^{\Delta aroE}$ or the empty control vector into yeast wild-type strain CEN.PK2-1C or strain CEN.PK2-1C$^{\Delta aroE}$. The cells were grown in selective SCD medium for 120 h, and PCA was measured by HPLC analysis of the supernatants of the cultures. As the levels of PCA varied in different experiments, the results of only a representative experiment are shown.

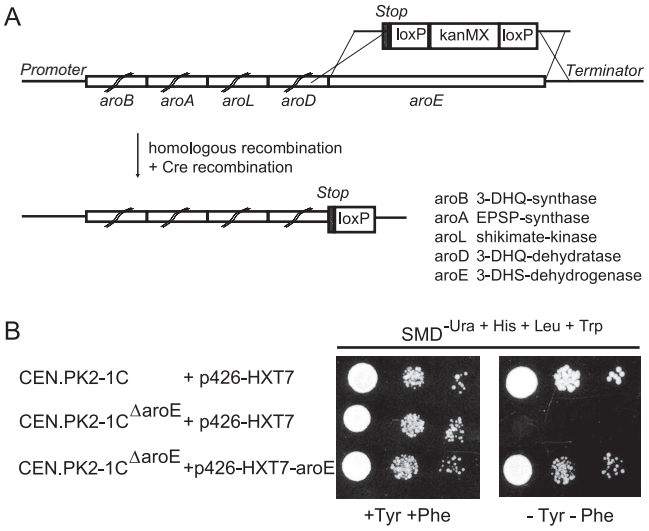

FIG 3 Deletion of the 3-DHS dehydratase domain of the Arom enzyme for optimized carbon flow into the CCM pathway. (A) Schematic representation of the *ARO1* gene, which encodes the pentafunctional enzyme Arom. The *E. coli* genes that encode the corresponding enzyme activities are indicated at the bottom. The AroE-encoding region was deleted with a *loxP-kanMX-loxP* cassette, introducing a stop codon after the AroD-encoding region. A residual *loxP* site remains in the terminator region after Cre-mediated recombination. 3-DHQ, 3-dehydroquinate; EPSP, 5-enolpyruvyl-shikimate-3-phosphate. (B) Complementation assay to verify that deletion of the AroE domain of Arom did not affect the enzymatic activities of the residual domains. Yeast cells transformed with either p426-HXT7-aroE or the respective empty vector were grown on SCD^{-Ura} medium to an OD_{600} of 1. Five-microliter volumes of diluted (1:1, 1:100, or 1:1,000) yeast cell suspensions were dropped onto the indicated agar plates on medium with or without tyrosine and phenylalanine. The plates were incubated at 30°C for 3 days.

separate coding regions. After transformation with the overexpression plasmid p426-HXT7-aroE or the respective empty control vector (p426-HXT7), the transformants were tested for the ability to grow on medium lacking tyrosine and phenylalanine. While the mutant transformed with the empty plasmid could not grow, the growth defect could be complemented by *aroE* from *E. coli*, indicating that the other domains of Arom$^{\Delta aroE}$ had not lost their activities (Fig. 3B).

To further increase the production of 3-DHS, the truncated *ARO1*$^{\Delta aroE}$ allele was cloned into an overexpression plasmid under the control of the strong *HXT7* promoter fragment, resulting in plasmid pRS42H-HXT7-ARO1$^{\Delta aroE}$. The various 3-DHS dehydratase overexpression plasmids were cotransformed with plasmid pRS42H-HXT7-ARO1$^{\Delta aroE}$ or the corresponding empty control vector pRS42H-HXT7 into strain CEN.PK2-1C$^{\Delta aroE}$. PCA production was measured by HPLC analysis of the supernatants of batch cultures grown in selective SCD medium (Fig. 2). The results show that the block of the aromatic amino acid biosynthesis pathway, together with overexpression of Arom$^{\Delta aroE}$, had a pronounced effect on PCA production levels. As in *E. coli*, the five enzymatic reactions of Arom are encoded on separate open reading frames. Alternatively, we also tested the overexpression of *aroB* (which encodes dehydroquinate synthase) and *aroD* (which encodes dehydroquinate dehydratase) from *E. coli* instead of *ARO1*$^{\Delta aroE}$ and found comparable PCA production levels (data not shown). The highest production rates were obtained with the 3-DHS dehydratases genes *Bt-aroZ*^{opt} and *Pa-aroZ*^{opt}. Interestingly, expression of *Bt-aroZ*^{opt} resulted in PCA levels only slightly higher than those obtained with the nonoptimized variant. In contrast, expression of *As-aroZ* resulted in the lowest PCA levels. On the basis of these results, *Bt-aroZ*^{opt} and *Pa-aroZ*^{opt} represent the most promising candidate genes for the heterologous CCM production pathway.

PCA decarboxylases. The conversion of PCA to catechol is catalyzed by nonoxidative 3,4-dihydroxybenzoate decarboxylases. These enzymes belong to the family of hydroxyarylic acid decarboxylases/phenol carboxylases and are composed of three different subunits encoded by the B, C, and D genes, which are usually organized in a cluster (33). Hydroxybenzoate-decarboxylating activities have been observed in various microorganisms. These enzymes differ greatly in substrate specificity, and just a few enzymes have been shown to specifically decarboxylate PCA (23, 24, 27, 31, 36, 38, 52). On the basis of the data in the literature, the enzymes from *Sedimentibacter hydroxybenzoicus*, *Enterobacter cloacae*, and *K. pneumoniae* seemed to be the most promising candidates for the CCM pathway, especially with respect to their substrate specificity. For our pathway, we chose the enzymes from *S. hydroxybenzoicus* and *K. pneumoniae*. The respective B, C, and D genes were cloned together into the high-copy-number yeast vec-

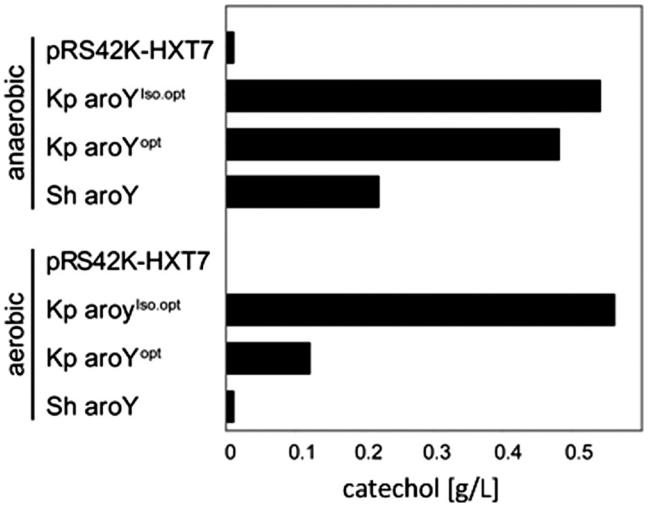

FIG 4 Analysis of PCA decarboxylase activities under anaerobic and aerobic conditions with externally added PCA. To analyze the activities of the different protocatechuate decarboxylases, the respective expression vectors and the empty control vector were transformed into wild-type strain CEN.PK2-1C. Yeast cells were grown in selective SCD medium supplemented with 5 mM PCA under aerobic and anaerobic conditions. Production of catechol was measured by HPLC analysis of the supernatants of the cultures after 120 h. As the levels of catechol varied in different experiments, the results of only a representative experiment are shown.

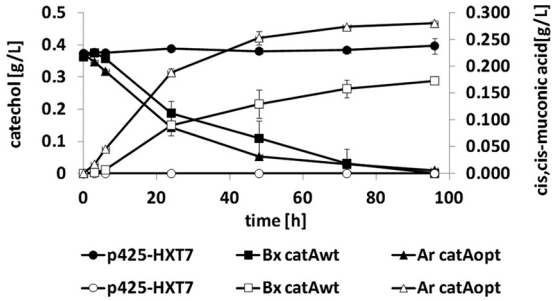

FIG 5 Analysis of catechol 1,2-dioxygenases with externally added catechol. To analyze the activities of the different catechol 1,2-dioxygenases, the respective expression vectors and the empty control vector were transformed into wild-type strain CEN.PK2-1C. The transformants were grown in selective SCD medium supplemented with 3.4 mM catechol. Conversion of catechol into CCM was measured by HPLC analysis of the supernatants of the cultures at up to 96 h. Fermentation experiments were performed three times by using the same precultures, and standard deviations are shown. wt, wild type.

tor pRS42K-HXT7. Each gene was controlled by its own promoter. The promoters were chosen from highly expressed yeast glycolytic genes (Table 2). For *S. hydroxybenzoicus* (*Sh-aroY*), we PCR amplified the B, C, and D genes directly from genomic DNA. In contrast, the *K. pneumoniae* (*Kp-aroY*) B, C, and D genes were cloned in synthetic, codon-optimized versions. We additionally performed detailed database searches and found another homolog for the C subunit encoded in the genome of *K. pneumoniae* (AB479384.1). This gene has been previously annotated by H. Ishioka and T. Sonoki in 2009, but experimental data have not been published. Our further analysis revealed that this gene is not organized in a BCD cluster. Sequence alignments showed that the protein sequence shares only up to 27% identity with the previously characterized C subunits of the BCD clusters of various organisms. However, typical core amino acids of the *aroY* C subunit are conserved (data not shown). Since it might represent a variant leading to an isomeric form of *aroY* with interesting properties, we included this subunit in our studies. We cloned this gene as a codon-optimized version together with the B and D subunit coding regions of the *K. pneumoniae* BCD cluster, resulting in the isomeric version named *Kp-aroY*$^{Iso.opt}$ (Iso, isoform).

To analyze the *in vivo* activities of the different enzymes, we performed fermentations in selective SCD medium with externally added PCA (5 mM) and growing cultures of CEN.PK2-1C transformed with the respective expression plasmids. Previous analysis indicated that several decarboxylases are oxygen sensitive (23, 24, 33, 48). To also test this possibility, we performed the experiments under aerobic and anaerobic conditions. The conversion of PCA to catechol was quantitatively monitored by HPLC analysis of the supernatants of the cultures (Fig. 4). Under anaerobic and aerobic conditions, yeast cells expressing *Kp-aroY*$^{Iso.opt}$ showed the highest catechol production rates and those expressing *Sh-aroY* showed the lowest ones. Under aerobic conditions, the respective overall activity of Sh-AroY was reduced almost completely whereas the activity of *Kp-aroY* was reduced 4.5-fold. These observations indicate that these proteins are oxygen sensitive. In contrast, *Kp-aroY*$^{Iso.opt}$ revealed no differences between the activities under the two conditions. Since the final reaction step in the CCM pathway from catechol to CCM requires molecular oxygen (see below) and because it had the highest conversion activity, the Kp-AroY$^{Iso.opt}$ variant represented the most promising candidate for the CCM pathway.

Catechol 1,2-dioxygenases. The conversion of catechol to CCM can be mediated by catechol 1,2-dioxygenases. These enzymes catalyze the intradiol cleavage of catechol by incorporation of molecular oxygen. By using sequence database analysis, we identified a *catA* gene in *Burkholderia xenovorans* (*Bx-catA*) (38). The gene was cloned by PCR amplification from genomic DNA of *B. xenovorans* and subsequent homologous recombination into the vector p425-HXT7. However, numerous 1,2-dioxygenases have been purified and characterized and it was shown that most of these enzymes have a broad substrate specificity, including activity on PCA (6, 44). This side activity would be undesirable for our intended CCM production pathway. Thus, we additionally cloned a codon-optimized version of *catA* from *Acinetobacter radioresistens* (*Ar-catA*^{opt}). The corresponding enzyme has been shown to have no unspecific activity on PCA (5, 7, 8). To verify its expression, as well as its activity with catechol, we performed fermentations with transformed CEN.PK2-1C cells in selective SCD medium with externally added catechol (about 3.4 mM) and measured the levels of catechol and CCM in the supernatants of the cultures. The HPLC analyses showed that both enzymes were actively expressed and able to convert catechol to CCM (Fig. 5). In contrast, no CCM was produced by cells with the empty control vector. As expected, the codon-optimized gene from *A. radioresistens* encoded the highest CCM production activity. Catechol was not quantitatively converted into CCM, which might indicate the formation of side products. To exclude the possibility that the enzymes have activity on PCA, we performed control experiments using externally added PCA (about 5 mM). For both enzymes, we did not observe any consumption of PCA (data not shown). On the basis of these results, both enzymes should be applicable for use in the CCM production pathway.

***De novo* production of CCM from glucose.** Our analysis iden-

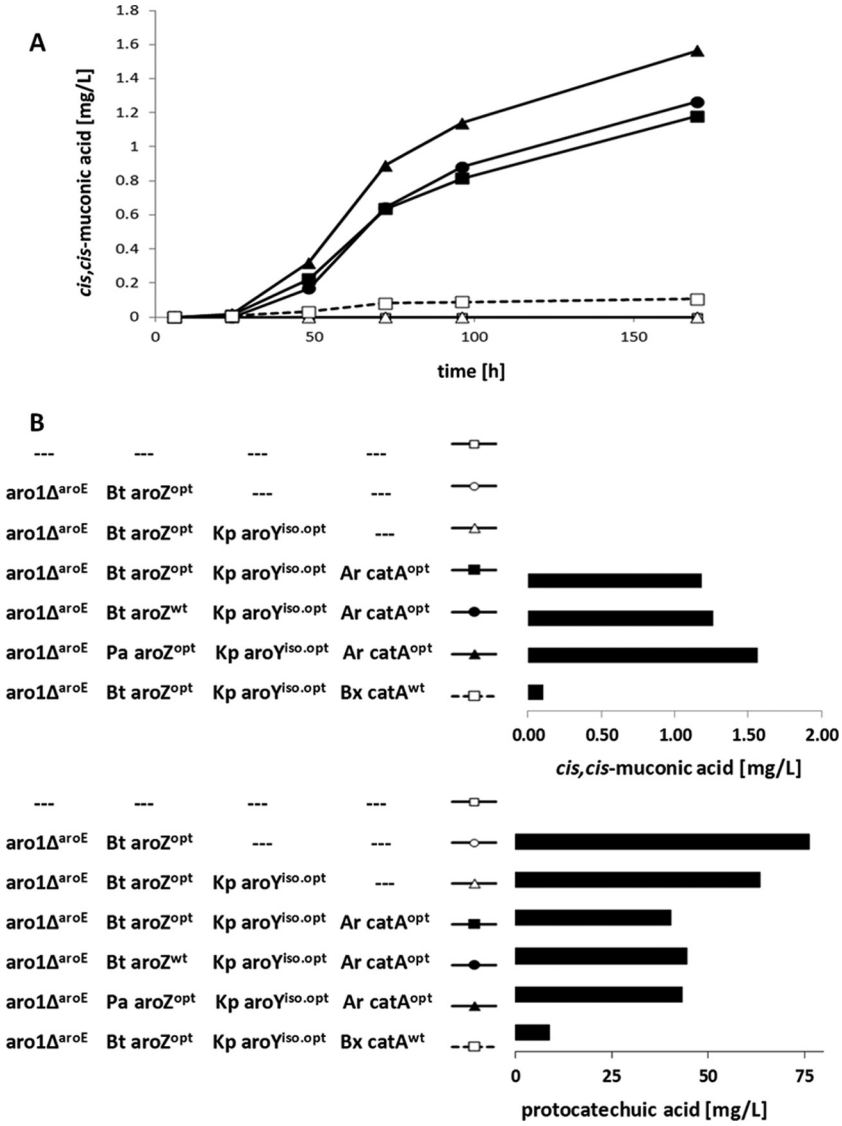

FIG 6 Analysis of *de novo* synthesis of CCM from glucose. For the expression of different enzyme combinations, the expression plasmids and respective empty control vectors were transformed into the CEN.PK2-1C$^{\Delta aroE}$ strain. Transformants were grown in selective SCD medium for up to 170 h. (A) The concentrations of CCM in the supernatants of the cultures were determined at different time points by HPLC analysis. (B) PCA and CCM levels after 170 h. As the levels of CCM varied in different experiments, the results of only a representative experiment are shown. wt, wild type.

tified promising enzymes that could be potentially combined in a CCM production pathway. To test this possibility, we transformed the expression vectors for the respective enzymes or their corresponding empty control vectors in different combinations into strain CEN.PK2-1C$^{\Delta aroE}$. We further cotransformed the vector pRS42H-HXT7-ARO1$^{\Delta aroE}$ to ensure high-level synthesis of the precursor 3-DHS. The different transformants were grown in batch cultures with selective SCD medium under aerobic conditions, and the levels of CCM in the supernatants of the cultures were measured by HPLC analysis (Fig. 6). Our analysis revealed that the highest production rates were obtained by the coexpression of Pa-AroZ^{opt}, Kp-AroY$^{Iso.opt}$, and Ar-CatA, which resulted in a final concentration of 1.56 mg/liter CCM after 170 h. In contrast, the combinations that included Sh-AroY revealed no detectable CCM production (data not shown) whereas the combinations with Kp-AroY^{opt} revealed very low CCM levels (up to 0.1 mg/liter). This observation can be explained by the presence of oxygen during the fermentations and is consistent with our analysis of the individual enzymes, which revealed the oxygen sensitivity of both enzymes (Fig. 4). Variation of the 3-DHS dehydratase isoforms had only slight effects on the CCM levels. This is in line with our observations above, which indicated only minor differences between the different enzymes (Fig. 2). In contrast, variation of the catechol 1,2-dioxygenase isoforms led to reduced CCM levels when Bx-CatA^{wt} was used. This observation was expected, as our previous analysis revealed higher activity of the optimized enzyme from *A. radioresistens*.

We further analyzed whether precursors of CCM accumulated in the broth (Fig. 6B). We detected high levels of the precursor PCA in nearly all fermentations with cells expressing 3-DHS dehydratase (AroZ) activity, indicating a bottleneck in AroY or CatA activity. As expected, the highest PCA level was observed in strains

expressing only Bt-AroZ^{opt}, while additional expression of Kp-AroY$^{Iso.opt}$ slightly reduced PCA levels. Although this might indicate further conversion of PCA into catechol, catechol was not detectable. Catechol was not detectable in any of the fermentations, irrespective of the different enzyme combinations. Also surprisingly, expression of Bx-CatA^{wt} together with the other enzymes resulted in not only lower levels of CCM but also lower levels of PCA. These results might be interpreted as meaning that either the different enzymes influence each other when expressed simultaneously or that side products which could not be measured with our HPLC analysis are synthesized. Nevertheless, our results show that it is possible to establish a CCM pathway in *S. cerevisiae*.

DISCUSSION

In this study, we demonstrate the first *de novo* production of CCM from glucose in *S. cerevisiae*. By coexpression of three bacterial enzymes, we assembled a heterologous production pathway that is similar to the previously established pathway in *E. coli* (38). Our analysis revealed that one key step in the successful engineering of CCM production in *S. cerevisiae* was the block of the aromatic amino acid synthesis pathway on the level of 3-DHS by deletion of the AroE domain of the pentafunctional Arom enzyme. This allows the conclusion that the heterologous 3-DHS dehydratases are in competition with the 3-DHS dehydrogenase reaction of the aromatic amino acid pathway or that increased levels of 3-DHS favor its conversion into PCA via 3-DHS dehydratases. However, in order to significantly increase the production of PCA, in addition to the deletion of the AroE domain of *ARO1* in the genome, the gene for the truncated enzyme with the residual enzyme activities (Arom$^{\Delta aroE}$) (Fig. 2) or the *E. coli aroB* and *aroD* genes had to be overexpressed. These results suggest either that the conversion of 3-DAHP to 3-DHS represents a limiting step in the aromatic amino acid synthesis pathway or that the deletion of the AroE domain at least partially affected the activity of the residual domains.

The different oxygen requirements of AroY and CatA represent a very critical step in the CCM pathway. Whereas molecular oxygen is essential for the final reaction step mediated by CatA, oxygen sensitivity has been reported for several AroY enzymes (23, 24, 33, 48). In agreement with these previous reports, we also observed oxygen sensitivity of Sh-AroY and Kp-AroY (Fig. 4). Whereas Sh-AroY completely lost its activity in the presence of oxygen, Kp-AroY lost at least 80%. Moreover, as we used only oxygen-limited conditions, the activities of both enzymes under strictly anaerobic conditions are probably even higher. By the way, it should be noted that it is not possible to directly compare the activities of Sh-AroY and Kp-AroY, as only in the latter case did we use a codon-optimized gene. AroY is composed of three different subunits which normally are encoded by three open reading frames organized into respective BCD clusters. Interestingly, we found another putative C subunit in the genome of *K. pneumoniae* which is not part of a BCD cluster. By using this alternative C subunit, we could establish a new enzyme complex (Kp-AroY$^{Iso.opt}$) which did not show oxygen sensitivity. Our results indicate that this alternative C subunit can replace the original C subunit in the *aroY* BCD cluster of *K. pneumoniae*. Thus, it is most likely that it is the C subunit which confers oxygen sensitivity on the AroY complex. Moreover, even under oxygen-limited conditions, Kp-AroY$^{Iso.opt}$ with the alternative C subunit performed slightly better than Kp-AroY with the original C subunit (Fig. 4). Therefore, in an industrial production process, this new isoform would be highly advantageous because the oxygen level would not need to be controlled so strictly. Interestingly, Niu et al. (38) also used an AroY enzyme from *K. pneumoniae* for the construction of a CCM pathway in *E. coli*. In their fermentation experiments, the oxygen level was kept at 10% dissolved oxygen air saturation, which was sufficient for the catechol 1,2-dioxygenase reaction and obviously had no deleterious effects on AroY of *K. pneumoniae*. Unfortunately, it is not clear which C subunit was used in their study.

Two different isoforms of CatA were tested in our study, one from *B. xenovorans* and the other a codon-optimized version from *A. radioresistens*. Whereas the yeast strain expressing no CatA activity did not consume any catechol and did not produce CCM, yeast cells containing either of the two heterologous isoenzymes consumed catechol at an initial concentration of 0.37 g/liter (3.4 mM) completely in about 100 h and in the case of Ar-CatA produced up to 0.28 g/liter (2.0 mM) CCM. Surprisingly, although all of the catechol was also consumed in the case of Bx-CatA, only 0.19 g/liter (1.3 mM) CCM was produced. The only explanation for the incomplete conversion of catechol into CCM would be either that the enzymes can also convert catechol into another product which was not detectable in our HPLC analyses or that their expression induces an activity in yeast that turns catechol into another product. This could also explain the observation that we could never detect catechol in the CCM fermentations, even when the conversion of PCA into catechol should be increased. Even more surprisingly, expression of Bx-CatA, compared to Ar-CatA, together with AroZ and AroY in the complete CCM pathway resulted not only in strongly reduced CCM production levels but also in very low PCA levels (Fig. 6).

When we combined the most promising enzymes into a complete CCM pathway in yeast, the yeast cells could indeed produce CCM. However, it is obvious that there are several bottlenecks in the pathway and that the capacity of carbon flow into and within the pathway must still be optimized substantially. Moreover, as we compared codon-optimized with nonoptimized genes, the choice of the individual enzymes need not necessarily reflect the best combination. The first revealing observation is the accumulation of PCA in the supernatant (Fig. 6) whereas no catechol could be detected. This suggests that either the conversion of PCA to catechol by AroY represents a rate-limiting step or that PCA is secreted out of the cells before it can be converted by AroY. Indeed, throughout our analyses, we observed that all of the intermediates and products of the CCM pathway could be detected in the supernatants of the cultures, and we could not find any indication that they accumulated in the cells. As mere diffusion of the compounds across the plasma membrane is unlikely, this suggests that they can be transported by yeast plasma membrane transporters. The draining of intermediates out of the cells clearly reduces the final CCM production levels. To circumvent this problem, either the transporters responsible must be identified and the genes that encode them deleted or enzymes with higher activities and higher affinities must be found.

Another problem, obviously, is end product repression and feedback inhibition of the committing step in the aromatic amino acid biosynthesis pathway, catalyzed by DAHP synthase (encoded by *ARO3* and *ARO4*) (25, 41). As the AroE domain of the Arom enzyme was deleted from our yeast strain, the resulting auxotrophy had to be complemented by the addition of aromatic amino

acids. These probably strongly reduced the expression and activity of DAHP synthase. Interestingly, it was shown previously (34) that genetic modifications leading to feedback-resistant mutant forms of DAHP synthase revealed a >4-fold increase in flux through the aromatic pathway with a >100-fold increase in the extracellular levels of aromatic compounds. Therefore, the overexpression of such mutant forms of DAHP synthase is a very promising strategy by which to further increase CCM yields. In addition, as was also shown in *E. coli* (10), increasing the supply of the substrates of the aromatic pathway, PEP and E4P, might further increase CCM production.

Nevertheless, compared to the CCM production titers in *E. coli* of up to 36.8 g/liter (38), the production titers obtained here with *S. cerevisiae* are much lower. In general, metabolic engineering of *S. cerevisiae* seems to be much more difficult than that of *E. coli* (29), as the metabolic network of *S. cerevisiae* is more rigidly resistant to modifications. This has also been observed, e.g., for the production of isobutanol (3), 1-butanol (43), and biodiesel (42). Much more effort is needed to finally achieve industrially relevant productivity (50). On the other hand, *S. cerevisiae* has several advantages in industrial production processes, especially in the production of organic acids at low pH values (26, 32, 50). Accordingly, we did not observe any toxic effects of CCM on *S. cerevisiae* cells up to its solubility level of 200 mg/liter at pH 3 to 6 (data not shown). Altogether, our results might pave the way for the engineering of *S. cerevisiae* for the industrial production of CCM and its further chemical hydrogenation into adipic acid.

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