Elimination of aromatic fusel alcohols as by-products of *Saccharomyces cerevisiae* strains engineered for phenylpropanoid production by 2-oxo-acid decarboxylase replacement

Else-Jasmijn Hassing, Joran Buijs, Nikki Blankerts, Marijke A. Luttik, Erik A.de Hulster, Jack T. Pronk, Jean-Marc Daran

Department of Biotechnology, Delft University of Technology, van der Maasweg 9, 2627 HZ, Delft, the Netherlands

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**ABSTRACT**

Engineered strains of the yeast *Saccharomyces cerevisiae* are intensively studied as production platforms for aromatic compounds such as hydroxycinnamic acids, stilbenoids and flavonoids. Heterologous pathways for production of these compounds use L-phenylalanine and/or L-tyrosine, generated by the yeast shikimate pathway, as aromatic precursors. The Ehrlich pathway converts these precursors to aromatic fusel alcohols and acids, which are undesirable by-products of yeast strains engineered for production of high-value aromatic compounds. Activity of the Ehrlich pathway requires any of four S. cerevisiae 2-oxo-acid decarboxylases (2-OADCs): Aro10 or the pyruvate-decarboxylase isoenzymes Pdc1, Pdc5, and Pdc6. Elimination of pyruvate-decarboxylase activity from *S. cerevisiae* is not straightforward as it plays a key role in cytosolic acetyl-CoA biosynthesis during growth on glucose. In a search for pyruvate decarboxylases that do not decarboxylate aromatic 2-oxo-acids, eleven yeast and bacterial 2-OADC-encoding genes were investigated. Homologs from *Kluyveromyces lactis* (KLPDC1), *Kluyveromyces marxianus* (KmpDC1), *Yarrowia lipolytica* (YlpDC1), *Zymomonas mobilis* (Zmpdc1) and *Glucanacetobacter diazotrophicus* (Gdpdc1.2 and Gdpdc1.3) complemented a Pdc− strain of *S. cerevisiae* for growth on glucose. Enzyme-activity assays in cell extracts showed that these genes encoded active pyruvate decarboxylases with different substrate specificities. In these in vitro assays, Zmpdc1.2 and Gdpdc1.3 had no substrate specificity towards phenylpyruvate. Replacing Aro10 and Pdc1,5,6 by these bacterial decarboxylases completely eliminated aromatic fusel-alcohol production in glucose-grown batch cultures of an engineered coumaric acid-producing *S. cerevisiae* strain. These results outline a strategy to prevent formation of an important class of by-products in ‘chassis’ yeast strains for production of non-native aromatic compounds.

1. Introduction

The aromatic amino acids L-phenylalanine and L-tyrosine are precursors of many industrially relevant compounds belonging to the phenylpropanoid family of aromatic compounds (Neelam et al., 2020), including hydroxycinnamic acids (Vannelli et al., 2007), stilbenoids (Trantas et al., 2009) and flavonoids (Falcone Ferreyra et al., 2012). These compounds have diverse applications in the food, chemical, pharmaceutical and cosmetic industries (Neelam et al., 2020). Current production processes mostly depend on petroleum-based chemical processes (Das et al., 2007) or direct extraction from plants (Trantas et al., 2015). However, the chemical processes involved are often inefficient and unsustainable (Chemler and Koffas, 2008; Bhan et al., 2013; Zha et al., 2019) while plant extraction processes are limited by biomass availability, low extraction yields and low purity of the final products (Zhang, 2007; Rodriguez et al., 2017). To overcome these pitfalls and meet the increasing demand for biologically and renewably sourced aroma and flavour compounds, microbial production from renewable feedstocks offers a promising alternative (Trantas et al., 2015).

Development of microbial platforms for *de novo* production of aromatic compounds has been intensively studied in the yeasts *S. cerevisiae* and *Y. lipolytica* (Liu et al., 2020). The yeast shikimate pathway for aromatic amino-acid biosynthesis, a focal point in these metabolic engineering studies, is initiated by condensation of phospho-enol-pyruvate...
(PEP) and erythrose-4-phosphate (E4P) to form 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP). This seven-carbon intermediate is converted into chorismate via a series of biochemical reactions (Fig. 1). At chorismate, the pathway for L-tryptophan synthesis branches off. For biosynthesis of L-phenylalanine and L-tyrosine, chorismate is converted to prephenate, from which either phenylpyruvate or p-hydroxyphenylpyruvate are formed. Transamination of these two aromatic 2-oxo acids then yields L-phenylalanine and L-tyrosine, respectively.

In addition to high-level functional expression of heterologous pathway enzymes (Liu et al., 2016, 2019; Yang et al., 2020), elimination of allosteric feed-back inhibition of the shikimate-pathway enzymes DHAP synthase (Aro3 and Aro4) and chorismate mutase (Aro7) (Hartmann et al., 2003; Krappmann et al., 2000; Luttik et al., 2008; Reifenth and Boles, 2018; Schnappauf et al., 1998), increasing the capacity of the shikimate pathway (Koopman et al., 2012; Paddon et al., 2013; Liu et al., 2016, 2019; Rodríguez et al., 2017; Levinson et al., 2018; Gu et al., 2020; Palmer et al., 2020; Sáez-Sáez et al., 2020; Yang et al., 2020) and improving supply of its precursors PEP and E4P (Liu et al., 2019; Gu et al., 2020; Yang et al., 2020) have enabled increased titers and yields of phenylpropanoid in S. cerevisiae. However, these metabolic engineering strategies also lead to increased formation of aromatic fusel alcohols (2-phenylethanol, p-hydroxyphenylethanol) (Koopman et al., 2012; Levinson et al., 2018; Liu et al., 2019) and fusel acids (phenylacetic acid, p-hydroxyphenylacetic acid) (Gu et al., 2020). During production of high-value phenylpropanoid such as hydroxycinnamic acids, stilbenoids and flavonoids, formation of these undesired by-products represents a drain of precursors and goes at the expense of product titers and yields.

Fusel alcohols and acids are formed via the Ehrlich pathway for degradation of branched-chain, aromatic, and sulfur-containing amino acids (Ehrlich, 1907; Hazelwood et al., 2008). In the Ehrlich pathway, transamination of amino acids yields the corresponding 2-oxo acids, which are subsequently decarboxylated. The resulting aldehyde is then either oxidised or reduced by yeast aldehyde dehydrogenases and alcohol dehydrogenases to yield fusel acids and fusel alcohols, respectively (Hazelwood et al., 2008). The irreversible decarboxylation reaction in the Ehrlich pathway is catalysed by thiamine-pyrophosphate-dependent 2-oxo acid decarboxylases (2-OADC), which in S. cerevisiae are encoded by PDC1, PDC5, PDC6 and ARO10. Pdc1, Pdc5 and Pdc6 show a preference for the linear-chain 2-oxo acids pyruvate, 2-oxobutyrate and 2-oxopentanoate (Romagnoli et al., 2012), while Aro10 shows no activity with linear chain 2-oxo acids, but does decarboxylate branched-chain and aromatic 2-oxo acids at high rates (Vuralhan et al., 2003, 2005; Romagnoli et al., 2012). Aro10 is a main contributor to 2-phenylethanol production by S. cerevisiae (Vuralhan et al., 2003; Romagnoli et al., 2012; Hassing et al., 2019), but Pdc5 also shows a distinct activity with phenylpyruvate (Romagnoli et al., 2012). Strains expressing heterologous pathways for flavonoid production from which ARO10, PDC5 and PDC6 were deleted, still exhibited residual 2-phenylethanol formation, indicating that Pdc1 still decarboxylated 2-phenylpyruvate at low rates (Koopman et al., 2012).

Deletion of the three pyruvate-decarboxylase genes PDC1, PDC5, PDC6 renders S. cerevisiae unable to grow on glucose in batch cultures. During aerobic glucose-limited growth, Pdc- strains are auxotrophic for Cα-compounds (ethanol or acetate) due to involvement of pyruvate decarboxylases in cytosolic acetyl-CoA synthesis (Flikweert et al., 1996, 1999). Although metabolic engineering strategies have been published to bypass this acetyl-CoA requirement (reviewed by Van Rossum et al. (van Rossum et al., 2016)) and to mitigate the glucose sensitivity of Pdc- strains (Oud et al., 2012), deletion of all four 2-OADC genes in S. cerevisiae is not a straightforward strategy to eliminate formation of aromatic fusel alcohols and acids. Still, complete elimination of these by-products, without negative impacts on growth on glucose or a need to rewire central carbon metabolism, would be an attractive attribute of S. cerevisiae ‘chassis’ strains for production of phenylpropanoid.

The goal of the present study was to identify heterologous pyruvate decarboxylases that show activity with pyruvate but not with aromatic 2-oxo acids and to investigate whether they can functionally replace the native yeast enzymes and thereby prevent formation of aromatic Ehrlich-pathway products. To this end, a set of 11 2-oxo acid decarboxylases from yeasts (K. lactis, K. marxianus and Y. lipolytica) and bacteria (Z. mobilis and G. diazotrophicus) were expressed in a 2-OADC-deficient S. cerevisiae strain. Enzyme assays with cell extracts of the resulting strains were used to assess substrate specificities and affinities of each of the decarboxylases for pyruvate and phenylpyruvate. Subsequently, they were used to replace the native 2-OADC in a S. cerevisiae strain engineered for the production of the phenylpropanoid compound, coumaric acid. The impact of replacing the native yeast 2-OADC with the two best-performing heterologous pyruvate-decarboxylases was evaluated in aerobic, pH-controlled bioreactor cultures.
2. Material and methods

2.1. Strains and growth media

*S. cerevisiae* strains used in this study were derived from the CEN.PK lineage (Entian and Köter, 2007; Salazar et al., 2017) (Table 1). Escherichia coli XL1 blue (Agilent Technologies, Santa Clara, CA) was used for plasmid propagation and storage. *S. cerevisiae* and *E. coli* strains were stored at −80 °C as described previously (Mans et al., 2015). Complex YP (yeast extract/peptone) medium was prepared and sterilized as described previously (Mans et al., 2015) and, when required, was supplemented with 200 mg L\(^{-1}\) hygromycin (InvivoGen, San Diego, CA). As a carbon source, YP medium was supplemented with either 20 g L\(^{-1}\) glucose (YPD) or a mixture of 2% (v/v) glycerol and 2% (v/v) ethanol.

Table 1

| Strain | Description | Genotype | Reference |
|--------|-------------|----------|-----------|
| CEN.PK113-7D | MATa MAL2-8c SUC2 | Entian and Köter (2007) |
| IMX2668 | pUDE1037 | Uralhan et al. (2015) |
| IMX2668 | pUDE1089 | Salazar et al. (2017) |

*Note:* *S. Saccharomyces cerevisiae, Km Kluyveromyces marxianus, K* Klykovermyces lactis, Y Yarrowia lipolytica, Gd Gluconacetobacter diazotrophicus, Zm Zymomonas mobilis, Rc Rhodobacter capsulatus, Pm Photobacterium luminescens, Co codon optimized, pr promoter, t terminator, pdc pdc5a pdc6a aro10a, Pd pdc1a pdc6a pdc8a aro10a, 3ABP aromatic acid biosynthetic pathway, COUM coumaric acid, FBR feedback resistant, 2 µm multicopy.*
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supplemented with either 20 g L\(^{-1}\) (YPEG) (Mans et al., 2017). Chemically defined synthetic medium (SM) containing mineral salts, trace elements and vitamins was prepared and compared by adding 20 g L\(^{-1}\) (SME) or a mixture of 2% (v/v) glycerol and 2% (v/v) ethanol (SMEG) (Mans et al., 2017). When required, 150 mg L\(^{-1}\) uracil (Sigma-Aldrich, St Louis, MO) (Prong, 2002) was supplied to synthetic media. Lysogeny broth (LB) for growth of E. coli strains was prepared as described by Bertani et al. (Bertani, 1951) and supplied with 25 mg L\(^{-1}\) chloramphenicol (Sigma-Aldrich), 100 mg L\(^{-1}\) ampicillin (Sigma-Aldrich) or 50 mg L\(^{-1}\) kanamycin (Sigma-Aldrich) as required. Solid media were prepared by adding 20 g L\(^{-1}\) Bacto Agar prior to autoclaving.

2.2. Molecular biology techniques

DNA templates for cloning were amplified with Phusion high-fidelity polymerase (Thermo Fisher Scientific, Landsmeer, Netherlands) according to manufacturer’s protocol, with the exception that a primer concentration of 200 nM and 0.04 μM of polymerase was used. The YeastStar genomic DNA kit (Zymo Research, Irvine, CA) was used to isolate genomic DNA as template for PCR amplification. The Zymoclean kit (Zymo Research) was used to purify PCR products by gel purification according to manufacturer’s recommendations using milliQ water as eluent. Alternatively, PCR products were first incubated for 1 h with DpnI FastDigest enzyme (Thermo Fisher Scientific) to digest template DNA and subsequently purified using the GenElute\textsuperscript{TM} PCR clean-Up Kit (Sigma-Aldrich). Diagnostic PCR was performed with DREAM Taq PCR mastermix (Thermo Fisher Scientific) and with oligonucleotide primers shown in Table S1. The GenElute plasmid mini prep kit (Sigma-Aldrich) was used to isolate plasmids from E. coli.

2.3. Construction of plasmids and expression cassettes

Plasmids used and constructed in this study are shown in Table 2. Constructed plasmids were transformed to E. coli (XL1-Blue) cells according to the supplier’s recommendations and grown under selective conditions.

Plasmids containing gRNAs for Cas9-mediated genome editing were constructed as described by Mans et al. (2015). The resulting gRNA plasmids pUDR406 (gRNA-PDCS/PDC6 and gRNA-ARO10), pUDR470 (gRNA-PDCX 2x) and pUDR599 (gRNA-X3 2x) (Hassing et al., 2019) were used to target PDC5, PDC6, PDC1, ARO10 and the X3-locus (Mikkelsen et al., 2012), respectively.

The expression cassettes used in this study were constructed using the Yeast Toolkit (Lee et al., 2015). In brief, promoter, gene and terminator fragments (parts) are amplified with part type specific overhangs containing restriction sites (BsmBI and BsaI). Using Golden Gate assembly, overhangs containing restriction sites (BsmBI and BsaI). Using Golden Gate assembly, the individual parts are initially assembled in an universal entry vector, resulting in a part plasmid. Next, a promoter, gene and terminator part are assembled to an expression cassette using BsmBI-mediated golden gate assembly, resulting in an expression cassette containing a transcriptional unit.

Initially, DNA fragments carrying the CYC1 terminator (CYC1t) and FBA1 promoter (FBA1pr) fragments were amplified from genomic DNA from S. cerevisiae CEN.PK113-7D with oligonucleotide primers 14039/14040 and 9419/9420 adding terminator (ATCC and CAGG) or promoter (AAGG and ATAC) part type specific overhangs (Table S1) (Lee et al., 2015). Open reading frames of genes from S. cerevisiae CEN.PK113-7D (Emtian and Kötter, 2007), Kluyveromyces marxianus NRRL 17777 (NITE Biological Resource Center, Japan) (Inokuma et al., 2015) or Kluyveromyces lactis CBS 2359 (Jugrens et al., 2018) were amplified from genomic DNA using primers with gene-pair type specific overhangs (TAAT and GGAT) (Table S1). Primers ASR_A023F/ASR_A023Rcorr, 13940/13941 and 16851/16852 and 17630/17631 were used to amplify KmPDC1, KIARO10, ScPDC1 and ScTHI3 respectively. The ORFs of KIPDC5, KIPDC1, KmPDCs and KmARO10 were amplified in several fragments to remove internal Bsal and BsmBI sites from the coding sequence (Hassing et al., 2019) using primer pairs 13932/13933 and 13934/13935 for KIPDC5, 13939/14138, 14137/13938 and 13937/13936 for KIPDC1, ASR_A024F/ASR_A024RM and ASR_A024MF/AR_A024Rcorr for KmPDC5 and ASR_A022F/ASR_A022MR and ASR_A022MF/ASR_A022Rcorr for KmARO10. CorRcatl was amplified using pUD1069 (Koopman et al., 2012) as template with primers 17825/17826. Correct removal of the internal Bsal/BsmBI sites of KmPDC5, KmARO10, KIPDC5 and KIPDC1 was confirmed by Sanger sequencing (BaseClear, Leiden, Netherlands). A codon-optimized, based on yeast glycolytic codon usage (Wiedemann and Boles, 2008), open reading frame of the phenylalanine lyase gene from Photorhabdus luminescens, CoPlsLA, was amplified from plasmid MV14 (CoPlsLA) (Gottardi et al., 2017) with primers ASR_N009F/ASR_N009R. Codon optimization of the Gdpdc1 and Zmpdc1 coding regions was performed using the Jcat Codon Adaptation Tool (Grote et al., 2005). The codon regions were custom-synthesized by Invitrogen GeneArt (Thermo Fisher Scientific) service. The sequence of Zmpdc1 was derived from the annotated genome of strain Zymomonas mobilis subsp. mobilis ATCC 10988 (Bioproject accession number PRJNA309987) (Pappas et al., 2011). Since three different sequences of Gdpdc1 have been reported for Gluconacetobacter diazotrophicus strain ATCC 49037, codon-optimized coding sequences for Gdpdc1.1 (van Zyl et al., 2014), Gdpdc1.2 (Bertalan et al., 2009) and Gdpdc1.3 (Giongo et al., 2010) were separately synthesized. Coding sequences were flanked upstream and downstream with the gene specific Yeast Toolkit flanks ‘AAGCATGCTCAGTCGCTCAT’ and ‘TTATGCCCTCACAGTCCAGAT’ respectively (Lee et al., 2015).

The amplified and synthesized fragments of CYC1t, KmARO10, KmPDC1, KmPDC5 and CoPlsLA were cloned into entry vector pYTko001 (Lee et al., 2015), via BsmBI Golden Gate assembly, obtaining part plasmids pGGKp182 (CYC1t), pGGKp183 (KmARO10), pGGKp184 (KmPDC1), pGGKp185 (KmPDC5) and JA_NM 1Sc_coPlsLA (CoPlsLA). FBA1pr, Zmpdc1, Gdpdc1.1-3, KIPDC1, KIARO10, ScPDC1, KIPDC5, CoRcatl and ScTHI3 were also assembled via BsmBI Golden Gate assembly into an entry vector pUD565 (Boonekamp et al., 2018), resulting in part plasmids pGGKp027 (FBA1pr), pGGKp211 (Zmpdc1), pGGKp212 (Gdpdc1.1), pGGKp213 (KIPDC5), pGGKp214 (KIARO10), pGGKp254 (Gdpdc1.2), pGGKp255 (Gdpdc1.3), (KIPDC1), pGGKp315 (ScTHI3), pGGKp327 (CoRcatl) and pGGKp337 (ScPDC1). Part plasmids were confirmed by colony PCR using primers 2012 and 2397 for the pUD565 entry vector and with primers 14036 and 14977 for YTK001 entry vector.

The GFP dropout plasmid pGGKd017 (URA3) (Wronska et al., 2020) was used as backbone to construct expression cassettes expressing a single 2-oxo acid decarboxylase. As example, the Golden Gate assembly of pGGKp035 (TDH3pr), pGGKp182 (CYC1t) and pGGKp211 (Zmpdc1) using pGGKd017 as a backbone resulted in the construction of pUD827 (URA3, TDH3pr-Zmpdc1-CYC1t). A full overview of all part plasmids that were used to construct the expression cassettes is presented in Table 2. Correct construction was verified by diagnostic PCR and restriction analysis.

Additionally, a multi-expression cassette plasmid expressing ScPDC1 and ScTHI3 was constructed. For this purpose, three additional GFP dropout plasmids were first constructed. The part plasmids pYTko02 and pYTko067 (ConLS and ConRL connectors), pYTko047 (GFP dropout), pYTko074 (URA3), pYTko078 (CoPlsLA) were assembled via BsmBI Golden Gate assembly and expressed as described by van Zyl et al. (2014), resulting in pGGKd071 (multigene cassette #1). Additionally pYTko03 and pYTko072 (ConLS and ConRL connectors), pYTko047, pYTko074, pYTko082 were assembled via BsmBI Golden Gate assembly (Lee et al., 2015) resulting in pGGKd072 (multigene cassette #2). Finally, pGGKd073, a GFP multigene dropout plasmid, was constructed by assembling pYTko08 and pYTko073 (ConLS and ConRL connectors), pYTko047, pYTko074, pYTko082 with pYTko084 (CoEl1 npdtII).

After this, using BsmI mediated golden gate assembly, pGGKd072
**Table 2**

Plasmids used in this study. Abbreviations: Sc Saccharomyces cerevisiae, Km Klyuyveromyces marxianus, Kl Klyuyveromyces lactis, Yl Yarrowia lipolytica, Gd Gluconacetobacter diazotrophicus, Zm Zymomonas mobilis, Rc Rhodobacter capsulatus, Pl Photobudobacter luminescens, Co codon optimized, pr promoter, t terminator, DO dropout.

| Name | Description | Part Type | Source |
|------|-------------|-----------|--------|
| JA_NM_1 | camR CoPlstIA | 3 | This study |
| pYTK001 | camR GFP entry vector | Insert | Lee et al. (2015) |
| pYTK002 | camR ConLS connector | 1 | Lee et al. (2015) |
| pYTK003 | camR ConLS connector | 1 | Lee et al. (2015) |
| pYTK008 | camR ConLS connector | 1 | Lee et al. (2015) |
| pYTK047 | camR GFP DO | 234r | Lee et al. (2015) |
| pYTK055 | camR ENO2t | 5 | Lee et al. (2015) |
| pYTK067 | camR ConR1 connector | 5 | Lee et al. (2015) |
| pYTK072 | camR ConRE connector | 5 | Lee et al. (2015) |
| pYTK073 | camR ConRE connector | 5 | Lee et al. (2015) |
| pYTK074 | camR URA3 | 6 | Lee et al. (2015) |
| pYTK082 | camR 2 μm | 7 | Lee et al. (2015) |
| pYTK084 | camR kanR-CoDE1 | 8 | Lee et al. (2015) |
| pUD565 | camR GFP entry vector | Insert | Boonekamp et al. (2018) |
| pGGKp025 | camR CDC1pr | 2 | Hassing et al. (2019) |
| pGGKp027 | camR RBA1pr | 2 | This study |
| pGGKp028 | camR ENO2pr | 2 | Hassing et al. (2019) |
| pGGKp035 | camR TDH3pr | 2 | Hassing et al. (2019) |
| pGGKp037 | camR ADH1t | 4 | Hassing et al. (2019) |
| pGGKp039 | camR TEF1t | 4 | Hassing et al. (2019) |
| pGGKp045 | camR PDC1t | 4 | Hassing et al. (2019) |
| pGGKp048 | camR GPM1t | 4 | Hassing et al. (2019) |
| pGGKp063 | camR SKTDE1pr | 2 | Hassing et al. (2019) |
| pGGKp074 | camR ScPDC1pr | 2 | Hassing et al. (2019) |
| pGGKp182 | camR CYC1t | 4 | This study |
| pGGKp183 | camR KmARO10 | 3 | This study |
| pGGKp184 | camR KmPDC1 | 3 | This study |
| pGGKp185 | camR putative KmPDC5 | 3 | This study |
| pGGKp211 | camR CoGdpdc1.1 | 3 | This study |
| pGGKp212 | camR CoGdpdc1.1 | 3 | This study |
| pGGKp213 | camR RKPDC5 | 3 | This study |
| pGGKp214 | camR KIA010 | 3 | This study |
| pGGKp254 | camR CoGdpdc1.2 | 3 | This study |
| pGGKp255 | camR CoGdpdc1.3 | 3 | This study |
| pGGKp314 | camR RKPDC1 | 3 | This study |
| pGGKp315 | camR ScTHI3 | 3 | This study |
| pGGKp327 | CoRctal1 | 3 | This study |
| pGGKp337 | ScPDC1 | 3 | This study |

**Expression cassettes**

| Plasmid | Genotype | Parts used | Source |
|---------|----------|------------|--------|
| pGGKd017 | 2 μm ampR-CoDE1 URA3 GFP DO | pYTK002, pYTK047, pYTK072, pYTK074, pYTK082, pYTK083 | Wronska et al. (2020) |
| pGGKd071 | 2 μm ampR-CoDE1 conLS conR1 URA3 GFP DO | pYTK002, pYTK047, pYTK067, pYTK074, pYTK082, pYTK083 | This study |
| pGGKd072 | 2 μm ampR-CoDE1 conLS conR1 URA3 GFP DO | pYTK003, pYTK047, pYTK072, pYTK074, pYTK082, pYTK083 | This study |
| pGGKd073 | 2 μm kanR-CoDE1 conLS camR5 URA3 GFP DO | pYTK008, pYTK047, YTK073, pYTK074, pYTK082, pYTK084 | This study |
| pUD827 | 2 μm ampR URA3 TDH3pr-CoaZmpdc1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp211 | This study |
| pUD828 | 2 μm ampR URA3 TDH3pr-KmA010-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp211 | This study |
| pUD829 | 2 μm ampR URA3 TDH3pr-KmPDC5-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp211 | This study |
| pUD832 | 2 μm ampR URA3 TDH3pr-CoGdpdc1.1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp212 | This study |
| pUD833 | 2 μm ampR URA3 TDH3pr-KPDC5-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp213 | This study |
| pUD834 | 2 μm ampR URA3 TDH3pr-KmPDC1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp214 | This study |
| pUD837 | 2 μm ampR URA3 TDH3pr-KmPDC1-CYC1t | Gibson assembly, pGGKd017, pGGKp035, pGGKp182 | This study |
| pUD838 | 2 μm ampR URA3 TDH3pr-KmPDC1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp214 | This study |
| pUD881 | 2 μm ampR URA3 TDH3pr-CoGdpdc1.2-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |
| pUD882 | 2 μm ampR URA3 TDH3pr-CoGdpdc1.3-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |
| pUD1019 | 2 μm ampR URA3 ScPDC1pr-CoPlstIA ENO2t | pGGKd017, pGGKp035, pGGKp314, pGGKp182 | This study |
| pUD1037 | 2 μm ampR URA3 TDH3pr-KPDC1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |
| pUD1049 | 2 μm ampR URA3 ENO2pr-ScTHI3-GPM1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |
| pUD1088 | 2 μm ampR URA3 SKTDE1pr-CoRctal1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |
| pUD1099 | 2 μm ampR URA3 TDH3pr-ScPDC1-CYC1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |
| pUD1100 | 2 μm kanR URA3 TDH3pr-ScPDC1-CYC1t, ENO2pr-ScTHI3-GPM1t | pGGKd017, pGGKp035, pGGKp182 pGGKp215 | This study |

**Cas9 Plasmids**

| Name | Relevant Genotype | Primer(s) used for gRNA | Source |
|------|-------------------|-------------------------|--------|
| pRS10 | 2 μm bla URA3 gRNA-CAN1.Y gRNA-ADE2.Y | N.A. | Mans et al. (2015) |

(continued on next page)
assembly. The gene
strain decahylase-expressing episomal (2
2.4. Strain construction
by restriction analysis.
terminator fragments. Correct construction of pUDE837 was confirmed
amplified from pGGKp035 and pGGKp182 using primers 14185/14186
from genomic DNA of
Yeast Chromosomes database (https://gryc.inra.fr)) was PCR amplified
restriction analysis.
ScPDC1, ScTHI3
were assembled using a BsmBI golden gate assembly into pUDE1101
et al., 2020) using primers 14187/14188. The
Finally, pGGkd073 (multigene dropout), pUDE1049 and pUDE1100
were assembled resulting in pUDE1100 (ScPDC1, ScTHI3) (Table 2). Final plasmid confirmation was done by restriction analysis.
The expression cassette bearing YIPDC1 was assembled using Gibson assembly. The gene YIPDC1 (YALI0D10131g, Genome Resources for Yeast Chromosomes database (https://gryc.inra.fr)) was PCR amplified from genomic DNA of Y. lipolytica W29 (Maganan et al., 2016; Wronska et al., 2020) using primers 14187/14188. The TDH3pr and CYC1t
were amplified from pGGKp035 and pGGKp182 using primers 14185/14186 and 14189/14190, respectively. The linear pGGkd073 backbone for construction was amplified using primers 14183/14184. The plasmid pUDE837 (YIPDC1) was constructed using Gibson assembly of the promoter, gene and terminator fragments. Correct construction of pUDE837 was confirmed by restriction analysis.

Table 2 (continued)

| Part Plasmids | Description | Part Type | Source |
|---------------|-------------|-----------|--------|
| pROS12       | 2 μm bla hphNT1 gRNA-CAN1.Y gRNA-DE2.Y | N.A. | E.-J. Hassing et al. (2015) |
| pUD406       | 2 μm bla URA3 gRNA-PDC5/PDC6 gRNA-ARO10 | 7246 & 13614, 6178 | This Work |
| pUD470       | 2 μm bla hphNT1 gRNA-PDC1 (2x) | 15832 | Hassing et al. (2019) |
| pUD599       | 2 μm bla hphNT1 gRNA-X3 (2x) | | |
| **Miscellaneous Plasmids** | | | |
| pUDE172       | Centromeric plasmid, URA3 TDH3pr-ApPAL1-CYC1t TP1p-CoAtC4H-ADHtr PGlpr-CoAtCPR1-PGl | | Koopman et al. (2012) |
| pUD069       | Integration plasmid, TRP1 TDH3pr-CoRctal1-CYC1t | | Koopman et al. (2012) |
| pL_MOG14     | 2 μm TDH3pr natN2 bla HXT7p,-CoPlstlA-CYC1t | | (Gottardi et al., 2017b) |

was co-transformed with pUDR599 (gRNA-X3) and the three expression cassettes CoRctal1, CoPlstlA and CoAtC4H/CoAtCPR1 containing homologous flanks to the X3 locus or a short homologous sequence (shr) (Kuipers et al., 2013) to allow homologous recombination of the flanks and integration into the edited X3 locus (Mikkelsen et al., 2012) resulting in strain IMX2656 after curing pUDR599. In the final step, PDC1 was deleted by co-transforming strain IMX2656 (coumaric acid producing pdc5Δ pdc6Δ aro10Δ) with pUDR470 (gRNA-PDC1) and a repair fragment consisting of annealed oligonucleotides 7719 and 7770, resulting in strain IMX2668 after curing the gRNA plasmid.

The resulting strain IMX2668 (coumaric acid producing, pdcΔ pdcΔ aro10Δ) was transformed with episomal plasmids expressing an individual 2-oxo-acid decarboxylase or with pGGkd017, an empty backbone plasmid, as negative control. This yielded strains IME656 (pGGkd017 (URA3, empty plasmid)), IME658 (pUDR827 (Zmpdc1)), IME659 (pUDE838 (KmPDC1)), IME660 (pUDE837 (YIPDC1)), IME661 (pUDE1037 (KIPDC1)), IME662 (pUDE881 (Gdpdc1.2t)), IME663 (pUDE882 (Gdpdc1.3t)) and IME677 (ScPDC1)).

2.5. Growth studies

Shake-flask cultures were grown in 500 mL shake flasks containing 100 mL medium and incubated at 30 °C in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ). Precultures on SMEG were inoculated from frozen stock cultures. These precultures were used to inoculate shake flasks containing SMEG and SMD, at an initial OD600 of 0.2. Independent duplicate cultures were grown for each combination of yeast strain and medium composition. Specific growth rates were calculated from a minimum number of six data points collected during exponential growth and covering 3–4 doublings of OD600. Ehrlich pathway products were quantified in supernatant samples of triplicate stationary phase (72 h) shake-flask cultures.

Aerobic bioreactor batch cultures on SMD supplemented with 0.2 g L−1 antifoam C (Sigma-Aldrich) were grown in 2L bioreactors (Applikon, Delft, Netherlands) with a working volume of 1.0 L. Oxygen was supplied by continuously sparging the culture with pressurized air at 0.5 L min−1. Exponentially growing shake-flask cultures on SMD were used to inoculate the bioreactors at an initial biomass concentration of around 0.1 g L−1. Cultures were grown at 30 °C and stirred at 800 rpm with a Rushton impeller. The culture pH was maintained at 5.0 by addition of 2 M KOH or 2 M H2SO4. Optical density at 660 nm was measured with a Jenway 7200 spectrophotometer (Jenway, Staffordshire, United Kingdom). Biomass dry weight was measured as described previously (Postma et al., 1989a). Off-gas from the bioreactors was cooled using a condenser and dried using a Permapure MD-110-48P-4 dryer (Permapure, Lakewood, NJ). CO2 and O2
concentrations in the off-gas were measured with a NGA 2000 Rosemount gas analyser (Rosemount, Analytical, Irvine, CA).

Concentrations of glucose, ethanol and extracellular organic acids in culture supernatants were measured by high performance liquid chromatography (HPLC) as described before (Hassing et al., 2019). The Ehrlich pathway metabolites 2-phenylethanol, p-hydroxyphenylethanol, phenylacetate, phenylpyruvate, coumaric acid and cinnamic acid were also measured by HPLC as described before (Hassing et al., 2019). Ar
tomatic compounds were detected by a diode-array multiple-wavelength detector (Agilent G1315C), at wavelengths of 200 nm for phenylacetate, 210 nm for phenylpyruvate, 214 nm for 2-phenylethanol and p-hydroxyphenylethanol, 270 nm for cinnamic acid and 280 nm for coumaric acid.

2.6. Enzyme-activity assays in cell extracts

Cell extracts of S. cerevisiae strains were prepared from late exponential phase (OD$_{600}$ of approximately 8) shake-flask cultures grown on SIMEG or SME medium. After 10 min centrifugation at 4696×g, cell pellets were washed twice with 20 mL 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, resuspended in 4 mL buffer and stored at −20 °C. Prior to the enzyme assays, biomass samples were thawed, resuspended and washed with 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM MgCl$_2$ and 2 mM dithiothreitol. When cell extracts were prepared for experiments to estimate kinetic parameters, which took several hours, complete(TM), Mini Protease Inhibitor Co. (Sigma-Aldrich) was added as protease inhibitor according to manufacturer’s recommendations. Cell extracts were prepared by sonication with 0.7 mm diameter glass beads using a MSE sonicator (150-W output, 7-nm peak-to-peak amplitude) at 0 °C. After four bursts of 30 s with 30 s cooling intervals, debris was removed by centrifugation using a Sorvall SS34-rotor (Thermo Fisher Scientific) for 20 min at 47.000 × g operated at 4 °C. The clear supernatants were used as cell extracts and kept on ice during experiments.

Pyruvate-decarboxylase activity in cell extracts was assayed as measured previously (Postma et al., 1989b). Phenylpyruvate-decarboxylase activity was assayed essentially as described before (Vuralhan et al., 2005) but with 5 mM instead of 2 mM phenylpyruvate. $K_m$ values for pyruvate were obtained by measuring pyruvate-decarboxylase activities at concentrations ranging from 0.1 mM to 50 mM, followed by nonlinear regression of the obtained results with GraphPad Prism (version 9.02, GraphPad Software, San Diego, CA). Datasets were fitted with Michaelis-Menten as well as allosteric sigmoidal kinetics.

### 2.7. Protein homology and phylogenetic tree

The amino acid sequences (Supplemental Dataset S.1) of the 2-oxo acid decarboxylases used in this study were aligned using Clustal Ω (Sievers et al., 2011). A heat map displaying sequence similarity was generated using GraphPad Prism. A phylogenetic tree of the aligned protein sequences was constructed with SeaView5 (Gouy et al., 2010) applying the LG model (Le and Gascuel, 2008) with default parameter settings using 100 Bootstrap replicates as support level for internal branches.

3. Results

### 3.1. Selection of heterologous pyruvate-decarboxylases with a potentially narrow substrate specificity

Heterologous pyruvate-decarboxylases with a potentially better substrate selectivity for pyruvate were selected based on three criteria: i) homology with S. cerevisiae pyruvate decarboxylases, ii) a demonstrated or proposed role in pyruvate decarboxylation, iii) absence of evidence for activity with aromatic 2-oxo acids. A resulting set of 11 decarboxylases was selected comprised of Klpdc1 (Dujon et al., 2004), KlPdc5 (Choo et al., 2018) and Klaro10 (Dujon et al., 2004) from K. lactis, KmPdc1, KmPdc5 and Kmaro10 from K. marxianus (Lertwattanasakul et al., 2015), Ylpdc1 from Y. lipolytica (Dujon et al., 2004) and four bacterial pyruvate decarboxylases: Zmpdc1 from Z. mobilis (Nale et al., 1998) and Gdpdc1.1 (van Zyl et al., 2014), Gdpdc1.2 (Bertalan et al., 2009) and Gdpdc1.3 (Giongo et al., 2010) from G. diazotrophicus (Table 3). In subsequent experiments, these heterologous enzymes were compared with the native S. cerevisiae 2-oxo acid decarboxylases ScPdc1, ScPdc5, ScPdc6 and Scaro10.

A phylogenetic tree of the amino-acid sequences of the selected 2-ODCs generated by multiple-sequence alignment using Clustal Ω (Sievers et al., 2011) showed a clear segregation of the eukaryotic (yeast) and bacterial sequences (Fig. 2). As anticipated, sequences of the S. cerevisiae pyruvate decarboxylases Pdc1 and Pdc5 clustered with those of the K. marxianus and K. lactis Pdc1 orthologs. Interestingly, KlPdc5 and KmPdc5 that had 76% similarity to one another showed only 34% similarity to ScPdc5. Despite the phylogenetic distance of the yeasts S. cerevisiae and Y. lipolytica, Ylpdc1 was more similar to the Pdc1 cluster comprising the Pdc1 orthologs from S. cerevisiae and Kluyveromyces species as well as the S. cerevisiae Pdc1 paralogs Pdc5 and Pdc6 than to the other selected proteins.

### Table 3

| Organism     | Gene    | Activity with pyruvate | Activity with PPY or pOHPPY | Co |
|--------------|---------|------------------------|-----------------------------|----|
| K. marxianus | KmPdc1  | Yes (Choo et al., 2018)| Unknown                     | No |
|              | KmPdc5  | No (Choo et al., 2018) | Unknown                     | No |
|              | Kmaro10 | Unknown                | Unknown                     | No |
| K. lactis    | KlPdc1  | Yes (Bianchi et al., 1996)| Unknown                     | No |
|              | KlPdc5  | Unknown                | Unknown                     | No |
|              | Klaro10 | Unknown                | Suggested (Uzunov et al., 2014)| No |
| Y. lipolytica| Ylpdc1  | Suggested (Beopoulus et al., 2009)| Unknown                     | No |
| Z. mobilis   | Zmpdc1  | Yes (Briner-Meyer et al., 1986)| Not PPY (Siegent et al., 2005), low pOHPPY (Siegent et al., 2005) | Yes |
| G. diazotrophicus | Gdpdc1.1 | Yes (van Zyl et al., 2014)| Not pOHPPY (van Zyl et al., 2014) | Yes |
|              | Gdpdc1.2 | Unknown                | Unknown                     | Yes |
|              | Gdpdc1.3 | Unknown                | Unknown                     | Yes |
3.2. In vivo pyruvate-decarboxylase activity of heterologous enzymes expressed in S. cerevisiae

To assess in vivo pyruvate decarboxylase activity of the 11 selected enzymes upon introduction in S. cerevisiae, they were expressed from an episomal plasmid and under control of the strong consecutive TDH3 promoter in the pyruvate-decarboxylase-negative strain CENPK711-7C (ura3Δ pdc1Δ pdc5Δ pdc6Δ aro10Δ thi3Δ). When precultures on SMEG were transferred to SMD, the empty-vector control strain IMZ001 (CENPK711-7C empty plasmid, URA3) and the ScAro10-expressing strain IMZ002 (CENPK711-7C ScARO10↑) failed to grow. This observation was consistent with the inability of pyruvate-decarboxylase-negative S. cerevisiae strains to grow on glucose as sole carbon source (Flikweert et al., 1996, 1999). Also strains IME418 (CENPK711-7C KIPDC5), IME423 (CENPK711-7C KmARO10↑), IME424 (CENPK711-7C KIPDC1↑) and IME425 (CENPK711-7C Gdpdc1.1↑), did not show growth on SMD after seven days of incubation, while the same strains were fully grown on SMEG. These results indicated that the heterologous genes introduced into these strains were either not functionally expressed or did not encode a functional pyruvate decarboxylase. In contrast, strains IME419 (CENPK711-7C YlPDC1↑), IME615 (CENPK711-7C KIPDC1↑), IME420 (CENPK711-7C KmPDC1↑), IME421 (CENPK711-7C Zmpdc1↑), IME422 (CENPK711-7C KmPDC5↑), IME474 (CENPK711-7C Gdpdc1.2↑), IME495 (CENPK711-7C Gdpdc1.3↑), and
the positive control strain IME667 (CEN.PK711-7C ScPDC1) all showed growth without lag-phase on SMD. Specific growth rates on SMD of these strains, including the positive-control strain IME667, were between 0.12 and 0.15 h\(^{-1}\). These growth rates were ca. 3-fold lower than that of the reference strain CEN.PK113-7D (0.42 ± 0.00 h\(^{-1}\)), which retains all 2-oxo acid decarboxylase genes in their native genetic context (Fig. 3).

The platform strain CEN.PK711-7C used to individually express the 2-oxo acid decarboxylases carried a deletion of THI3, a gene that was originally assumed to encode a fifth S. cerevisiae 2-oxo acid decarboxylase (Dickinson et al., 1998, 2000; Vuralhan et al., 2003) but was later shown to instead encode a protein involved in thiamine homeostasis (Mojzita and Hohmann, 2006; Nosaka et al., 2008). To investigate if inactivation of THI3 was responsible for the unexpectedly low specific growth rate of the tested strains, strain IME668 (CEN.PK711-7C ScTHI3) was constructed. Its specific growth rate on SMD was only 10% higher than that of strain IME667 (CEN.PK711-7C ScPDC1) and therefore still much lower than that of strain CEN.PK113-7D (Fig. 3).

3.3. In vitro comparison of substrate specificity of 2-oxo acid decarboxylase from various origin

To assess the substrate specificities of the selected heterologous pyruvate decarboxylases, enzyme activity assays were performed in cell extracts. In view of the goal of this study to eliminate production of aromatic fusel alcohols and acids, these assays focused on their activities with pyruvate and phenylpyruvate as substrates. Absence of pyruvate decarboxylase activity in cell extracts of strains IMZ002 (ScAro10\(^{-}\)), IME423 (KmAro10\(^{-}\)), IME424 (KiaAro10\(^{-}\)), IME418 (KlpDc) and IME422 (KmPdc5\(^{-}\)) correlated with their inability to grow on SMD. In contrast, cell extracts of strains expressing YpPdc1 (IME419), KmPdc1 (IME420), KlpDc1 (IME615), ZmPdc1 (IME421), GdPdc1.2 (IME674) or GdPdc1.3 (IME495), as well as the strains expressing ScPdc1 (IME667), ScPdc5 (IMZ024) and ScPdc6 (IMZ031) all showed pyruvate-decarboxylase activities (Fig. 4 and Table S2). The highest activities, above 3 μmol (mg protein\(^{-1}\)) \(^{-1}\) min\(^{-1}\), were observed in cell extracts of strains expressing yeast Pdc1 orthologs (Fig. 4 and Table S2). Cell extracts of strains expressing either of the two G. diazotrophicus decarboxylases (GdPdc1.2 and GdPdc1.3) exhibited a 35-fold lower pyruvate-decarboxylase activity than those of a strain expressing ScPdc1. However, pyruvate-decarboxylase activities of cell extracts of the strain expressing ZmPdc1, the other bacterial pyruvate decarboxylase, were close to those observed with the ScPdc1-expressing strain IME667 (Fig. 4).

As anticipated, cell extracts of strains expressing yeast Aro10 orthologs showed phenylpyruvate-decarboxylase activity, although activities were two orders of magnitude lower than pyruvate-decarboxylase activities observed in cell extracts of strains expressing yeast or Z. mobilis Pdc1 homologs (Fig. 4 and Table S2). Three of the heterologous 2-oxo acid decarboxylases with demonstrated in vivo and in vitro pyruvate-decarboxylase activity upon expression in S. cerevisiae (GdPdc1.2, GdPdc1.3 and ZmPdc1) showed no activity with 5 mM phenylpyruvate as substrate (Table 4). These enzymes were therefore for pyruvate as substrate and <20 nmol mg of protein\(^{-1}\) min\(^{-1}\) for phenylpyruvate as substrate. This results in the visualization of 4 classes: enzymes with decarboxylase activity for A) PPY but not pyruvate, B) both PPY and pyruvate C) no activity for either substrates and D) activity for pyruvate, but not PPY.

![Fig. 4. Specific decarboxylase activities for pyruvate and phenylpyruvate (PPY) in cell extracts of CEN.PK711-7C (pdc1A pdc5A pdc6A aro10A thi3A) expressing individual 2-oxo acid decarboxylase genes from a multicopy plasmid.](image)

All strains were grown duplicates at 30 °C on synthetic medium containing 2% (v/v) glycerol and 2% (v/v) ethanol as carbon source (SMEG). Black: S. cerevisiae, blue: K. marxianus, purple: Y. lipolytica, red: K. lactis, orange: Z. mobilis, green: G. diazotrophicus. Strains tested were IMZ001 (pdc1A pdc5A pdc6A aro10A thi3A URA3), IMZ002 (ScAro10\(^{-}\)), IMZ024 (ScPdc5\(^{-}\)), IMZ031 (ScPdc6\(^{-}\)), IME419 (KmAro10\(^{-}\)), IME420 (KmPdc1\(^{-}\)), IME422 (KmPdc5\(^{-}\)), IME423 (KmAro10\(^{-}\)), IME418 (KlpDc\(^{-}\)), IME421 (ZmPdc1\(^{-}\)), IME425 (GdPdc1.1\(^{-}\)), IME474 (GdPdc1.2\(^{-}\)), IME405 (GdPdc1.3\(^{-}\)). The dotted line indicates the detection limit for decarboxylase activity, which was <0.04 μmol mg protein\(^{-1}\) min\(^{-1}\).

Table 4

Specific pyruvate decarboxylase activity, \(K_0\) and the Hill coefficient for cell free extracts of S. cerevisiae strain CEN.PK711-7C (pdc1A pdc5A pdc6A aro10A thi3A) expressing individual 2-OADC genes. All strains were grown aerobically at 30 °C, 200 RPM in shake flasks containing 100 mL synthetic medium with 2% w/v ethanol as carbon source (SME). The cell extracts were prepared from late-exponential-phase shake-flask cultures. Different pyruvate concentrations were used as substrate for measuring pyruvate decarboxylase activity ranging from 0.1 mM to 50 mM. Enzyme activities were assayed from duplicate cultures.

| Strain  | Genotype | \(K_0\) (mM) ± SD | \(V_{max}\) (μmol min\(^{-1}\) mg of protein\(^{-1}\)) ± SD | Hill coefficient ± SD | \(V_{max}/K_0\) ratio |
|---------|----------|-------------------|---------------------------------|----------------------|----------------------|
| IME667  | ScPdc1   | 2.5 ± 0.0         | 11.2 ± 0.0                      | 2.4 ± 0.1            | 4.5                   |
| IME420  | KmPdc1   | 2.9 ± 0.1         | 7.8 ± 0.1                       | 2.4 ± 0.1            | 2.7                   |
| IME419  | YpPdc1   | 1.3 ± 0.0         | 2.5 ± 0.0                       | 1.3 ± 0.0            | 1.9                   |
| IME615  | KlPdc1   | 3.1 ± 0.1         | 8.9 ± 0.1                       | 2.3 ± 0.0            | 3.2                   |
| IME421  | ZmPdc1   | 0.6 ± 0.0         | 4.5 ± 0.1                       | 1.3 ± 0.1            | 8.8                   |
| IME474  | GdPdc1.2 | 0.8 ± 0.1         | 0.4 ± 0.0                       | 1.0 ± 0.0            | 0.5                   |
| IME495  | GdPdc1.3 | 0.8 ± 0.0         | 0.2 ± 0.0                       | 1.0 ± 0.0            | 0.3                   |

\(V_{max}\) and \(K_0\) values were obtained by performing a non-linear regression of specific decarboxylase activity over the substrate concentration using either a Michaelis-Menten model or a sigmoidal allosteric model. The Hill coefficients were calculated using the Hill equation; A Hill coefficient of 2.0 indicates positive cooperativity.
identified as promising candidates for replacing the native 2-OADCs in *S. cerevisiae* strains engineered for production of phenylpropanoid (Fig. 4).

To estimate the Michaelis constant (K_m) of the heterologous pyruvate decarboxylases for pyruvate, enzyme activity assays with cell extracts of strains expressing the prokaryotic enzymes and ScPdc1 yeast orthologs (KIPdc1, Kmpdc1 and Ylpdc1) were performed at pyruvate concentrations ranging from 0.1 to 50 mM (Fig. S1). To investigate whether, similar to *S. cerevisiae* pyruvate decarboxylase (Hübner et al., 1978), the heterologous pyruvate decarboxylases exhibit cooperativity, the data was fitted by non-linear regression to substrate-saturation Michaelis-Menten kinetics as well as to sigmoidal allosteric Hill kinetics (Table 4). Consistent with literature (Romagnoli et al., 2012), cell extracts containing ScPdc1 showed a Hill coefficient of 2.4, while a similar cooperativity was observed for cell extracts containing the *Kluyveromyces* enzymes KIPdc1 and Kmpdc1. In contrast, assays with cell extracts containing either *Y. lipolytica* Pdc1 or one of the three bacterial enzymes (Zmpdc1, Gdpdc1.2 or Gdpdc1.3), yielded a Hill coefficient close to one and absence of a sigmoidal relation between substrate concentration and enzyme activity (Table 4, Fig. S1), thus indicating absence of cooperativity (Table 4). In these assays, the *Z. mobilis* pyruvate decarboxylase Zmpdc1 showed a 4-fold lower K_m than ScPdc1 and a higher V_{max}/K_m ratio than the *G. diazotrophicus* pyruvate decarboxylases.

3.4. Decarboxylase swapping in a coumarin acid-producing *S. cerevisiae* strain

To investigate whether replacement of the native yeast 2-OADCs (Pdc1, Pdc5, Pdc6 and Aro10) by heterologous pyruvate decarboxylases (‘decarboxylase swapping’) can eliminate formation of by-products in *S. cerevisiae* strains engineered for phenylpropanoid production, a tester strain producing coumaric acid was constructed. To this end, ARO10, PDC5 and PDC6 were first deleted from the previously constructed strain IMX1593, which overexpresses feedback-insensitive alleles of the DAHP synthase and chorismate mutase (aro34 ARO4Δk2296,1 ARO7Δ72204Δ) (Hassing et al., 2019). Subsequently, expression cassettes for *PlatA*, *Rctal1t*, *AtC4H* and *AtCPR1*, which encode for respectively, a phenylalanine alanine lyase, tyrosine alanine lyase, cinnamic acid hydroxylase and its cytochrome p450 reductase, required for the activation of the cytochrome P450, were integrated at the X3 locus on CHRX (Mikkelsen et al., 2012). Deletion of the pyruvate decarboxylase gene *PDC1* yielded the 2-OADC-negative, coumaric acid producing platform strain IMX2668. This strain was transformed with multi-copy plasmids carrying expression cassettes for the different 2-oxo acid decarboxylases with specificity for pyruvate. All these strains grew on SMD in shake-flask cultures, albeit slower than the

| Strain | Genotype | AMH (h⁻¹) |
|--------|----------|-----------|
| CEN.PK113-7D | Ref. | 0.39 ± 0.00 |
| IME656 | IMX2668 URA3 | 0.00 ± 0.00 |
| IME677 | IMX2668 ScPDC1 | 0.27 ± 0.00 |
| IME658 | IMX2668 Zmpdc1 | 0.18 ± 0.00 |
| IME659 | IMX2668 Kmpdc1 | 0.22 ± 0.00 |
| IME660 | IMX2668 Ylpdc1 | 0.17 ± 0.00 |
| IME661 | IMX2668 ScPDC1 | 0.11 ± 0.00 |
| IME662 | IMX2668 Gdpdc1.2 | 0.22 ± 0.00 |
| IME663 | IMX2668 Gdpdc1.3 | 0.12 ± 0.00 |

Fig. 5. Metabolite profile of the coumaric acid producing reference strain IMX2668 (ScPdc1+1, Scpdc1Δ1, Scpdc1Δ2, Scpdc1Δ3). 

To more accurately quantify the impact of decarboxylase swapping on coumaric acid production, the coumaric acid-producing reference strain IMX2667 (ScPDC1), as well as strains IME658 (Zmpdc1Δ1) and IME663 (Gdpdc1.3Δ1) were grown aerobically on SMD in pH-controlled bioreactors. Under these conditions, the reference strain IME677 produced 2.2 mM of coumaric acid and displayed the typical diauxic growth pattern of aromatic glucose-grown batch cultures of *S. cerevisiae*, with an initial respiro-fermentative growth phase followed by a respiratory ethanol consumption phase (De Deken, 1966) (Fig. 6A.I). As observed in shake-flask cultures, strain IME677 (ScPDC1) produced 2-phenylethanol (0.10 mM) and p-hydroxyphenylethanol (0.15 mM) (Fig. 6A.II), which together corresponded to 12% of the total extracellular aromatic metabolites. In addition, this strain excreted detectable amounts of

Table 4

| Strain | Genotype | AMH (h⁻¹) |
|--------|----------|-----------|
| CEN.PK113-7D | Ref. | 0.39 ± 0.00 |
| IME656 | IMX2668 URA3 | 0.00 ± 0.00 |
| IME677 | IMX2668 ScPDC1 | 0.27 ± 0.00 |
| IME658 | IMX2668 Zmpdc1 | 0.18 ± 0.00 |
| IME659 | IMX2668 Kmpdc1 | 0.22 ± 0.00 |
| IME660 | IMX2668 Ylpdc1 | 0.17 ± 0.00 |
| IME661 | IMX2668 ScPDC1 | 0.11 ± 0.00 |
| IME662 | IMX2668 Gdpdc1.2 | 0.22 ± 0.00 |
| IME663 | IMX2668 Gdpdc1.3 | 0.12 ± 0.00 |
phenylpyruvate (0.1 mM) (Fig. 6A.II).

Although growing 30% slower than strain IME677, strain IME658 (Zmpdc1) also displayed a respiro-fermentative growth (Fig. 6B.I). In contrast to the reference strain, secreted the aromatic precursor cinnamic acid (0.1 mM). In the absence of aromatic fusel alcohol production, strain IME658 (Zmpdc1) therefore excreted 14% more cinnamic acid and 24% more aromatic acid precursors than the reference strain IME677 (ScPDC1). These improvements were also observed in the molar yields of cinnamic acid on glucose (Y_COUM) in these strains, which were 22.0 ± 0.2 mmol mol⁻¹ for strain IME658 and 18.4 ± 0.7 mmol mol⁻¹ for strain IME677 (Table 6).

Strain IME663 (Gdpdc1.3) showed a 50% lower specific growth rate in the bioreactor cultures than strain IME677 (ScPDC1) (Table 6). In comparison to the other two cinnamic acid-producing strains, it did not produce detectable amounts of ethanol and reached 30% higher final biomass concentrations. In contrast to the shake-flask cultures of strain IME663, the bioreactor cultures consumed all glucose. Although aromatic fusel alcohols were not detected in culture supernatants, strain IME663 reached a lower coumaric acid titer than strain IME677 (1.5 mM vs 2.2 mM) and, additionally, produced nearly three-fold higher extracellular phenylpyruvate concentrations (0.3 mM).

### 4. Discussion

Microbial thiamine-pyrophosphate-dependent pyruvate decarboxylases (EC 4.1.1.1) exhibit different kinetic properties and substrate specificities (Vuralhan et al., 2005; Romagnoli et al., 2012; Milne et al., 2015; Stribny et al., 2016). By exploring this diversity, we identified bacterial pyruvate decarboxylases that did not, or very slowly, decarboxylate phenylpyruvate in vitro and could functionally replace the native S. cerevisiae pyruvate decarboxylases in glucose-grown cultures in vivo. Replacing all native S. cerevisiae 2-OADCs in a cinnamic acid producing strain by bacterial decarboxylases from G. diazotrophicus or Z. mobilis eliminated formation of aromatic by-products via the Ehrlich pathway. Moreover, the cinnamic acid-producing strain S. cerevisiae...
IME658, in which the native yeast 2-OADCs were replaced by Z. mobilis pdc1, did not produce aromatic fuel alcohols and showed a higher coumaric acid yield than the congenic strain IME677 that instead expressed ScPDC1.

While our study provided a clear proof of principle for the ‘decarboxylase swapping’ approach, the Zmpdc1-expressing strain grew 30% slower than the ScPDC1 expressing strain. These different growth rates occurred despite high and similar pyruvate-decarboxylase activities in cell extracts of S. cerevisiae strain expressing pdc1Δ pdc6Δ aro10Δ thi3Δ strains carrying the same Zmpdc1 and ScPDC1 expression vectors (Table S2 and Fig. 4). Slower growth of the Zmpdc1-expressing strain may be related to a reported 20-fold higher sensitivity of Zmpdc1 to inhibition by its product acetaldehyde (Goetz et al., 2001). It would therefore be interesting to express the acetaldehyde-tolerant variant Zmpdc1W392M (Bruhn et al., 1995; Yun and Kim, 2008). Alternatively, as proposed earlier for a ScPDC1-overexpressing S. cerevisiae strain (van Hoek et al., 1998), reduced growth rates on SMD of strains expressing pyruvate-decarboxylase genes from epimol-omucopy plasmids may reflect protein-burden effects. Further metabolic engineering and/or adaptive laboratory evolution (Mans et al., 2018) can be applied to identify optimal expression levels of these pyruvate-decarboxylases. When impacts on specific growth rate can be prevented, the 2-oxo acid decarboxylase swapping strategy outlined in this study should be applicable for reduction of by-product formation by yeast strains engineered for production of a wide range of phenylpropanoids including stilbenoids, flavonoids and hydroxycinnamic acids.

Applicability of Zmpdc1 in yeast biotechnology may extend beyond prevention of aromatic by-product formation. Previous research showed that, in contrast to ScPdc1 (Romagnoli et al., 2012), Z. mobilis pyruvate decarboxylase does not decarboxylate the 2-oxo acids 3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate (Chang et al., 2000; Siegert et al., 2005), which are derived from isoleucine, leucine and valine, respectively. Elimination of these volatile fuel alcohol by-products during yeast-based ethanol production may enable reduced processing costs (Mayer et al., 2015).

Expression of 2-OADCs from an episomal-multicopy plasmid in the CEN.PK711–7C background (pdc1Δ pdc6Δ aro10Δ thi3Δ) resulted in specific growth rates for all strains between 0.12 and 0.15 h⁻¹ (Fig. 3). When CEN.PK113-7D is grown on synthetic medium with glucose as carbon source and ammonium sulphate as nitrogen source, ScPDC1 transcript levels are 10 fold higher compared to the other decarboxylases (Vuralhan et al., 2005), and serves as the main decarboxylase and/or acetaldehyde dehydrogenase (Vuralhan et al., 2005) and serves as the main decarboxylase and/or acetaldehyde dehydrogenase (Vuralhan et al., 2005). Further research should establish the physiological relevance of ScPdc1 in its native host.

The present study, which explored only a fraction of the natural diversity of 2-OADCs, illustrates for further screening, mutagenesis and targeted protein engineering to tailor catalytic and regulatory properties of these key enzymes to specific applications in biotechnology.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mec.2021.e00183.

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