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ARTICLE

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# Production of octanoic acid in *Saccharomyces cerevisiae*: Investigation of new precursor supply engineering strategies and intrinsic limitations

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## Abstract

The eight-carbon fatty acid octanoic acid (OA) is an important platform chemical and precursor of many industrially relevant products. Its microbial biosynthesis is regarded as a promising alternative to current unsustainable production methods. In *Saccharomyces cerevisiae*, the production of OA had been previously achieved by rational engineering of the fatty acid synthase. For the supply of the precursor molecule acetyl-CoA and of the redox cofactor NADPH, the native pyruvate dehydrogenase bypass had been harnessed, or the cells had been additionally provided with a pathway involving a heterologous ATP-citrate lyase. Here, we redirected the flux of glucose towards the oxidative branch of the pentose phosphate pathway and overexpressed a heterologous phosphoketolase/phosphotransacetylase shunt to improve the supply of NADPH and acetyl-CoA in a strain background with abolished OA degradation. We show that these modifications lead to an increased yield of OA during the consumption of glucose by more than 60% compared to the parental strain. Furthermore, we investigated different genetic engineering targets to identify potential factors that limit the OA production in yeast. Toxicity assays performed with the engineered strains suggest that the inhibitory effects of OA on cell growth likely impose an upper limit to attainable OA yields.

**KEYWORDS**
acetyl-CoA, octanoic acid, phosphoketolase, phosphotransacetylase

## 1 | INTRODUCTION

Fatty acids (FAs) with various chain lengths and their derivatives with different functional groups are important compounds in modern industry, which have numerous applications like fuels, cosmetics, pharmaceuticals, and food additives. Recently, engineering microbial FA production has attracted attention as an alternative to established methods such as petrochemistry or oil palm cultivation, which have lately been criticized for their environmental impact.

In *Saccharomyces cerevisiae*, biosynthesis of FAs in the cytosol is catalyzed by the large multidomain fatty acid synthase (FAS) complex, which naturally generates long-chain fatty acid (LCFA, C14-C18) as building blocks of membranes or for storage lipids. The biosynthesis is initiated by cytosolic acetyl-CoA (AcCoA) and maturing FAs are elongated by AcCoA-derived malonyl-CoA until

reaching their final length. NADPH is required during the process for reductive power (for review, see Baumann et al., 2020). In *S. cerevisiae*, AcCoA is compartmentalized in the cytosol, mitochondria, and peroxisomes and there is no free exchange of the intermediate across organelle membranes (Chen et al., 2012; Krivoruchko et al., 2015). The majority of cytosolic AcCoA for FA biosynthesis is generated by the cytosolic pyruvate dehydrogenase bypass (PDH-bypass), in which pyruvate is decarboxylated to acetaldehyde by the pyruvate decarboxylases followed by conversion of acetaldehyde to acetate by aldehyde dehydrogenases. Finally, acetate is ligated to CoA by AcCoA synthetase at the expense of two ATP equivalents (Van Rossum et al., 2016).

For improved production of various AcCoA-derived products, engineering the precursor supply has been the approach of choice, and many studies have targeted an increased flux through the PDH-bypass (De Jong et al., 2014; Krivoruchko et al., 2013; Li et al., 2014; Lian et al., 2014; Shiba et al., 2007). Due to the Crabtree effect in yeast, the largest fraction of acetaldehyde is converted into ethanol by dominant alcohol dehydrogenases even under aerobic conditions, which competes with the aldehyde dehydrogenase reaction (Nielsen, 2014) and thereby decreases cytosolic AcCoA formation from pyruvate. Indeed, it was demonstrated that deletion of the dominant alcohol dehydrogenase gene *ADH1* improves LCFA production (Li et al., 2014). Other strategies relied on the overexpression of PDH-pathway genes, which resulted in increased carbon flux through the PDH-bypass (De Jong et al., 2014; Krivoruchko et al., 2013; Li et al., 2014; Lian et al., 2014; Shiba et al., 2007). Increased acetate and NADPH accumulation was achieved by overexpressing the aldehyde dehydrogenase *ALD6*. Additionally, expression of a heterologous AcCoA synthetase with an L641P substitution ($^{Se}ACS^{L641P}$) to prevent its inactivation by acetylation (Starai et al., 2005) proved beneficial compared to the overexpression of endogenous variants (Shiba et al., 2007). In a subsequent study targeting FA ethyl ester (FAEE) production, concomitant overexpression of the alcohol dehydrogenase *ADH2* to increase acetate formation from ethanol, *ALD6*, and $^{Se}ACS^{L641P}$ led to increased product formation (De Jong et al., 2014). Very recently, the AcCoA and NADPH supply were engineered by introducing a heterologous ATP-citrate lyase, a mitochondrial citrate transporter, a cytosolic malate dehydrogenase, and a cytosolic malic enzyme to optimize the production of LCFA (Yu et al., 2018), short- and medium-chain fatty acid (SMCFA; Zhu et al., 2020) and FA derivatives (Zhou et al., 2016). These modifications were introduced in addition to the endogenous PDH bypass.

Van Maris and coworkers (Van Rossum et al., 2016) calculated the theoretical maximum yields for palmitic acid (C16) production via different AcCoA providing pathways. The highest FA yield was predicted for a heterologous phosphoketolase (PK)/phosphotransacetylase (PTA) pathway when NADPH formation occurs via the pentose phosphate pathway (PPP). In this scheme, glucose-6-P is converted to xylulose-5-P by four sequential reactions catalyzed by glucose-6-P-dehydrogenase (Zwf1), 6-phospogluconolactonase (Sol3), 6-phosphogluconate dehydrogenase (Gnd1), and ribulose-5-P-epimerase (Rpe1). In the two dehydrogenase reactions, NADPH is formed, resulting in two moles NADPH for one mole of xylulose-5-P. Cytosolic AcCoA is then provided by the consecutive reactions of a xylulose-5-P specific PK (xPK) which converts xylulose-5-P to acetyl-P (AcP) and glyceraldehyde-3-P and conversion of acetyl-P to AcCoA by a PTA. Expression of the xPK/PTA pathway proved to increase the formation of FAEE (De Jong et al., 2014) and, more recently, of farnesene (Meadows et al., 2016). In the latter study, it was shown that AcP formed by PK can be hydrolyzed by endogenous glycerol-3-P phosphatases (*GPP1* alias *RHR2*) and deletion of *GPP1* is therefore beneficial.

To the best of our knowledge, engineering precursor and cofactor supply via the PPP/xPK/PTA pathway have not yet been employed for FA production in *S. cerevisiae*, but heterologous xPK/PTA expression in the oleaginous yeast *Yarrowia lipolytica* led to significantly higher lipid yields (Niehus et al., 2018; Xu et al., 2016). For the proof of concept in baker's yeast, we targeted the production of octanoic acid (OA), an eight-carbon (C8) FA. Production of OA (and other SMCFA) can be achieved by a minimal invasive strategy through mutations in the FAS enzyme, which favor premature termination of the FA elongation cycle (Gajewski et al., 2017). For instance, a single R1834K substitution in the malonyl–palmitoyl transferase domain of Fas1 (herein referred to as $FAS^{RK}$) allows for a favored biosynthesis of OA.

Here, we investigated if PDH-bypass engineering and PK/PTA pathway expression are applicable to increase OA production by $FAS^{RK}$ in *S. cerevisiae*. The targets for employed genetic engineering are summarized in Figure 1. We show that known PDH-bypass engineering targets have no positive effect on OA production, whereas the expression of xPK/PTA in combination with interventions that increase the flux through the oxidative PPP improve the production of OA.

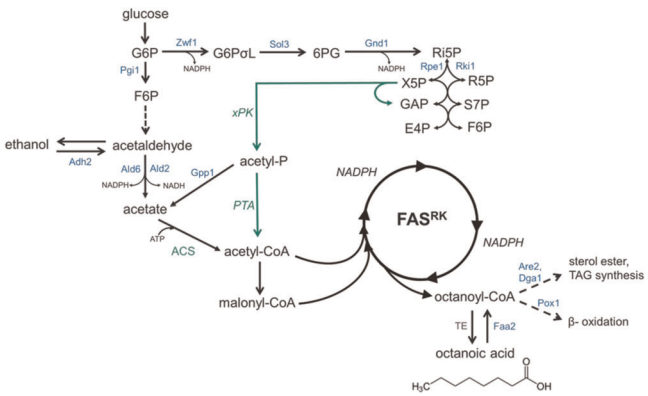


**FIGURE 1** Overview of genetic engineering targets. The metabolic intermediates and enzymes relevant for this study are shown. Dashed lines depict multiple metabolic steps. The endogenous enzymes, whose genes were overexpressed, knocked down or knocked out as described in Section 3 are shown in blue. Heterologously expressed enzymes are shown in green. The multiple endogenous enzymes with thioesterase activity are depicted with a generic abbreviation (TE). ACS, acetyl-CoA synthetase; PTA, phosphotransacetylase; xPK, xylulose-5-P specific phosphoketolase [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 1** Yeast strains used in this study

| Strain name | Relevant genotype | Reference/source |
|---|---|---|
| BY4741 | *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | Brachmann et al. (1998) |
| SHY34 | *MATα ura3Δ0 his3Δ0 leu2Δ0 TRP1 lys2Δ0 MET15 Δfas1 Δfas2 Δfaa2* | Wernig et al. (2020) |
| CEN.PK2-1C | *MATa; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2* | Euroscarf, Germany |
| VGY2 | CEN.PK2-1C *FAS1p:: HXT7p*$^{-1\text{--}392}$*-FAS1*$^{R1834K}$, *FAS2p::pHXT7*$^{-1\text{--}392}$*-FAS2* | This study |
| LBY38 | VGY2 *Δfaa2* | This study |
| FWY32 | LBY38 *Δpox1* | This study |
| FWY36 | FWY32 *Δald6* | This study |
| FWY37 | FWY36 *ZWF1p::HXT7p*$^{-1\text{--}392}$ | This study |
| FWY38 | FWY37 *PGI1p::COX9p* | This study |
| FWY40 | FWY32 *Δare2* | This study |
| FWY41 | FWY32 *Δdga1* | This study |
| FWY43 | LBY38 *Δgpp1::HXT7p*$^{-1\text{--}392}$*-*$^{Ca}$*xPK-FAB1t-HHF2p-*$^{Bs}$*PTA-SSA1t* | This study |
| FWY45 | FWY38 *Δgpp1::HXT7p*$^{-1\text{--}392}$*-*$^{Ca}$*xPK-FAB1t-HHF2p-*$^{Bs}$*PTA-SSA1t* | This study |
| FWY46 | FWY38 *Δgpp1::HXT7p*$^{-1\text{--}392}$*-*$^{Ca}$*xPK-FAB1t-HHF2p-*$^{Se}$*eutD-SSA1t* | This study |
| FWY47 | FWY45 *Δura3::PFK1p-RPE1-RPE1t* | This study |
| FWY48 | FWY47 *Δfaa2::HXT7p*$^{-1\text{--}392}$*-*$^{Ca}$*xPK-FAB1t-HHF2p-*$^{Bs}$*PTA-SSA1t* | This study |
| FWY50 | FWY45 *Δald2* | This study |
| FWY58 | FWY45 *Δura3::ADH2p-ALD6* | This study |

Abbreviation: xPK, xylulose-5-P specific phosphoketolase.

## 2 | MATERIALS AND METHODS

### 2.1 | Strain construction and transformation

Yeast strains used in this study are listed in Table 1. CRISPR/Cas9 was used to modify strains with deletions, integrations, and exchange of promoters as described previously (Generoso et al., 2016). For this, yeast strains were transformed with the CRISPR/Cas9 plasmid encoding Cas9 and guide RNA together with an appropriate donor DNA (synthetic double-stranded DNA for deletions, polymerase chain reaction-amplified expression cassettes or promotors for integrations). Donor DNA carried at least 30 base pairs (bp) overhangs to the desired locus. Specific gRNA sequences were selected with the freely available tool (https://www.atum.bio/eCommerce/cas9/) to cut in the desired gene or promoter region. Gene deletions of *POX1*, *ALD6*, *ALD2*, *ARE2*, and *DGA1* and integrations into the gene loci of *GPP1* and *URA3* were carried out by removing the entire gene ORF. The native promoters of *ZWF1* (from −500 to 0 bp), and *PGI1* (from −405 to 0 bp) were replaced by the truncated *HXT7p*$^{-1\text{--}392}$ (amplified from plasmid pRS62-K) and *COX9p* amplified from genomic DNA of CEN.PK2-1C, respectively. For the construction of strain FWY47, a *PFK1p-RPE1-RPE1t* cassette was amplified from the plasmid pHD8 (Demeke et al., 2013) and integrated into the *URA3* locus. $^{Ca}$xPK (Bergman et al., 2016) and either $^{Bs}$PTA (De Jong et al., 2014) or $^{Se}$eutD (Brinsmade & Escalante-Semerena, 2004) were amplified from plasmids FWV163, FWV164, or FWV165 and integrated into the *GPP1* locus, which was thereby deleted. Oligonucleotides used for amplification or deletions (synthetic double-stranded DNA) are listed in Table S1. Relevant gene sequences are listed in the Supporting Information. Transformations were performed following the frozen competent cell protocol (Gietz & Schiestl, 2007), whereas SHY34 was transformed by a slightly modified method previously described (Gajewski et al., 2017). Transformed yeasts were plated on solid yeast extract, peptone, dextrose (YPD) (2% [wt/vol] peptone, 1% [wt/vol] yeast extract, 2% [wt/vol] glucose) containing appropriate antibiotics hygromycin (100 mg/L) or G418 (200 mg/L) for plasmid selection and grown at 30°C for 2–4 days.

## 2.2 | Plasmid construction

Plasmids were constructed via homologous recombination in yeast (Oldenburg et al., 1997) or Golden Gate cloning (Lee et al., 2015; PTA genes) by standard procedures. Plasmid fragments were amplified by PCR using oligonucleotides listed in Table S1. The assembled plasmids were propagated in and extracted from *Escherichia coli* DH10B by standard procedures. For the construction of FWV171, the dominant marker *hphNT1* was completely replaced by *kanMX4* in plasmid TWRV1 by amplification of *kanMX4* from plasmid pRS52-K and insertion into the NdeI cut site of *hphNT*.$^{Ca}$xPK, $^{Bs}$PTA, and $^{Se}$EutD were provided by Dr. Arun Rajkumar, cloned into the PYTK001 backbone of the Golden Gate system. $^{Ca}$xPK was subcloned into the plasmid pRS62-H by homologous recombination. $^{Bs}$PTA and $^{Se}$EutD under the control of *HHF2p* and *SSA1t* by Golden Gate cloning (Lee et al., 2015). All plasmids are listed in Table S2.

## 2.3 | Media and cultivation

If not stated otherwise, *S. cerevisiae* liquid cultures were inoculated to an optical density at 600 nm ($OD_{600}$) of 0.1 and grown in shake flasks at 30°C and 180 rpm in YPD medium without supplementation of free FA or with supplementation of oleic acid (0.5 mM and 1% [vol/vol] Tergitol NP-40 solution; Sigma Aldrich) for the FAS-deficient strain SHY34. For maintaining plasmids with *hphNT1* or *kanMX4* marker appropriate antibiotics hygromycin (100 mg/L) or G418 (200 mg/L) were used. The medium was additionally buffered with 100 mM potassium phosphate and adjusted to a pH of 6.5. If not stated otherwise, glucose was used as a carbon source at concentrations of 20 g/L. Samples for compound extraction were taken at given time points. Growth was either monitored by measurement of $OD_{600}$ or online by use of a Cell Growth Quantifier; Aquila Biolabs (Bruder et al., 2016).

## 2.4 | Compound extraction and derivatization

Extraction of free FAs in the culture medium was performed as described before (Henritzi et al., 2018). Cells were separated from the medium by centrifugation (3500 rcf, 10 min) and 10 ml of culture supernatant was mixed with an internal standard (0.2 mg heptanoic acid), 1 ml of 1 M HCl, and 2.5 ml of methanol:chloroform (1:1) solution. After phase separation (3000 rcf, 5 min) the organic phase layer was taken and evaporated in a vacuum concentrator (Concentrator 5301; Eppendorf). FAs were methylated for gas chromatography (GC) analysis as described before (Ichihara & Fukubayashi, 2010). The extract was dissolved in 200 µl toluene, mixed with 1.5 ml of methanol, and 300 µl of 8.0% (wt/vol) HCl solution and incubated at 100°C for 3 h to form fatty acid methyl esters (FAME). FAMEs were extracted from the mixture by the addition of 1 ml $H_2O$ and 1 ml hexane. The organic phase was taken for GC analysis.

## 2.5 | Protein extraction and enzyme assays

For protein extraction, 100–150 $OD_{600}$ units of cells were collected from a shake flask culture by centrifugation (3000 rcf, 4°C, 10 min), washed with water, and stored at −80°C until further processing. After thawing on ice, the cells were mechanically disrupted in assay buffer (50 mM imidazole, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid [EDTA]) containing 1X concentrated Protease Inhibitor Cocktail Complete, EDTA-free (Roche Diagnostics) by shaking (10 min at 4°C) with glass beads (0.45-mm diameter) using a Vibrax cell disruptor (Janke & Kunkel). The cell debris was subsequently removed by centrifugation (15,000 rcf, 5 min, 4°C). Protein concentration of clear crude extracts was determined by the Bradford method, using bovine serum albumin as a standard. The reaction mixtures for enzyme assays contained (final concentration) 0.67 mM $NADP^+$ and 2.5 mM substrate (glucose-6-P for glucose-6-P dehydrogenase or fructose-6-P for phosphoglucose isomerase). In phosphoglucose isomerase assays, the mixture additionally contained 2 U of glucose-6-phosphate dehydrogenase for coupling. All assay components were purchased from Merck. The final reaction volume was 200 µl in assay buffer (see above). The reactions were started by adding 20 µl of 25 mM substrate solution. The reduction of $NADP^+$ was recorded by measuring the change of the absorbance at 340 nm. The specific activities were calculated as milli-units per milligram protein.

## 2.6 | Gas chromatography

The GC analysis was performed on a Perkin Elmer Clarus 400 system (Perkin Elmer) equipped with an Elite-5MS capillary column (Ø: 0.25 mm; length: 30 m; film thickness: 1.00 µm) and a flame ionization detector (Perkin Elmer). One microlitre of sample was analyzed after split injection (1:10) and helium was used as carrier gas (90 kPa). For FAME quantification, the temperatures of the injector and detector were set to 200°C and 250°C, respectively. The following temperature program was applied: run time 42.67 min, start at 50°C and hold for 5 min; ramp at 10–120°C and hold for 5 min, ramp at 15–220°C and hold for 10 min, ramp at 20–300°C and hold for 5 min. FAMEs were identified and quantified by comparison with authentic standard substances.

## 2.7 | Quantificaion of glucose and ethanol by high-performance liquid chromatography

Samples for analysis were centrifuged (16,000 rcf, 5 min) and 450 µl of the supernatant was mixed with 50 µl of 50% (wt/vol) 5-sulphosalicylic acid for protein precipitation. The supernatant was analyzed by high-performance liquid chromatography (Dionex; Thermo Fisher Scientific) equipped with a HyperREZ XP Carbohydrate $H^+$ column (300 × 700 mm, 8 µ; Thermo Fisher Scientific) at 30°C; 0.5 mM $H_2SO_4$ was used as mobile phase with a constant flow

of 0.6 ml/min. Metabolites were identified and quantified by the use of authentic standards.

### 2.8 | OA toxicity assay

For the toxicity assay, a preculture of the strains was inoculated into fresh YPD medium to an $OD_{600}$ of 0.2 and cultivated for 5–6 h until an $OD_{600}$ of 0.8–1.0 was reached. The culture was rediluted in YPD medium to an $OD_{600}$ of 0.05 and 50 µl was used to inoculate 200 µl of YPD medium in 96-well plates with a dilution series of OA. OA was dissolved in YPD medium at the highest concentration, filtered for sterility (0.2 µm), and subsequently diluted to desired concentrations. Cultivations were performed for 18 h at 30°C without agitation and cells were mixed thoroughly before $OD_{600}$ measurement in a plate reader (ClarioStar; BMG Labtech).

## 3 | RESULTS AND DISCUSSION

### 3.1 | Base strain construction and elimination of SMCFA degradation

In our previous work on OA production by a mutated FAS in *S. cerevisiae*, we have been using the FAS deficient strain SHY34 (Δ*fas1*Δ*fas2*Δ*faa2*), derived from the BY-series (Wernig et al., 2020). However, SHY34 depends on supplementation of FAs like oleic acid, since *FAS1* and *FAS2* genes are essential (Giaever et al., 2002) and their knockout leads to auxotrophy for LCFA, which are necessary for cell survival. Although growth can be restored by LCFA (e.g., oleic acid) supplementation, *FAS* gene knockout resulted in a strongly reduced growth rate and a lower final cell density compared to a BY4741 WT strain (Figure 2a). Due to these characteristics, handling of the strain is difficult, resulting in low transformation efficiencies and extended experimental times, which is an undesired trait for a biotechnological application. When the FAS deficient strain is transformed with a plasmid for the production of OA (SHV61 carrying $FAS1^{RK}$/*FAS2*), oleic acid supplementation becomes redundant due to leaky production of LCFA (Baumann et al., 2021; Gajewski et al., 2017), but growth is still strongly impaired (Figure 2b). Therefore, we sought to generate a superior OA producer strain by eliminating these drawbacks. We chose the strain CEN.PK2-1C, belonging to the popular CEN.PK-series that is used for both academic (e.g., Van Dijken et al., 2000) and industrial applications (e.g., Meadows et al., 2016) as a parental strain. Using CRISPR/Cas9, we introduced a mutation encoding the amino acid exchange R1834K into the genomic *FAS1*. To increase the expression of the FAS genes, we exchanged the endogenous promoters of both *FAS1* and *FAS2* by the strong $HXT7p^{-1\text{--}392}$ promotors (Hamacher et al., 2002). The resulting strain VGY2 is simpler in handling (such as for plasmid transformations), has twice the maximum growth rate of SHY34 + $FAS1^{RK}$/*FAS2* (0.65 ± 0.08 vs. 0.33 ± 0.05) and grows to higher final cell densities (Figure 2b). To eliminate degradation of SMCFA, which was observable after longer incubation periods (48–72 h) of VGY2 (Figure S1), we knocked out the medium-chain fatty acyl-CoA synthetase *FAA2* (Henritzi et al., 2018; Leber et al., 2016), resulting in strain LBY38. LBY38 maintained OA amounts constant even 72 h after inoculation (Figure S1) and accumulated increased OA amount compared to its precursor strain VGY2 (Figure 2c).

FA biosynthesis depends on cytosolic AcCoA and derived malonyl-CoA as precursor and elongation units, respectively, and utilizes NADPH as reduction equivalent. To increase the production of OA by a mutated FAS, we aimed to enhance the precursor and cofactor supply and investigated the PDH-bypass as a first engineering target in the LBY38 background. Although decreased alcoholic fermentation, achieved by deletion of the main alcohol

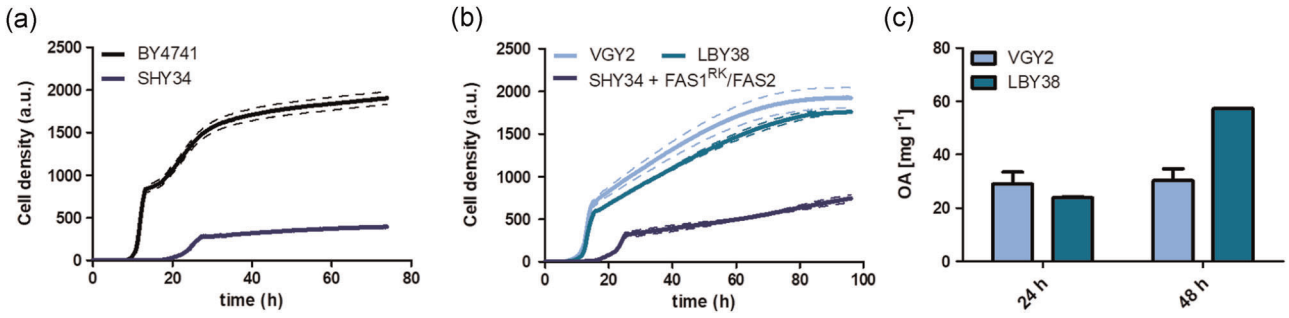


**FIGURE 2** Growth behavior and octanoic acid production of strains expressing a mutated fatty acid synthase. In (a), growth of the BY-derived SHY34 strain (Δ*fas1*Δ*fas2*Δ*faa2*) is compared to the BY4741 wild-type in YPD media supplemented with oleic acid. In (b) growth of strains VGY2 (Δ*FAS1p*::$HXT7p^{-1\text{--}392}$::$FAS1^{R1834K}$, Δ*FAS2p*::$HXT7p^{-1\text{--}392}$::*FAS2*), LBY38 (VGY2 Δ*faa2*) and SHY34 transformed with plasmid SHV61 ($FAS1^{RK}$/FAS2) in YPD medium without oleic acid supplementation is shown. Growth analyses were performed on a Cell Growth Quantifier (Aquila Biolabs), which measures backscattered light intensities. 2000 U corresponds to approximately $OD_{600}$ = 12.5 as determined by offline measurements. The curves represent the mean (solid lines) and standard deviation (dashed lines) of three biological replicates. Octanoic acid (OA) production of strains VGY2 and LBY38 grown in phosphate-buffered YPD medium is shown in (c). The data represent mean and standard deviation of two biological replicates. $OD_{600}$, optical density at 600 nm [Color figure can be viewed at wileyonlinelibrary.com]

dehydrogenase *ADH1*, proved to be beneficial for LCFA biosynthesis (Li et al., 2014) we rejected this approach for OA production, considering its strong negative effect on cell growth rates (Paquin & Williamson, 1986), which we also observed in preliminary experiments that resulted in very low OA titers. Instead, we decided to enhance the utilization of ethanol (by overexpressing *ADH2*) and the PDH bypass (by overexpressing *ALD6* and $^{Se}ACS^{L641P}$) since this strategy was reported to improve the biosynthesis of LCFA-derived compounds (De Jong et al., 2014). However, neither the overexpression of *ADH2* alone nor in combination with *ALD6* and $^{Se}ACS^{L641P}$ had a positive effect on OA production (Figure S2). On the contrary, when the cells were transformed with the $^{Se}ACS^{L641P}$/*ALD6* plasmid, growth and OA titers decreased (Figure S2a,b), possibly due to the accumulation of toxic acetate as a consequence of increased Ald6 activity (Shiba et al., 2007). The specific OA titers (defined as OA production over $OD_{600}$), which were calculated to take the tradeoff between growth and production into account (Figure S2c), were comparable to the empty vector control, indicating that the decrease in titers was mainly due to the growth defect. It is noteworthy that LBY38 transformed with two empty high copy plasmids (2 μ origin) accumulated about 50% less OA in comparison to plasmid-free LBY38 (compare Figure 2c at 48 h and Figure 3b), possibly due to plasmid burden effects (Karim et al., 2013).

Improvements in FA production have been previously reported for deletion of the acyl-CoA oxidase (*POX1*) gene, catalyzing the first step of β-oxidation (Leber et al., 2016; Runguphan & Keasling, 2014) and for the knockouts of the nonessential storage lipid formation of steryl esters (AcCoA sterol acyltransferase, *ARE2*) or triacylglycerols (diacylglycerol acyltransferase, *DGA1*; Valle-Rodríguez et al., 2014). Deletion of *POX1* in LBY38 increased OA titers in the resulting strain FWY32 (Figure S3). In contrast, in FWY32-derived FWY40 (Δ*are2*) or FWY41 (Δ*dga1*) no further positive effect was observed (Figure S3), suggesting that Are2 and Dga1 are specific for LCFA and do not utilize OA as substrate. Thus, FWY32 was used to further investigate the influence of precursor and cofactor supply engineering strategies on OA production.

### 3.2 | Rerouting the flux of glucose through the oxidative PPP and expression of a heterologous PK/PTA shunt

In our previous work and initial experiments described above, we observed that OA production mainly occurs after exhaustion of glucose, that is, during the utilization of ethanol. This can be readily explained by the fact that ethanol catabolism must occur via AcCoA formation, whereas only a minor fraction of pyruvate produced from glucose is entering the PDH bypass (Pronk et al., 1996). The abovementioned observation that overexpressing *ADH2*, $^{Se}ACS^{L641P}$, and *ALD6* does not lead to increased OA titers (Figure S2), suggests that the capacity of the endogenous PDH bypass enzymes is not limiting and low productivity on glucose is rather due to inherent physiological constraints. We, therefore, reasoned that a higher yield of OA on glucose—which is highly desirable from a biotechnological prospective—could be achieved by modifying the sugar metabolism. Among different alternative pathways for AcCoA (and NADPH) supply, the highest yields of FAs were predicted for concomitantly rerouting glucose flux to the oxidative PPP (for NADPH production) and expressing heterologous xPK and PTA (Van Rossum et al., 2016, see introduction). We, therefore, decided to test this scheme for OA production.

The Zwf1/Gnd1 (oxidative PPP) and Ald6 catalyzed reactions are the main source for NADPH supply so that a *zwf1 ald6* double deletion mutant is not viable (Grabowska & Chelstowska, 2003). To enhance the metabolic flux over the PPP and increase NADPH formation, overexpression of *ZWF1* alone is not sufficient (Kwak et al., 2019; Yu et al., 2018). Therefore, we forced the metabolism into the direction of the PPP by deleting the *ALD6* gene in strain FWY32, leaving the Zwf1 reaction as the only source of NADPH. Next, we overexpressed *ZWF1* by exchanging its promoter by the strong $HXT7p^{-1\text{--}392}$ (Hamacher et al., 2002). To increase the availability of the Zwf1 substrate glucose-6-P, we exchanged the promotor of the phosphoglucose isomerase gene (*PGI1*) by the weak *COX9p*, as described previously (Yu et al., 2018). This approach is advantageous over *PGI1* deletion, which causes a severe growth

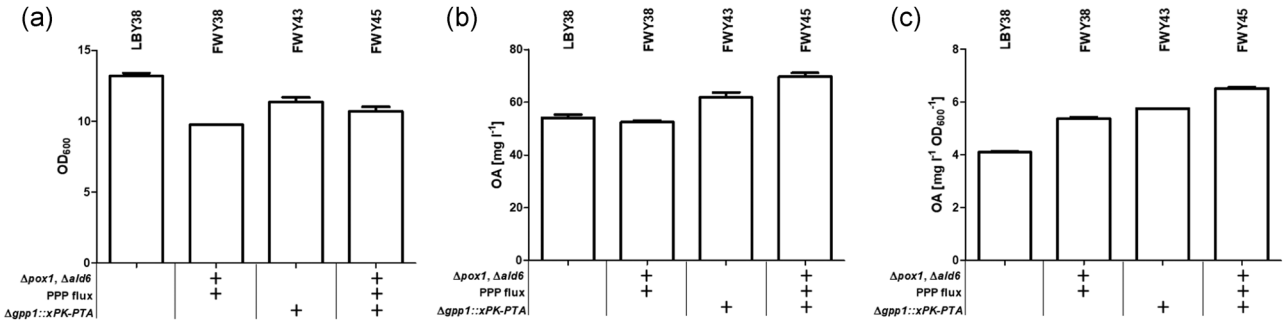


**FIGURE 3** Increasing OA production by PPP and xPK/PTA pathway engineering. Growth (as final $OD_{600}$, a), OA titers (b) and specific OA titers (normalized to $OD_{600}$, c) of engineered strains are shown. Strains were grown in potassium phosphate-buffered YPD media and samples were taken after 48 h of fermentation. Values and error bars represent mean and standard deviation of two biological replicates. OA, octanoic acid; $OD_{600}$, optical density at 600 nm; PPP, pentose phosphate pathway; PTA, phosphotransacetylase; xPK, xylulose-5-P specific phosphoketolase

phenotype (Aguilera, 1986). The resulting strain FWY38 harboring the combined strategy of *ALD6* deletion, *PGI1* knockdown, and *ZWF1* overexpression (denoted as PPP flux in the figures) showed slightly reduced growth (Figure 3a), comparable OA titers to parental strain LBY38 (Figure 3b) but increased specific titers of OA (Figure 3c).

Several different PKs have been functionally expressed in *S. cerevisiae* before (Bergman et al., 2016; De Jong et al., 2014; Meadows et al., 2016). We genomically integrated the PK gene of *Clostriudium acetobutylicum* (under the control of the $HXT7p^{-1\text{--}392}$ promoter), which showed the highest specific activity for xylulose-5-P (xPK) in a previous report (Bergman et al., 2016), and a PTA gene from *Bacillus subtilis* ($^{Bs}$PTA; under the control of the *HHF2p* promoter; De Jong et al., 2014) into the *GPP1* locus of strains LBY38 and FWY38. Thereby, *GPP1*, which encodes for a glycerol-3-P-phosphatase and is able to dephosphorylate acetyl-P produced by a PK (Meadows et al., 2016), was deleted. The resulting strain FWY43 showed 18% increased OA titers (Figure 3b) and about 33% increased specific titers compared to the parental strain LBY38 (Figure 3c). Combined strategies of PPP flux increase and xPK/PTA expression (strain FWY45) led to an increase in OA titers by 29% (Figure 3b) and in specific titers by about 45% (Figure 3c) compared to LBY38. Besides $^{Bs}$PTA expression, we chose to test and compare an additional promising PTA, *eutD* from *Salmonella enterica* ($^{Se}$*EutD*; Brinsmade & Escalante-Semerena, 2004), but the titers of OA did not differ between the variants (Figure S4).

To confirm that the promoter replacements intended to enhance the flux of glucose-6-P towards the oxidative PPP indeed led to the desired alterations, the activities of the phosphoglucose isomerase (Pgi1) and glucose-6-P dehydrogenase (Zwf1) were measured in the series of the engineered strains up to FWY45 (Figure 4). The samples for protein extraction were taken in different stages of fermentation (i.e., during glucose and ethanol consumption phases). The deletion of *ALD6* apparently had no influence on the expression of *ZWF1* on either carbon source, suggesting that the endogenous level of Zwf1 is sufficient to provide NADPH for cellular maintenance. Replacement of the native *ZWF1* promoter by $HXT7p^{-1\text{--}392}$, led to a more than seven-fold and fourfold increase in Zwf1 activity on glucose and ethanol, respectively. These levels were not further affected by downstream strain modifications, that is the down-regulation of *PGI1* and introduction of xPK/PTA (Figure 4a). The insertion of the *COX9* promoter reduced the *PGI1* expression up to 2.5-fold on glucose but had not changed the inherently lower Pgi1 activity on ethanol (Figure 4b). In summary, an approximately 17-fold increase of the Zwf1/Pgi1 activity ratio was achieved in FWY45 on glucose compared to the starting strain FWY32.

## 3.3 | Growth phase and carbon source dependent production of OA

As explained above, one of the premises of redirecting carbon flux via the PPP and xPK/PTA pathway was to increase OA yield during the glucose consumption phase. To investigate this hypothesis, we analyzed the growth phase-dependent production of OA (Figure 5) and compared the parental strain LBY38 with strains expressing the xPK/PTA pathway without (FWY43) or with the enhanced Zwf1/Pgi1 activity ratio (FWY45). Interestingly, about two-thirds of total OA are produced, regardless of the strain background, in the late phase of fermentation during ethanol consumption. This behavior is in accordance with the production of LCFA in yeast, which can be increased in late growth phases due to loss of competition between production and biomass formation (Yu et al., 2018). To get a clearer insight into the flux distribution changes by the introduced genetic modifications, we calculated

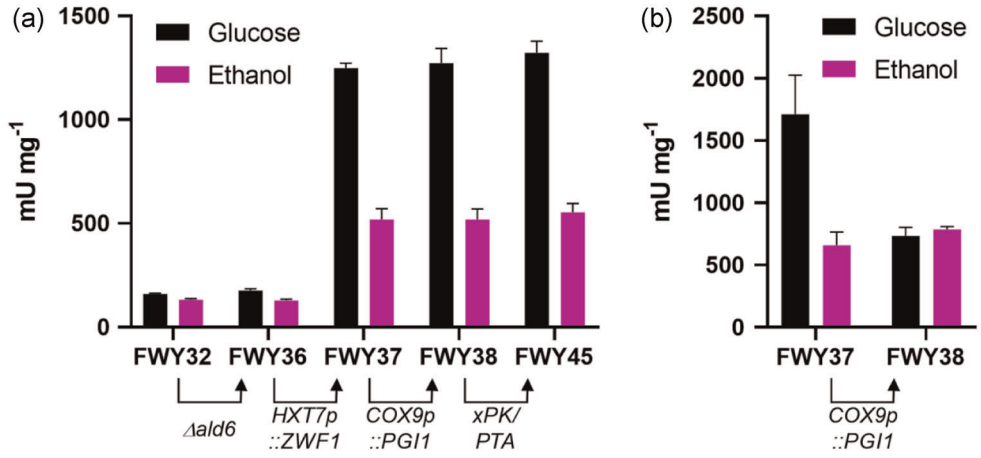


**FIGURE 4** Glucose-6-P dehydrogenase and phosphoglucose isomerase activities in engineered strains. The activities of the glucose-6-P dehydrogenase/Zwf1 (a) and phosphoglucose isomerase/Pgi1 (b) were measured in protein extracts from the indicated strains. The consecutive modification steps of the genotype are indicated below the strain names. Cells were grown in potassium phosphate-buffered YPD media and samples were taken after 15 h (glucose) and 39 h (ethanol) of fermentation. The activities are calculated as milli-units per milligram of total protein. Values and error bars represent mean and standard deviation of two technical replicates from two independent cultures for each genotype (each column represents four measurements) [Color figure can be viewed at wileyonlinelibrary.com]

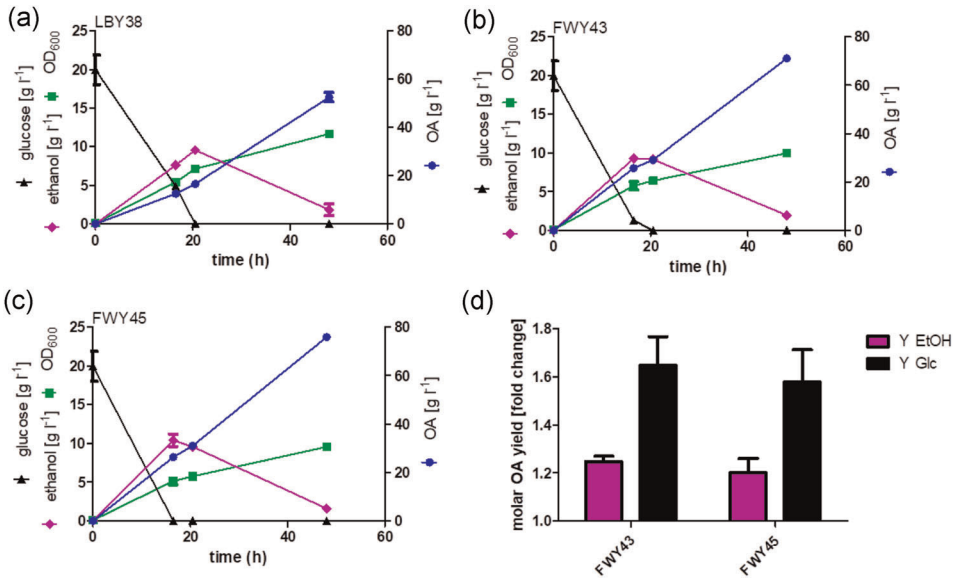


**FIGURE 5** Growth phase-dependent production of octanoic acid in strains LBY38, FWY43, and FWY45. Strains LBY38 (a), FWY43 (b), and FWY45 (c) were grown in potassium phosphate-buffered YPD media. Samples for OA, glucose and ethanol measurements were taken at indicated time points. (d) Yields of OA per theoretical AcCoA equivalents from consumed glucose or ethanol were calculated as fold change over the parental strain LBY38. Values and error bars represent mean and standard deviation of two biological replicates. AcCoA, acetyl-CoA; OA, octanoic acid; $OD_{600}$, optical density at 600 nm [Color figure can be viewed at wileyonlinelibrary.com]

the yields of OA (mol OA per mol AcCoA equivalents) at late stages of glucose (16.5 h) and of ethanol consumption (48.0 h; Figure 5d). Yields achieved by the engineered strains FWY43 and FWY45 increased by about 25% on ethanol but even more considerably by approximately 65% during the glucose consumption phase (Figure 5d) in comparison to LBY38, which is consistent with the expectation. The increased yields of OA on ethanol in FWY43 and FWY45 compared to LBY38 could be explained by the entrance of glucose-6-P and xylulose-5-P that are also generated via gluconeogenesis into the xPK/PTA shunt. Interestingly, an upregulation of the *PCK1* and *PYC1* genes, encoding two essential enzymes of gluconeogenesis, was observed upon the expression of a PK in *S. cerevisiae* (Bergman et al., 2019). Since the activity of Pgi1 on ethanol is not altered by the promoter exchange (see Figure 4b), the gluconeogenic flux is not negatively affected by our engineering strategy. Moreover, the loss of the Ald6 activity in the strain series comprising the "PPP flux" modifications can be compensated by the enhanced Zwf1 activity (Figure 4a).

Despite the higher relative yield on glucose, the strains FWY43 and FWY45 still produce two-thirds of OA during ethanol consumption. We, therefore, investigated whether cultivating the cells on ethanol as the sole carbon source could increase OA production. In the course of glycolysis and PDH-bypass, 1 mole of consumed glucose theoretically results in the formation of 2 moles AcCoA (two AcCoA equivalents) while catabolism of 1 mole ethanol via PDH-bypass results in 1 mole AcCoA equivalents. To account for this, strains LBY38 and FWY45 were cultivated in double molar amounts of ethanol (corresponding to 9.3 g/L) compared to glucose (corresponding to 20 g/L). Both strains showed barely any growth and OA production on ethanol with our standard cell inoculum (starting $OD_{600}$ 0.1; Figure S5), although the used ethanol concentration is known to be sufficient for the growth of a wild-type strain. As a known enhancer of OA toxicity (Legras et al., 2010), ethanol in combination with intrinsic OA production apparently has a strong negative effect on cell proliferation. OA production became detectable by increasing the cell inoculum of fermentation (high OD fermentation, starting $OD_{600}$ of 8.0), but titers remained below those with glucose as a carbon source (Figure S5).

## 3.4 | Systematic analysis of reactions limiting OA production

Expression of the xPK/PTA pathway and enhanced PPP-flux successfully increased OA biosynthesis, but various reactions in the pathway could be limiting OA titers. Hence, we systematically analyzed potential targets in the pathway. Higher accumulation of xylulose-5-P and following reaction of xPK/PTA towards AcCoA were targeted by overexpression of ribulose-5-P-epimerase *RPE1*, which was achieved by exchanging its native promoter by the strong *PFK1p*, and integration of a second genomic copy of the heterologous xPK/PTA cassette in strain FWY45. The resulting strains (FWY47 and FWY48, respectively) did not show changed growth or production compared to parental strain FWY45 (Figure 6a,b). Interestingly, *RPE1* and *TKL1* genes were found to be upregulated in a strain expressing a PK (Bergman et al., 2019), possibly as a compensatory mechanism in response to a reduced level of xylulose-5-P and perturbed pools of other sugar phosphates. This upregulation could have

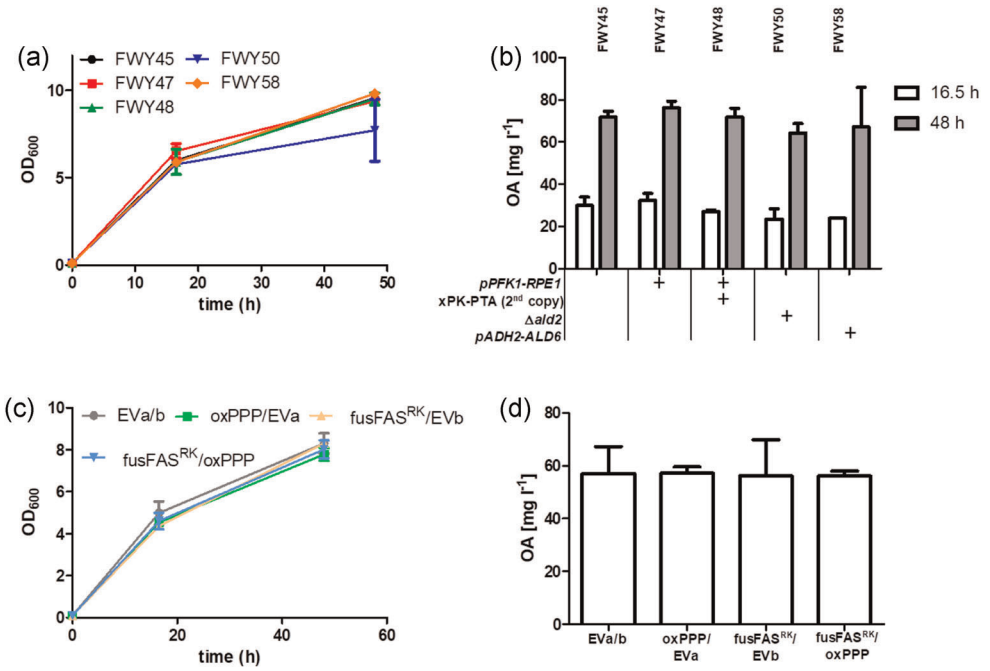


**FIGURE 6** Systematic investigation of reactions that potentially limit AcCoA supply. Growth (a and c) and OA production (b and d) of engineered strains or strain FWY45, which was transformed with plasmids FWY169 (oxPPP) for overexpression of three enzymes of the oxidative PPP (*TEF2p-ZWF1*, *CCW12p-SOL3*, *TEFF1p-GND1)* and/or with the plasmid FWY133 (*FAS1p*-fusFAS$^{RK}$) for overexpression of an additional mutated FAS copy are shown. Appropriate empty vectors (Ev a and b) were used for controls. Strains were grown in potassium phosphate-buffered YPD media and samples were taken after 16.5 (late glucose consumption phase) or 48 h (late ethanol consumption phase) (b) or after 48 h (d). Values and error bars represent the mean and standard deviation of two biological replicates. AcCoA, acetyl-CoA; OA, octanoic acid; $OD_{600}$, optical density at 600 nm [Color figure can be viewed at wileyonlinelibrary.com]

occurred in FWY45, too, making an overexpression of *RPE1* redundant. In strain FWY45 the gene encoding the NADPH dependent aldehyde dehydrogenase Ald6 is deleted, leaving the $NAD^+$-dependent Ald2 as the main aldehyde dehydrogenase of the PDH-bypass. To create a stronger driving force for rerouting carbon flux to the PPP/xPK/PTA pathway, *ALD2* gene was additionally deleted in the strain FWY45, thereby decreasing AcCoA production via the PDH bypass. However, the new strain FWY50 showed slightly reduced OA titers compared to the parental strain FWY45 (Figure 6b), demonstrating that some flux through PDH-bypass is important even when xPK/PTA is expressed. Furthermore, we reasoned that, even if OA formation increases in the strain FWY45 (mainly) during the glucose growth phase, this advantage should be diminished after the diauxic shift from glucose to ethanol consumption. For increased metabolic flux and NADPH formation during ethanol degradation, we reintegrated *ALD6* under the control of the glucose repressed *ADH2p* into FWY45. However, the resulting strain FWY58 did not show any increase in OA compared to the other strains (Figure 6b). This supports the above hypothesis that the increased activity of Zwf1 fully compensates the deletion of *ALD6* in the FWY45 background by providing NADPH through oxidation of glucose-6-P derived from gluconeogenesis.

We next targeted further up and downstream reactions of OA biosynthesis. This includes the oxidative part of PPP consisting of three reactions catalyzed by Zwf1, Gnd1, and Sol3. To evaluate whether a limitation originates from one of these reactions, we concomitantly overexpressed *ZWF1*, *GND1*, and *SOL3* from a high copy plasmid with strong promoters (module named oxPPP [plasmid FWV171]) in FWY45. To test a possibly limiting role of FAS, we overexpressed a superior mutated FAS variant (fusFAS$^{RK}$), consisting of a fusion construct of *FAS1*$^{RK}$ and *FAS2* (Wernig et al., 2020) in addition to the oxPPP module or separately. Surprisingly, the additional expression of fusFAS$^{RK}$ in strain FWY45 did not influence OA production nor did the combined expression of oxPPP and fusFAS$^{RK}$ (Figure 6d). Hence, although the individual expression of fusFAS$^{RK}$ (Wernig et al., 2020) and PPP/xPK/PTA (Figure 3) increase OA production, these effects appear not to be additive.

Collectively, the results presented here suggest that some intrinsic factor(s) limit(s) higher OA accumulation. One possibility is the strong inhibitory effect of OA on yeast growth (Alexandre et al., 1996; Borrull et al., 2015; Henritzi et al., 2018; Legras et al., 2010; Viegas et al., 1989; Wernig et al., 2020). SMCFA inhibitory effects are attributed to different mechanisms. They act as weak acids, which enter the cell in a protonated form by passive diffusion and dissociate intracellularly, thereby acidifying the cytosol (Cabral et al., 2001; Viegas & Sá-Correia, 1997). This triggers the activity of $H^+$-ATPase (Cabral et al., 2001), causing a strong energetic effort. Additionally, OA is known to disturb the integrity of the plasma membrane and cause its leakiness (Borrull et al., 2015; Liu et al., 2013). The membrane-related stress can be counteracted by supplementation of

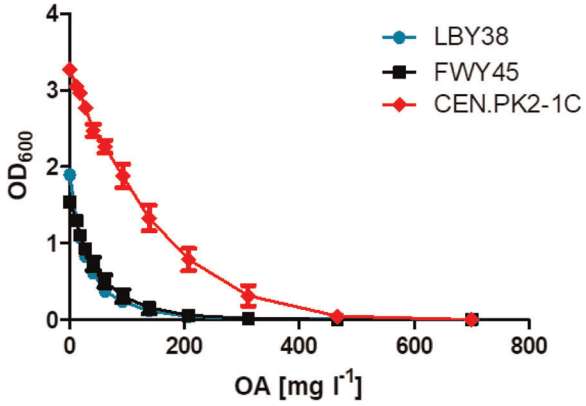


**FIGURE 7** Strain robustness of strains LBY38, FWY45, and CEN.PK2-1C towards octanoic acid. Strains were grown in 96-well plates in different concentrations of supplemented octanoic acid and cell growth was analyzed by $OD_{600}$ measurement in a plate reader after 18 h. Values show mean and standard deviation of three biological replicates. $OD_{600}$, optical density at 600 nm [Color figure can be viewed at wileyonlinelibrary.com]

LCFA like oleic acid (Besada-Lombana et al., 2017; Liu et al., 2013), which increases membrane stability. Furthermore, it was shown that the expression of a deregulated form of the AcCoA-carboxylase ($Acc1^{S1157A}$) increases the resistance to OA by favoring the production of LCFA (Besada-Lombana et al., 2017). To test the sensitivity of engineered strains to external OA, we exposed LBY38 and FWY45 as well as the wild-type CEN.PK2-1C to increasing OA concentrations in microtiter plates and measured the growth of the cells as a function of OA concentration. As shown in Figure 7, the producer strains are almost completely growth-inhibited already at 200 mg/L of supplemented OA in contrast to the wild-type cells. This observation can be readily explained by (i) the additive toxic effect of internally produced and external OA, (ii) compromised synthesis of LCFA, and (iii) the inability of the producer strains to degrade OA (due to the *faa2/pox1* deletions).

This suggests that the inhibitory effect of OA might be the main limitation for higher OA production and hinder an additive effect of different engineering strategies. However, other limiting factors cannot be ruled out at present. In the context of an OA producing strain, engineering strategies that counteract the toxicity by favoring the production of LCFA, such as the expression of $Acc1^{S1157A}$, would not be feasible as the chain elongation inevitably leads to decreased OA yields (Besada-Lombana et al., 2017; Zhu et al., 2020). However, it is possible that the cells intrinsically upregulate factors that drive the production of LCFA (e.g., Acc1). Therefore, adaptive laboratory evolution, as a possible approach to increase the OA tolerance, should be accompanied by high-throughput methods capable of measuring the intrinsic OA production, such as the recently developed biosensors (Baumann et al., 2018), to prevent the selection of adapted strains exhibiting an unfavorable FA product profile. Uncoupling biomass generation from production is another promising strategy to improve the production of toxic products (Yu et al., 2017), that could also be tested for OA biosynthesis in follow-up studies.

## 4 | CONCLUSION

In this study, we engineered a robust, CEN.PK-based strain for OA production that has clear advantages in respect to handling and genetic manipulation in comparison to the previously developed BY-derived strains. We demonstrated that the engineering of precursor supply routes for cytosolic AcCoA and NADPH are important targets to increase the production of OA by mutant FAS. To the best of our knowledge, the heterologous xPK/PTA in combination with increased flux over PPP was tested for the first time in the context of FA production in *S. cerevisiae*. This strategy is favorable over PDH-bypass engineering, especially for obtaining higher yields of OA from glucose, which is consistent with theoretical considerations and a desirable trait from an industrial point of view. However, our experiments show that further improvements of OA yields are likely constrained by product toxicity. Therefore, improving strain robustness or process development for in situ product removal will be important challenges to reach industrial-scale OA production in the future.

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## AUTHOR CONTRIBUTIONS

Florian Wernig and Leonie Baumann performed the experiments, analyzed the data, and drafted the manuscript. Eckhard Boles and Mislav Oreb were involved in the experimental design. Mislav Oreb guided the project and finalized the manuscript, which was approved by all authors. The authors thank Arun Stephen Rajkumar for providing plasmids pB14, pB15, and pB20 and Sandra Born for providing strain VGY02.

## DATA AVAILABILITY STATEMENT

The authors will make available all data (underlying the described findings) without restriction.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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