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Metabolic engineering of *Saccharomyces cerevisiae* to improve 1-hexadecanol production

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ABSTRACT

Fatty alcohols are important components of a vast array of surfactants, lubricants, detergents, pharmaceuticals and cosmetics. We have engineered *Saccharomyces cerevisiae* to produce 1-hexadecanol by expressing a fatty acyl-CoA reductase (FAR) from barn owl (*Tyto alba*). In order to improve fatty alcohol production, we have manipulated both the structural genes and the regulatory genes in yeast lipid metabolism. The acetyl-CoA carboxylase gene (*ACC1*) was over-expressed, which improved 1-hexadecanol production by 56% (from 45 mg/L to 71 mg/L). Knocking out the negative regulator of the *INO1* gene in phospholipid metabolism, RPD3, further enhanced 1-hexadecanol production by 98% (from 71 mg/L to 140 mg/L). The cytosolic acetyl-CoA supply was next engineered by expressing a heterologous ATP-dependent citrate lyase, which increased the production of 1-hexadecanol by an additional 136% (from 140 mg/L to 330 mg/L). Through fed-batch fermentation using resting cells, over 1.1 g/L 1-hexadecanol can be produced in glucose minimal medium, which represents the highest titer reported in yeast to date.

1. Introduction

Fatty alcohols have been widely used in producing detergents, emulsifiers, lubricants, and cosmetics (Knaut and Richtler, 1985; Mudge et al., 2008), and have a billion-dollar market globally (Runguphan and Keasling, 2013; Rupilius and Ahmad, 2006; Youngquist et al., 2013). Currently, fatty alcohols are mainly synthesized from vegetable oils or petrochemical feedstock. When produced from vegetable oils, the triglycerides derived from vegetable oils are hydrolyzed or trans-esterified, followed by hydrogenation to produce fatty alcohols (Rupilius and Ahmad, 2007). When produced from petrochemical feedstock, fatty alcohols are synthesized chemically through oligomerization of ethylene followed by oxidation (Rupilius and Ahmad, 2007). Environmental concerns have been raised against using either vegetable oils or petrochemical feedstock for fatty alcohol production. The increased demand for using vegetable oils such as palm oils has led to extensive deforestation of rainforest to plant palm trees, which has compromised the biodiversity and led to other ecological problems (Fitzherbert et al., 2008). Additionally, the dependence on petroleum products for fatty alcohol production has raised significant concerns about greenhouse effects and climate change (IPCC, 2007).

As an alternative and more sustainable route for fatty alcohol production, microbial production of fatty alcohols from renewable feedstock has recently achieved successes in both *Escherichia coli* and *Saccharomyces cerevisiae* via metabolic engineering. In *E. coli*, fatty alcohols have been produced by either expressing the heterologous enzymes such as carboxylic acid reductase (CAR) (Akhtar et al., 2013) and fatty acyl-CoA reductase (FAR) (Steen et al., 2010; Zheng et al., 2012), or expressing the endogenous enzymes acyl-CoA reductase (ACR) and aldehyde reductases (AR) (Liu et al., 2013; Youngquist et al., 2013). Via a series of engineering approaches that manipulate the structural genes (e.g., over-expression of a thioesterase and elimination of β-oxidation) in fatty acid metabolism, the C12–C14 alcohols have been produced in *E. coli*, with the highest titer reaching 1650 mg/L in fed-batch reactor (Youngquist et al., 2013). In *S. cerevisiae*, a mouse FAR has recently been expressed to produce 1-hexadecanol (Runguphan and Keasling, 2013). Through over-expression of acetyl-CoA carboxylase (ACC1) and fatty acid synthases (FAS1 and FAS2), the engineered *S. cerevisiae* strain produced 98.0 mg/L hexadecanol from 20 g/L hexose sugars in batch culture in minimal medium. In spite of the lower titer than that in *E. coli*, fatty alcohol production using yeast as the host can be advantageous since the enzymatic pathway for fatty alcohol production in yeast is more direct

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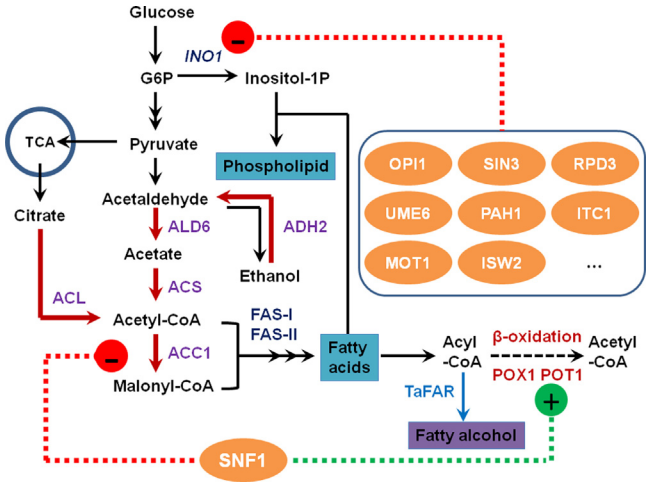

Fig. 1. Scheme of engineering yeast lipid metabolism for fatty alcohol production. Fatty alcohols can be produced by expressing a fatty acyl-CoA reductase from *Tyto alba* (TaFAR). In order to improve the fatty alcohol production, the *ACC1* gene encoding acetyl-CoA carboxylase (ACC1) was over-expressed and β-oxidation enzymes POX1 and POT1 were knocked out. In addition, the SNF1 protein that down-regulates the *ACC1* expression and up-regulates β-oxidation was knocked out, while the negative regulators of *INO1* were knocked out as well. To enhance the supply of cytosolic acetyl-CoA, two pathways were used independently: 1) a previously reported pathway that can efficiently provide acetyl-CoA in the cytoplasm by expressing ADH2, ALD6, and a codon-optimized ACS (Chen et al., 2013); and 2) expression of ACL from either *Arabidopsis thaliana* or *Yarrowia lipolytica*.

(Runguphan and Keasling, 2013) and phage contamination can be potentially avoided in yeast when producing chemicals at an industrial level (Łos et al., 2004; Runguphan and Keasling, 2013).

The low titer of fatty alcohol in spite of thorough manipulation of the structural genes in yeast lipid metabolism (Runguphan and Keasling, 2013) indicated that the other factors, such as the regulation of lipid metabolism and the supply of key precursors, could play important roles when engineering yeast for producing fatty acid-derived biofuels and chemicals. Therefore, in this study, we chose the regulatory genes of yeast lipid metabolism as the novel targets for manipulation and increased the acetyl-CoA availability in order to improve fatty alcohol production in *S. cerevisiae*. We first engineered *S. cerevisiae* to almost exclusively produce 1-hexadecanol by expressing a bird FAR. We also manipulated the structural genes in yeast lipid metabolism, which enhanced the 1-hexadecanol production by 56% to a similar level as previously reported (Runguphan and Keasling, 2013). Then, we systemically knocked out a series of repressors of phospholipid synthesis (Fig. 1) and successfully further doubled the 1-hexadecanol titer. Next, we enhanced the supply of cytosolic acetyl-CoA by expressing different heterologous pathways, and increased the 1-hexadecanol titer by an additional 136%, reaching 330 mg/L from 20 g/L glucose in minimal medium. By using fed-batch fermentation with resting cells, over 1100 mg/L 1-hexadecanol was produced from glucose, which is the highest titer reported in yeast to date.

2. Material and methods

2.1. Yeast strains, media, and transformation

The yeast strains used in this study were derived from BY4741, BY4742 or BY4743 (Table 1). The knockout strains, including Δ*POX1*, Δ*POT1*, Δ*SNF1*, Δ*OPI1*, Δ*SIN3*, Δ*RPD3*, Δ*PAH1*, Δ*UME6*, Δ*ISW2*, Δ*ITC1*, and Δ*MOT1*, were purchased from the Yeast Knockout Collection from Thermo Scientific Inc. (Waltham, MA). The yeast knockout strain of Δ*SNF1*Δ*SIN3* was generated by knocking out the *SNF1* gene in Δ*SIN3* strain via a previously reported gene disruption cassette used in *S. cerevisiae* (Gueldener et al., 2002). The disruption cassette was cloned using forward primer FP_Snf1_KO and reverse primer RP_Snf1_KO (Table S1). The knock-out strain was confirmed by sequencing the PCR products of the *SNF1* region amplified from the extracted genomic DNA. Similarly, to knock out the *OPI1* gene in the Δ*RPD3* strain, the disruption cassette was cloned by using forward primer FP_Opi1_KO and reverse primer RP_Opi1_KO (Table S1), followed by a similar confirmation step of the gene knock-out.

Yeast and bacterial strains were stored in 25% glycerol at −80 °C. *E. coli* was grown in Luria–Bertani medium. Ampicillin at 100 μg/mL was added to the medium when required. Yeast strains BY4741, BY4742, and BY4743 were cultivated in YPD medium. Yeast cells were transformed with plasmids listed in Table 1 using the LiAc/PEG method as described previously (Gietz and Schiestl, 2007a, 2007b). To select the yeast transformants, synthetic complete (SC) medium was used, containing 6.7 g/L of yeast nitrogen base plus 20 g/L of glucose, 20 g/L of agar, and the appropriate amino acid drop out mix (MP Biomedicals, Solon, OH). A single colony was picked and cultivated in 3 mL SC medium containing 20 g/L glucose. The cells were cultivated at 30 °C in disposable culture tubes shaken at 250 rpm for 2 days.

2.2. Plasmid construction

To construct the pTaFAR plasmid, the fatty acyl-CoA reductase gene from barn owl, *Tyto alba* (*TaFAR*, GenBank Accession number JN638549) (Hellenbrand et al., 2011), was synthesized using gBlocks from Integrated DNA Technologies and cloned using forward primer (FP_TaFAR) and reverse primer (RP_TaFAR). The PCR products were flanked with a 40bp region homologous to a constitutive yeast promoter, *TEF1p*, and a terminator, *TEF1t*, at the 5′ and 3′ ends, respectively. The DNA Assembler method (Shao et al., 2009) was then used to construct the *TEF1p-TaFAR-TEF1t* cassette in a multi-copy pRS425 helper plasmid containing *TEF1p* and *TEF1t*, using LEU2 as the auxotrophic marker (Lian and Zhao, 2014).

To construct the pTaFAR_ACC1 plasmid, the *ACC1* gene of *S. cerevisiae* was PCR-amplified from genomic DNA using forward primer (FP_ACC1) and reverse primer (RP_ACC1), which was flanked with a 40bp region homologous to a constitutive yeast promoter, *PGK1p*, and a terminator, *HXT7t*, at the 5′ and 3′ ends, respectively. The DNA Assembler method was used to construct the *PGK1p-ACC1-HXT7t* cassette in a multi-copy pRS425 helper plasmid containing *PGK1p* and *HXT7t*, using LEU2 as the auxotrophic marker (Lian and Zhao, 2014). The *TEF1p-TaFAR-TEF1t* cassette and *PGK1p-ACC1-HXT7t* cassette were next PCR-amplified using two pairs of primers: 1) forward primer (FP_TEF_cassette) and reverse primer (RP_TEF_cassette) for amplifying the *TEF1p-TaFAR-TEF1t* cassette and 2) forward primer (FP_PGK_cassette) and reverse primer (RP_PGK_cassette) for amplifying the *PGK1p-ACC1-HXT7t* cassette. The DNA Assembler method was next used to construct the pTaFAR_ACC1 plasmid harboring pRS425-TEF1p-TaFAR-TEF1t-PGK1p-ACC1-HXT7t.

To construct the pPex30 plasmid, the *PEX30* gene of *S. cerevisiae* was PCR-amplified from genomic DNA using forward primer (FP_Pex30) and reverse primer (RP_Pex30), which was flanked with a 40bp region homologous to a constitutive yeast promoter, *PGK1p*, and a terminator, *HXT7t*, at the 5′ and 3′ ends, respectively. The DNA Assembler method was used to construct the *PGK1p-PEX30-HXT7t* cassette in a multi-copy pRS426 helper plasmid containing *PGK1p* and *HXT7t*, using URA3 as the auxotrophic marker.

Table 1
Plasmids and strains used in this study.

Plasmids used in this study			
Plasmids	Description		Reference
pTaFAR	pRS425-TEF1p-TaFAR-TEF1t		This study
pTaFAR_ACC1	pRS425-TEF1p-TaFAR-TEF1t-PGK1p-ACC1-HXT7t		This study
pPex30	pRS426-PGK1p-PEX30-HXT7t		This study
pIYC08	pRS423-ADH2- ALD6-SeAcs^{L641P}Opt-ERG10		Chen et al. (2013)
pAtACL	pRS423-TPI1p-AtACL1-TPI1t-TEF1p-AtACL2-TEF1t		Lian et al. (2014)
pYlACL	pRS423-TPI1p-YlACL1-TPI1t-TEF1p-YlACL2-TEF1t		Lian et al. (2014)
Strains used in this study			
Strains	Phenotype	Plasmids	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	–	*Saccharomyces* Genome Deletion
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	–	Project
BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ*	–	
BY4741 Δ*POX1*: pTaFAR	BY4741 in which *POX1* has been deleted	pTaFAR	This study
BY4741 Δ*POT1*: pTaFAR	BY4741 in which *POT1* has been deleted	pTaFAR	This study
BY4741 Δ*SNF1*: pTaFAR	BY4741 in which *SNF1* has been deleted	pTaFAR	This study
BY4741 Δ*OPI1*: pTaFAR	BY4741 in which *OPI1* has been deleted	pTaFAR	This study
BY4741 Δ*SIN3*: pTaFAR	BY4741 in which *SIN3* has been deleted	pTaFAR	This study
BY4742 Δ*RPD3*: pTaFAR	BY4742 in which *RPD3* has been deleted	pTaFAR	This study
BY4743 Δ*PAH1*: pTaFAR	BY4743 in which *PAH1* has been deleted	pTaFAR	This study
BY4741 Δ*UME6*: pTaFAR	BY4741 in which *UME6* has been deleted	pTaFAR	This study
BY4741 Δ*ISW2*: pTaFAR	BY4741 in which *ISW2* has been deleted	pTaFAR	This study
BY4741 Δ*ITC1*: pTaFAR	BY4741 in which *ITC1* has been deleted	pTaFAR	This study
BY4743 Δ*MOT1*: pTaFAR	BY4743 in which *MOT1* has been deleted	pTaFAR	This study
BY4741 Δ*SNF1* Δ*SIN3*: pTaFAR	BY4741 in which *SNF1* and *SIN3* have been deleted	pTaFAR	This study
BY4741 Δ*POX1*: pTaFAR_ACC1	BY4741 in which *POX1* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*POT1*: pTaFAR_ACC1	BY4741 in which *POT1* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*SNF1*: pTaFAR_ACC1	BY4741 in which *SNF1* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*OPI1*: pTaFAR_ACC1	BY4741 in which *OPI1* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*SIN3*: pTaFAR_ACC1	BY4741 in which *SIN3* has been deleted	pTaFAR_ACC1	This study
BY4742 Δ*RPD3*: pTaFAR_ACC1	BY4742 in which *RPD3* has been deleted	pTaFAR_ACC1	This study
BY4743 Δ*PAH1*: pTaFAR_ACC1	BY4743 in which *PAH1* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*UME6*: pTaFAR_ACC1	BY4741 in which *UME6* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*ISW2*: pTaFAR_ACC1	BY4741 in which *ISW2* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*ITC1*: pTaFAR_ACC1	BY4741 in which *ITC1* has been deleted	pTaFAR_ACC1	This study
BY4743 Δ*MOT1*: pTaFAR_ACC1	BY4743 in which *MOT1* has been deleted	pTaFAR_ACC1	This study
BY4741 Δ*SNF1*Δ*SIN3*: pTaFAR_ACC1	BY4741 in which *SNF1* and *SIN3* have been deleted	pTaFAR_ACC1	This study
XF1	Same as BY4742 Δ*RPD3*: pTaFAR_ACC1		This study
XF2	XF1	pAtACL	This study
XF3	XF1	pYlACL	This study
XF4	XF1	pIYC08	This study
XF5	XF3 in which *OPI1* has been deleted		This study
XF6	XF3	pPex30	This study

2.3. Determining 1-hexadecanol production and phospholipid content

For screening the 1-hexadecanol production in different strains, the engineered yeast strains were pre-cultured in 3 mL synthetic complete (SC) medium including all the appropriate nucleotides and amino acids, with 2% glucose for three days until saturation. The cells were then centrifuged and washed twice with double-distilled water. The cell pellets were next inoculated into 5 mL fresh SC medium with 2% glucose in glass disposable tubes overlaid with 10% dodecane to prevent the evaporation of fatty alcohols, according to previous report (Runguphan and Keasling, 2013). The concentrations of 1-hexadecanol were quantified at 96 h using a previously described method (Runguphan and Keasling, 2013). In general, the glass tubes of yeast cultures were allowed to sit for 2 min until the organic layer could be clearly visualized. Then, 10 μL of dodecane was withdrawn from the organic layer and mixed with 990 μL of ethyl acetate and analyzed by GC–MS (Shimadzu GC-MS-QP2010) with a DB-Wax column with a 0.25 μm film thickness, 0.25 mm diameter, and 30 m length (Agilent Inc., Palo Alto, CA). Tridecane at a concentration of 2 mg/L was used as the internal standard. The GC program was as follows: an initial temperature of 50 °C was maintained for 1.5 min, followed by ramping to 180 °C at a rate of 25 °C/min. The temperature was then ramped to 250 °C at a rate of 10 °C/min, where the temperature was held for 3 min. To analyze the phospholipid concentration, the yeast cells were centrifuged and washed twice with double-distilled water, followed by using a phospholipid assay kit (Abnova, Taipei, Taiwan) to determine the concentration of phospholipids according to the manufacturer's instructions.

2.4. Screening for the genes with differentiated expression levels

A database for the transcriptional analysis of *ΔRPD3*, *ΔISW2*, *ΔSIN3*, and *ΔUME6* (Fazzio et al., 2001) was used to screen for the genes that were differentially expressed between *ΔRPD3* and *ΔISW2*, *ΔSIN3*, or *ΔUME6* strains. The genes whose expression levels were statistically different in each of the knock-out strains were selected. Among them, the genes with expression levels at least three-fold higher than those in the control (i.e., wild-type strain without gene knock-out) were identified as the "up-regulated genes," while the genes with expression levels at least three-fold lower than those in the control were identified as the "down-regulated genes." The genes that were identified as "up-regulated" or "down-regulated" in the *ΔRPD3* strain but not in the *ΔISW2*,

ΔSIN3, and *ΔUME6* strains were selected as the target genes. Gene ontology analysis of the target genes was performed by using a generic GO term mapper developed by Princeton University (http://go.princeton.edu/cgi-bin/GOTermMapper).

2.5. Batch and fed-batch fermentation for 1-hexadecanol production

To measure the kinetics of 1-hexadecanol production in batch fermentation using high yeast cell density, the engineered yeast strains were first grown in 100 mL SC medium including all the appropriate nucleotides and amino acids, with 2% glucose for 3 days until saturation. Then, cells from 5 mL of culture were centrifuged, washed twice with double-distilled water, and inoculated into 5 mL fresh SC medium with 2% glucose in glass disposable tubes overlaid with 10% dodecane (giving an initial OD_{600} of ~3.0). Samples were taken at various time points to measure the 1-hexadecanol concentration, OD_{600}, and glucose concentration. At each time point, the glass tubes of yeast cultures were allowed to sit for 2 min until the organic layer could be clearly visualized. To measure the 1-hexadecanol concentration, 10 μL of dodecane was withdrawn from the organic layer and then mixed with 990 μL of ethyl acetate, followed by the analysis using the aforementioned GC–MS protocol. To monitor OD_{600}, 20 μL of yeast culture was taken from the water layer and mixed with 180 μL of double-distilled water, followed by measuring the absorbance at 600 nm using a Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT). To measure the concentration of glucose, 20 μL of yeast culture was taken from the water layer and mixed with 180 μL of double-distilled water, which was then centrifuged at 15,000 rpm for 5 min. The supernatant was taken and analyzed by Shimadzu HPLC (Columbia, MD) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and Shimadzu RID-10A refractive index detector. The column was kept at 65 °C, and 0.5 mM sulfuric acid solution was used as a mobile phase with a constant flow rate of 0.6 mL/min. Each data point represents the mean of triplicate samples.

To measure the kinetics of 1-hexadecanol production in fed-batch fermentation with resting cells, the engineered yeast strains were first grown in 100 mL SC medium including all the appropriate nucleotides and amino acids, with 2% glucose for 3 days until saturation. Then, cells from 25 mL culture were centrifuged, washed twice with double-distilled water, and inoculated into 5 mL fresh SC medium with 4% glucose in glass disposable tubes overlaid with 10% dodecane (giving an initial OD_{600} of ~12.0). In this discontinuous fed-batch fermentation, additional glucose (0.5 mL with concentration of 200 g/L) and dodecane (0.05 mL) were fed every 12 h. Samples were taken at various time points to measure 1-hexadecanol concentration, OD_{600}, and glucose concentration by following a similar protocol as that used for sample analysis in batch fermentation.

3. Results

3.1. Manipulating structural genes in yeast lipid metabolism for fatty alcohol production

In order to produce fatty alcohols, we cloned a *FAR* gene from barn owl *Tyto alba* (i.e., *TaFAR*) and expressed it in yeast using a constitutive *TEF1* promoter. It was reported previously (Hellenbrand et al., 2011) that TaFAR can be functionally expressed in *S. cerevisiae* and led to the production of a series of fatty alcohols of which 80% was 1-hexadecanol. As detected in the dodecane layer of the yeast culture, we found that fatty alcohols were produced at ~45 mg/L (Fig. 2) in wild-type BY4741 strains, a similar starting point as previously reported using a mouse FAR (Runguphan and Keasling, 2013). The fatty alcohols produced were mainly in the form of 1-hexadecanol (>97%) with little tetradecanol (<3%) and an absence of octadecanol. As illustrated in many studies (Liu et al., 2010; Tehlivets et al., 2007), the conversion of acetyl-CoA to malonyl-CoA by ACC1 is the bottleneck step in fatty acid synthesis. We therefore over-expressed the *ACC1* gene using a strong constitutive *PGK1* promoter previously identified in our group (Sun et al., 2012) and found that the 1-hexadecanol production was enhanced by 56% (Fig. 2). To further increase the 1-hexadecanol production, we aimed at deleting the β-oxidation pathway to remove the competition for the fatty acyl-CoA precursors. We used the strategy of knocking out two key genes in the β-oxidation pathway: *POX1*, which encodes the first enzyme in the β-oxidation pathway that oxidizes acyl-CoA to trans-2,3-dehydroacyl-CoA; and *POT1*, which encodes the enzyme that cleaves 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA during β-oxidation of fatty acids. It was surprising to find that down-regulating the β-oxidation pathway had an adverse effect on fatty alcohol production, decreasing the fatty alcohol titer from 45 mg/L to less than 15 mg/L. Interestingly, the growth rates and final biomass of *ΔPOX1* and *ΔPOT1* strains (growth rate at 0.16 h^{-1} and final OD_{600} at 2.62 for *ΔPOX1*; growth rate at 0.16 h^{-1} and final OD_{600} at 3.00 for *ΔPOT1*) showed no significant difference compared to the wild-type BY4741 strain expressing TaFAR (growth rate at 0.17 h^{-1} and final OD_{600} at 3.13), indicating that the reduced production of fatty alcohol was unlikely due to the slower growth. The combination of over-expressing *ACC1* and knocking out *POT1* can restore the fatty alcohol production to 61 mg/L. However, no similar effect was found when over-expressing *ACC1* in the context of the *ΔPOX1* strain. Overall, by manipulating the structural genes in fatty acid synthesis and degradation pathways, 1-hexadecanol can be produced at up to 70.8 mg/L, which is at a similar level as that in the recent report (Runguphan and Keasling, 2013) on yeast metabolic engineering for fatty alcohol production.

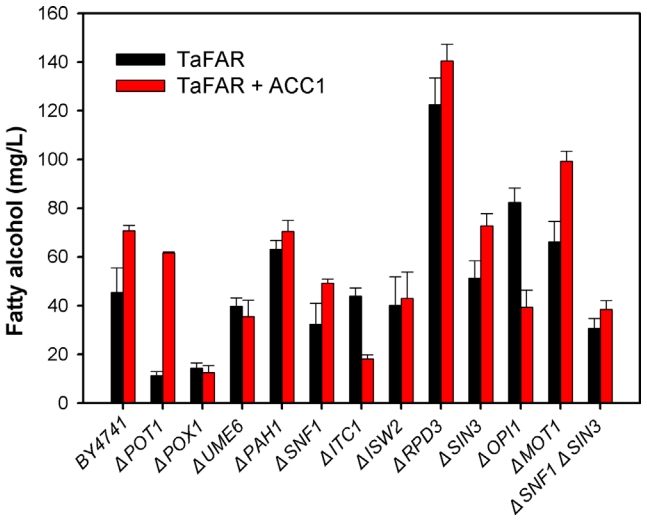

Fig. 2. 1-Hexadecanol produced by engineered *S. cerevisiae* strains. All strains were cultured in minimal medium lacking the appropriate amino acids (i.e., SC-LEU) with 2% glucose. Values are the mean of three biological replicates ± standard deviation ($n=3$) after 96 h. TaFAR: the TaFAR protein expressed by pTaFAR plasmid; TaFAR+ACC1: the TaFAR protein expressed and *ACC1* gene over-expressed by pTaFAR_ACC1 plasmid.

3.2. Manipulating regulatory genes in yeast lipid metabolism for fatty alcohol production

Since only limited success was achieved to enhance fatty alcohol production via manipulating the structural genes, we next sought to develop novel methods that can increase the flux towards fatty alcohol production. One of the reasons that yeast

lipid metabolism is difficult to engineer is the complex transcriptional and post-transcriptional regulations required to maintain homeostasis (Nielsen, 2009; Petranovic et al., 2010). We hypothesized that the metabolic fluxes could be re-distributed if the regulatory machineries were properly perturbed. Therefore, we decided to knock out the negative regulators that have been reported to down-regulate lipid synthesis based on previous transcriptional studies (Fazzio et al., 2001; Goldmark et al., 2000; Kadosh and Struhl, 1997; Usaite et al., 2009) and narrowed down our targets to two families of transcriptional factors: SNF1 protein and regulators of *INO1*. The SNF1 protein belongs to the AMP-activated protein kinase (AMPK) family, participating in cell adaptation to glucose limitation and use of alternative carbon sources (Lo et al., 2001; Woods et al., 1994). Subjected to SNF1 regulation, *ACC1* gene is down-regulated while the β-oxidation pathway is up-regulated (Usaite et al., 2009). Transcriptomic studies of the Δ*SNF1* strain have found that the expression level of *ACC1* was increased while key genes in the β-oxidation pathway (e.g., *POX1* and *POT1*) were significantly down-regulated. A recent report has shown that the deletion of *SNF1* in an oleaginous yeast, *Yarrowia lipolytica*, led to increased accumulation of fatty acids by more than two-fold (Seip et al., 2013). Another study that abolished the SNF1-binding site in the *ACC1* gene via site-directed mutagenesis has successfully improved the activity of ACC1 and fatty acid ethyl ester (FAEE) production in *S. cerevisiae* (Shi et al., 2014). Inspired by the successful applications of manipulating SNF1 to enhance the production of fatty acid-derived chemicals, we chose to knock out SNF1 protein in this study to test its effect on fatty alcohol production. The regulators of *INO1*, on the other hand, have been well known to transcriptionally control phospholipid synthesis by binding to a *cis*-regulatory element called UAS_{INO} in the promoter region of the *INO1* gene, which encodes inositol-1-phosphate synthase as the first step for synthesis of inositol phosphates and inositol-containing phospholipids. The family of regulators of *INO1* consists of a dozen transcription factors that interact with each other to control the expression level of the *INO1* gene (Ambroziak and Henry, 1994; Greenberg and Lopes, 1996; White et al., 1991). Since phospholipid synthesis requires fatty acids as precursors, we expected that the deletion of negative regulators of phospholipid metabolism would enhance phospholipid production and hence increase fatty acid synthesis. In this study, we chose PAH1, RPD3, SIN3, OPI1, UME6, ITC1, ISW2, and MOT1 as the target regulators to knock out since all of them can decrease *INO1* expression in phospholipid production.

We profiled 1-hexadecanol production in the context of various knock-out strains. Cell growth was found to be unaffected in all of the knock-out strains compared to the wild type strains (data not shown). The Δ*SNF1* strain expressing TaFAR did not result in higher 1-hexadecanol production, while the effects of knocking out regulators of *INO1* were found to be divergent. For Δ*PAH1*, Δ*RPD3*, Δ*MOT1*, and Δ*OPI1*, the 1-hexadecanol production was enhanced by 60–170% compared to the wild-type *S. cerevisiae* strain with only TaFAR expressed, with the highest titer reaching 122 mg/L for Δ*RPD3*. The other knock-out strains, including Δ*UME6*, Δ*ITC1*, Δ*SIN3*, and Δ*ISW2*, failed to increase the 1-hexadecanol production significantly. In order to find out if cooperation between the two families of regulators (i.e., SNF1 protein and regulators of *INO1*) can be achieved in terms of 1-hexadecanol production, we also created a double-deletion mutant Δ*SNF1*Δ*SIN3* strain, considering the master role of SIN3 in regulating *INO1* expression (Chen et al., 2007). In fact, SIN3 protein physically contacts most of the other regulators, namely RPD3, ITC1, ISW2, OPI1, and UME6, to form protein complexes in controlling *INO1* expression. Therefore, the double-deletion mutant *ΔSNF1ΔSIN3* can be representative of the cooperation between the two families of regulators. However, we did not find the increased 1-hexadecanol production in *ΔSNF1ΔSIN3* expressing TaFAR, compared to the two single-deletion mutants: *ΔSNF1* and Δ*SIN3* expressing TaFAR. This indicated that cooperation between SNF1 protein and regulators of *INO1* may not be achieved in terms of fatty alcohol production.

We next attempted to explore the possibility of coordinating the manipulation of both structural genes and regulatory genes. As the strategy of over-expressing the *ACC1* gene was found to be the most effective way to boost 1-hexadecanol production among all the strategies of manipulating structural genes, we over-expressed the *ACC1* gene in the context of all the strains with the knock-out of regulatory genes. For the Δ*SNF1* strain, the 1-hexadecanol production was enhanced by 50% with over-expression of *ACC1*. For the strains with knock-out of regulators of *INO1*, the 1-hexadecanol production by Δ*RPD3*, Δ*SIN3*, and Δ*MOT1* was further improved by 19%, 32%, and 43%, respectively, when *ACC1* was over-expressed. However, the over-expression of *ACC1* could not enhance 1-hexadecanol production in the Δ*UME6*, Δ*PAH1*, and Δ*ISW2* strains, and even decreased the titer in Δ*ITC1* and Δ*OPI1* strains (Fig. 2). The enhancement of *ACC1* over-expression was found to be marginal for the Δ*SNF1*Δ*SIN3* strain. Such phenomena clearly indicated that the effects of manipulating structural and regulatory genes are intertwined in a complex manner that cannot be simplified as orthogonal approaches. Overall, by manipulating both structural genes and regulatory genes in lipid metabolism, 1-hexadecanol can be produced at 140.4 mg/L, which is an additional 98% increase compared to the best yeast strain obtained by manipulating the structural genes only.

3.3. Comparative analysis on transcriptional profiles of single deletions of regulators of INO1

It is worth noting that the 1-hexadecanol production between *ΔRPD3* (122.4 mg/L) and *ΔISW2* (40.2 mg/L), *ΔSIN3* (51.2 mg/L), *ΔUME6* (39.9 mg/L) was significantly different (p-value < 0.01). Based on previous transcriptional studies (Fazzio et al., 2001; Goldmark et al., 2000; Kadosh and Struhl, 1997), the RPD3, ISW2, SIN3 and UME6 proteins can coordinate to form a protein complex in controlling the expression of *INO1*. However, the *ΔRPD3* strain produced nearly two fold higher 1-hexadecanol than its counterparts. Therefore, it would be interesting to identify the genes that resulted in such different metabolic behavior as the potential targets for host engineering towards improved 1-hexadecanol production. In order to find the genes responsible for the discrepancy in 1-hexadecanol production between *ΔRPD3* and *ΔISW2*, *ΔSIN3*, or *ΔUME6*, we solicited a transcriptional study that compared the gene expression levels of *ΔRPD3*, *ΔISW2*, *ΔSIN3*, and *ΔUME6* strains to the wild-type strain (Fazzio et al., 2001). As shown in Fig. 3, a total of 18 genes demonstrated different expression levels between *ΔRPD3* and *ΔISW2*, *ΔSIN3*, or *ΔUME6*, nine of which were up-regulated in the *ΔRPD3* but not in the *ΔISW2*, *ΔSIN3*, or *ΔUME6* strains. The other nine genes were down-regulated in the *ΔRPD3* but not in the *ΔISW2*, *ΔSIN3*, or *ΔUME6* strains. Gene Ontology (GO) analysis of the differentially expressed genes (Table 2) indicated that most of the genes were involved in response to chemical stimuli (GO:0042221) and cellular amino acid metabolic processes (GO:0006520). In sum, knocking out different regulators has diverse effects on 1-hexadecanol production, involving different cell-wide transcriptional and metabolic adjustments.

Among the gene targets identified from the comparative transcriptional analysis, several pathways were revealed as being potentially involved in improving fatty alcohol production. For example, the *PEX30* gene involved in negative regulation of the size and number of peroxisomes (Vizeacoumar et al., 2004) for

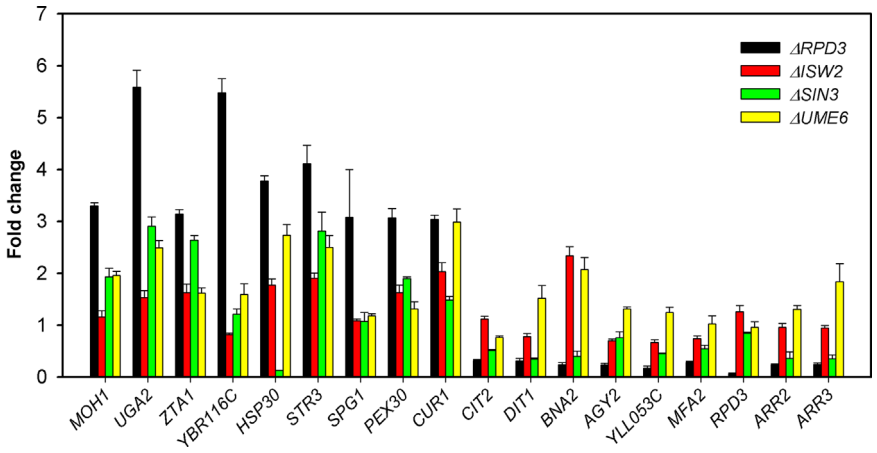

Fig. 3. Differentiated gene expression levels between *ΔRPD3* and *ΔISW2*, *ΔSIN3*, or *ΔUME6*. The transcriptional analysis from Fazzio et al. (2001) was solicited. The genes whose expression levels were statistically different in each of the knock-out strains were selected. Among them, the genes with expression levels of at least three-fold higher than those in the control (i.e., wild-type strain without gene knock-out) were identified as the "up-regulated genes," while the genes with expression levels of at least three-fold lower than those in the control were identified as the "down-regulated genes." The genes that were identified as "up-regulated" or "down-regulated" in the *ΔRPD3* strain but not in the *ΔISW2*, *ΔSIN3*, and *ΔUME6* strains were selected as the target genes. The fold changes of gene expressions in the knock-out strains (i.e., *ΔRPD3*, *ΔISW2*, *ΔSIN3*, and *ΔUME6*) compared to those of the control strain (i.e., wild-type *S. cerevisiae*) are represented as the mean of three biological replicates ± standard deviation ($n=3$).

Table 2
Gene Ontology (GO) analysis of differentially expressed genes between *ΔRPD3* and *ΔISW2*, *ΔSIN3*, or *ΔUME6*.

GO term (GO ID)	Genes annotated to the GO term
Response to chemical stimulus (GO:0042221)	*ARR2, ARR3, MFA2, UGA2, ZTA1*
Cellular amino acid metabolic process (GO:0006520)	*BNA2, CIT2, STR3, UGA2*
Ion transport (GO:0006811)	*ARR3, HSP30*
Response to oxidative stress (GO:0006979)	*UGA2, ZTA1*
Nucleobase-containing small molecule metabolic process (GO:0055086)	*BNA2, HSP30*
Mitotic cell cycle (GO:0000278)	*RPD3*
DNA replication (GO:0006260)	*RPD3*
Transcription from RNA polymerase I promoter (GO:0006360)	*RPD3*
Histone modification (GO:0016570)	*RPD3*
Meiotic cell cycle (GO:0051321)	*RPD3*
DNA-dependent transcription, elongation (GO:0006354)	*RPD3*
Signaling (GO:0023052)	*MFA2*
Carbohydrate metabolic process (GO:0005975)	*CIT2*
Cellular respiration (GO:0045333)	*CIT2*
Cofactor metabolic process (GO:0051186)	*BNA2*
Peroxisome organization (GO:0007031)	*PEX30*
Protein folding (GO:0006457)	*CUR1*
Cell wall organization or biogenesis (GO:0071554)	*DIT1*
Regulation of DNA metabolic process (GO:0051052)	*RPD3*
Generation of precursor metabolites and energy (GO:0006091)	*CIT2*
Sporulation (GO:0043934)	*DIT1*
Response to heat (GO:0009408)	*RPD3*
Transcription from RNA polymerase II promoter (GO:0006366)	*RPD3*
Response to starvation (GO:0042594)	*RPD3*
Chromatin organization (GO:0006325)	*RPD3*
Regulation of cell cycle (GO:0051726)	*RPD3*
Conjugation (GO:0000746)	*MFA2*
DNA recombination (GO:0006310)	*RPD3*

fatty acid degradation was up-regulated in *ΔRPD3*, suggesting that the up-regulated *PEX30* could decrease the size and number of peroxisomes and hence decreased fatty acid degradation through β-oxidation in *ΔRPD3*. We then over-expressed *PEX30* in the host engineering effort discussed below.

3.4. Host engineering to enhance the supply of cytosolic acetyl-CoA

The biosynthesis of fatty acid and fatty acid-derived chemicals requires cytosolic acetyl-CoA as the primary precursor. Therefore, to further increase 1-hexadecanol production, we next chose the yeast strain in which the *ACC1* gene was over-expressed and *RPD3* gene was knocked out as the parent strain (i.e., XF1), and applied two strategies to enhance the supply of acetyl-CoA in the cytosol (Fig. 4). The first strategy was expressing the ATP-dependent citrate lyase (ACL) from either *Arabidopsis thaliana* or *Yarrowia lipolytica*, which converted the citrate to acetyl-CoA and oxaloacetate in the cytosol and hence increased the availability of cytosolic acetyl-CoA. As expected, the resulting XF2 and XF3 strains, expressing ACL from *A. thaliana* and *Y. lipolytica*, respectively, achieved a 55% and 136% increase in terms of 1-hexadecanol production compared to the parent strain (XF1). Beside the expression of ACL, we also tried the PDH-bypass pathway in which ADH2 and ALD6 were expressed and a codon-optimized heterologous ACS mutant was used (Chen et al., 2013) to enhance acetyl-CoA production in the cytosol. The resulting strain XF4 led to a 69% increase in 1-hexadecanol production. As indicated by another study in our group, combining the expression of ACL from *Y. lipolytica* and the PDH-bypass pathway with codon-optimized ACS cannot

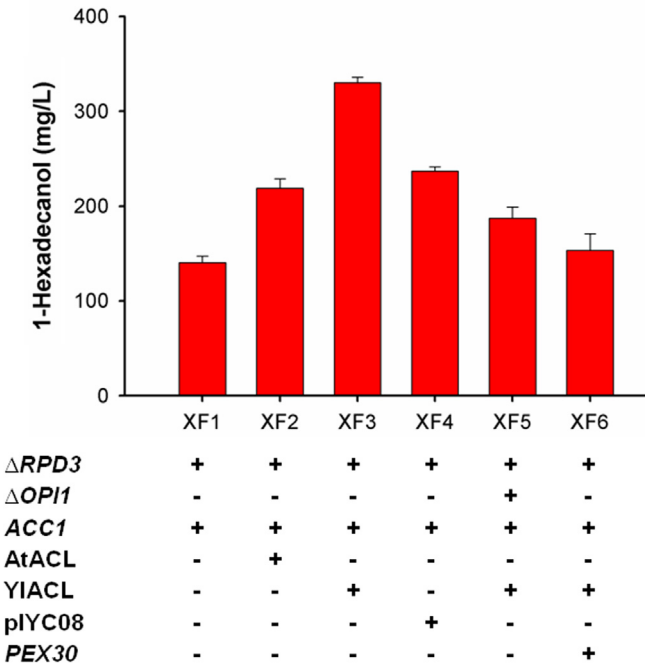

Fig. 4. Host engineering to improve 1-hexadecanol production. *ΔRPD3*: knock-out of *RPD3* gene; *ΔOPI1*: knock-out of *OPI1* gene; *ACC1*: over-expression of *ACC1* gene; AtACL: expression of ACL from *Arabidopsis thaliana*; YlACL: expression of ACL from *Yarrowia lipolytica*; pIYC08: expression of the pathway that has been previously reported to enhance cytosolic acetyl-CoA (Chen et al., 2013); *PEX30*: over-expression of *PEX30* gene. All strains were cultured in minimal medium lacking the appropriate amino acids with 2% glucose. Values are the mean of three biological replicates ± standard deviation ($n=3$) after 96 h.

improve the production of acetyl-CoA based chemicals, e.g., *n*-butanol (Lian et al., 2014). Therefore, we did not combine the two strategies in this study for producing 1-hexadecanol.

Since the expression of ACL from *Y. lipolytica* led to the highest titer of 1-hexadecanol in tube culture, we next tried to further improve the fatty alcohol production by knocking out another negative regulator, OPI1 protein, in the context of XF3. The knock-out of *OPI1* alone was found to increase 1-hexadecanol production by 81% when engineering the regulatory genes of yeast lipid metabolism (Fig. 2). However, the *OPI1* deletion failed to improve the 1-hexadecanol titer in the context of simultaneous deletion of the *RPD3* gene, over-expression of the endogenous *ACC1* gene and the heterologous expression of ACL from *Y. lipolytica* (strain XF5, Fig. 4). We also tried to over-express another endogenous gene, *PEX30*, in the context of XF3. Up-regulation of the *PEX30* gene was found to decrease fatty acid degradation through β-oxidation (Vizeacoumar et al., 2004) and was also suggested as a potential target by the comparative transcriptional analysis. However, over-expression of the *PEX30* gene in XF3 cannot further increase the 1-hexadecanol production (strain XF6, Fig. 4), possibly due to the complex control of the size and number of peroxisomes. Besides PEX30, there are over 20 regulators coordinated in controlling the size and number of peroxisomes. Therefore, it is possible that other regulators could play more significant roles than PEX30 in fatty acid degradation. Overall, by engineering the supply of acetyl-CoA in the cytosol, 1-hexadecanol can be produced at 330.2 mg/L, which is an additional 136% increase compared to the best yeast mutant obtained by manipulating both structural genes and regulatory genes of yeast lipid metabolism.

3.5. Batch and fed-batch fermentation for 1-hexadecanol production

With XF3 as our best strain to produce 1-hexadecanol, we next characterized its 1-hexadecanol production using both batch and fed-batch fermentation. In batch culture, we inoculated the cells with initial OD_{600} as high as ~3.0, while in fed-batch fermentation, we used resting cells with initial OD_{600} at ~12.0 (Fig. 5). By using high cell density or even resting cells, yeast growth can be prevented so that 1-hexadecanol can be more efficiently converted from glucose. Within 36 h in the batch culture, 655 mg/L 1-hexadecanol was produced from 20 g/L glucose in XF3, while 533 mg/L 1-hexadecanol was produced in the control strain, XF1 (i.e., the strain that was engineered by manipulating both structural genes and regulatory genes in yeast lipid metabolism, but without enhancing acetyl-CoA supply in the cytosol). The highest titer of 1-hexadecanol was achieved using fed-batch fermentation with resting cells, in which the XF3 strain produced 1111 mg/L 1-hexadecanol while the control strain XF1 produced 684 mg/L in 48 h. Interestingly, during the fed-batch fermentation of XF3, most of the 1-hexadecanol (~90%) was produced within the first 24 h, while little fatty alcohol was generated afterward. A similar production profile was also reported in another study of fed-batch fermentation using an engineered *E. coli* strain for producing C12/C14 alcohol (Zheng et al., 2012). Such a phenomenon was hypothesized to be attributed to the diversion of carbon flux to fatty aldehydes instead of fatty alcohols at the late stage of fed-batch fermentation (Zheng et al., 2012). Also, we found that 1-hexadecanol can be metabolized by *S. cerevisiae*, as detected by a growth assay (Fig. S1). Therefore, the decreased productivity of 1-hexadecanol and the degradation of fatty alcohol could limit the accumulation of 1-hexadecanol after 24 h. Overall, through fed-batch fermentation with resting cells, 1-hexadecanol was produced by XF3 at the highest reported titer of 1111 mg/L, a 10-fold increase compared to the previous report on engineering yeast for fatty alcohol production (Runguphan and Keasling, 2013). The yield and productivity of 1-hexadecanol production achieved in this study were 30 mg 1-hexadecanol/g glucose and 42 mg/L/h, respectively.

4. Discussion

4.1. Mechanistic studies of manipulating regulatory genes for fatty alcohol production

In this study, we have identified new target genes for engineering yeast lipid metabolism to produce fatty acid-derived biofuels and chemicals. The idea of manipulating transcription factors has been illustrated in previous reports (Nevoigt, 2008; Ro et al., 2006) to improve biochemical production. The global transcription machinery engineering (gTME) approach (Alper et al., 2006) was also developed to evolutionarily alter key transcription factors to improve yeast fermentation performance. However, when engineering yeast lipid metabolism for producing biofuels and chemicals, structural genes have heretofore been the only targets subject to genetic manipulations (Runguphan and Keasling, 2013). As demonstrated in this study, manipulating the appropriate transcription factors in yeast lipid metabolism can be advantageous and cooperative with the strategies of manipulating structural genes.

To understand why knocking out regulators of *INO1* led to improved productivity of fatty alcohol, we hypothesized that knocking out the negative regulators resulted in the up-regulation of phospholipid synthesis and created a driving force for synthesizing fatty acids as the precursors for phospholipid production. As more flux was diverted into fatty acid synthesis, the availability of fatty acyl-CoA increased and hence led to enhanced production of fatty alcohol. If so, the fatty alcohol production would be correlated with the phospholipid content of the engineered strains. As determined by the phospholipid assay, the phospholipid content in different knock-out strains was almost linearly correlated with the 1-hexadecanol production ($R^2=0.87$, Fig. 6). The only outlier was the *ΔUME6* strain, which was predicted to produce over 80 mg/L 1-hexadecanol based

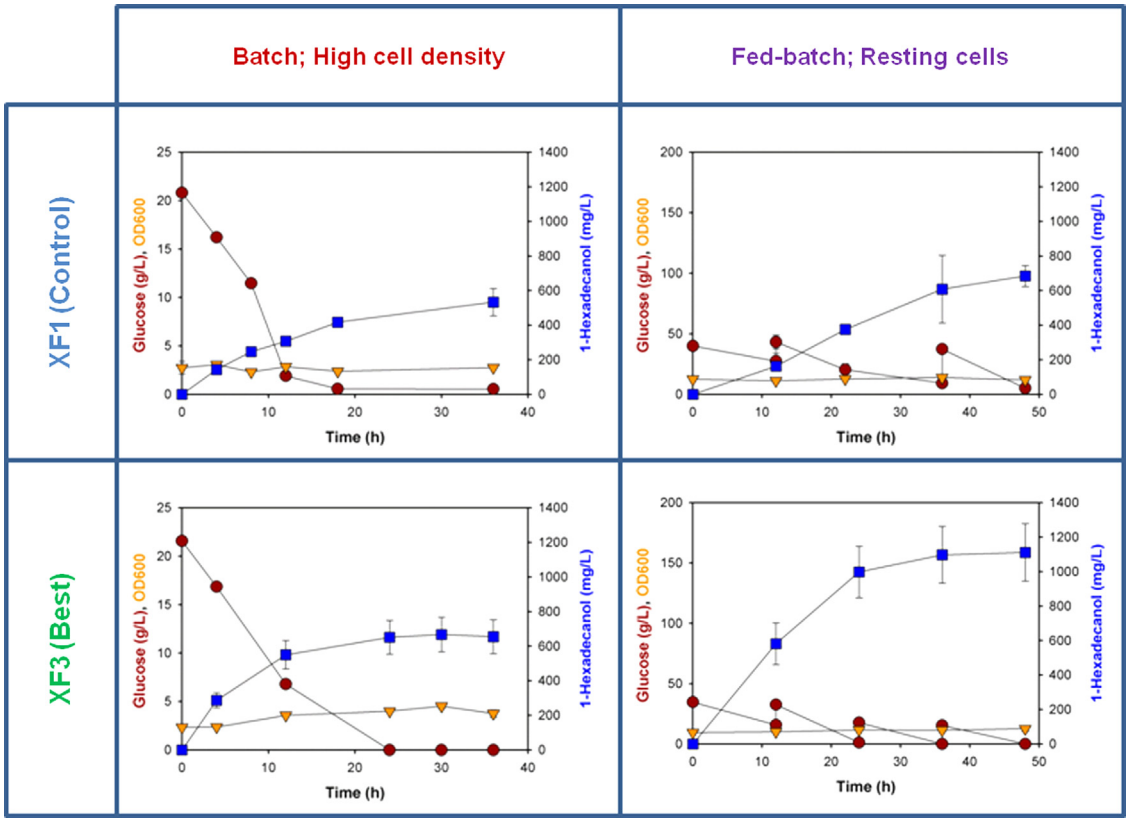

Fig. 5. Batch fermentation and fed-batch fermentation for 1-hexadecanol production. Two strains were chosen for the fermentation: XF1 strain, the control strain in which the *ACC1* gene was over-expressed and the *RPD3* gene was knocked out, without enhancing the cytosolic acetyl-CoA supply; XF3 strain, currently the best strain in which the *ACC1* gene was over-expressed, the *RPD3* gene was knocked out, and ACL from *Yarrowia lipolytica* was expressed to enhance cytosolic acetyl-CoA supply. The batch fermentation for XF1 and XF3 used high cell density (i.e., OD_{600} maintained at ~3.0). The fed-batch fermentation for XF1 and XF3 used resting cells (i.e., OD_{600} maintained at ~12.0). All strains were cultured in minimal medium lacking the appropriate amino acids. Values are the mean of three biological replicates ± standard deviation ($n=3$) at each of the time points.

on the correlation between phospholipid content and 1-hexadecanol production, but only produced 40 mg/L instead. The poor fit of *ΔUME6* into the phospholipid-1-hexadecanol correlation indicated that the regulatory mechanisms for 1-hexadecanol production are still too complex to be elucidated completely, and other metabolic processes must be involved in regulating 1-hexadecanol production.

One interesting question that naturally arises in this study is whether or not the deletion of multiple regulatory genes could cooperate with the manipulation of structural genes. As shown in this study (Fig. 2), over-expression of the *ACC1* gene and deletion of a single regulatory gene demonstrated nonlinear behaviors as 1-hexadecanol was produced at different titers. Since it is prohibitively resource-intensive to construct all of the possible double- and triple-deletions of regulatory genes (i.e., 72 double-deletions and 504 triple-deletions), we did not pursue such work in our current study. However, deletion of more than one regulatory gene may not be beneficial to fatty alcohol production. As we discovered, the main reason that the deletion of regulatory genes could increase fatty alcohol production was the enhancement of phospholipid production, which generated a strong pulling flux towards fatty acyl-CoA and fatty alcohol production. Phospholipid production is tightly controlled through regulating the expression of the *INO1* gene. The regulators of *INO1* that were engineered in this study, e.g., RPD3 and SIN3, have been proved to be functional in the same regulatory pathway, in which multiple regulators form a protein complex that binds to a *cis*-regulatory element of *INO1* (Chen et al., 2007). Therefore, the double- and triple-deletion of regulators of *INO1* would lead to no more severe phenotype than the single deletion, as previously reported (Kasten et al., 1997). To further prove such hypothesis, we have constructed a double-deletion strain by deleting the *RPD3* gene

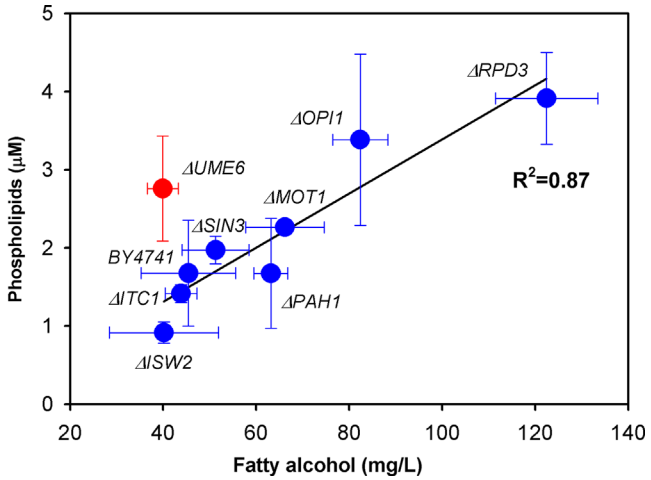

Fig. 6. Correlation between phospholipid production and 1-hexadecanol production in engineered *S. cerevisiae* strains. All strains were cultured in minimal medium lacking the appropriate amino acids (i.e., SC-LEU) with 2% glucose. Values are the mean of three biological replicates ± standard deviation ($n=3$) after 96 h.

and *OPI1* gene. The *ΔRPD3* and *ΔOPI1* strains were ranked as the first and second best mutants, respectively, in terms of 1-hexadecanol production (Fig. 2). However, when both of the regulators are deleted, the 1-hexadecanol production cannot be further improved (Fig. 4), indicating that the regulatory pathway has been sufficiently disturbed, leaving no room for further manipulation. We also created another double-deletion stain, *ΔSNF1ΔSIN3*, since the SNF1 protein and the regulators of *INO1* employ different mechanisms in

regulating fatty acid metabolism. It turns out that the *ΔSNF1ΔSIN3* double-deletion did not increase fatty alcohol production either, confirming the hypothesis that the main mechanism for the improvement of fatty alcohol production was the pulling flux caused by phospholipid production. It is also worth noting that the effects of deleting multiple structural genes or regulatory genes could be different. As reported in many metabolic engineering applications, the additional deletion of certain structural genes could actually ameliorate or even reverse the non-optimal effects of previous mutants. For example, the deletion of *fnr* gene in addition to *ΔadhΔldhΔfrdΔpta* increased the 1-butanol production in *E. coli* by over 65% (Atsumi et al., 2008). Such a phenomenon may result from better balancing of cofactor usage as the topology of the metabolic network was modified. However, a similar phenomenon may not be achieved when the regulatory genes are the targets to be engineered, as these genes cannot change the architecture of the metabolic network.

4.2. The rate-limiting steps in producing fatty alcohol

It has been well studied that the first reaction in fatty acid synthesis catalyzed by *ACC1* was one of the rate-limiting steps when engineering yeast for producing fatty acid and fatty acid-derived chemicals (Runguphan and Keasling, 2013; Tehlivets et al., 2007). Consistent with previous reports, we also confirmed the important role of over-expressing the *ACC1* gene in terms of 1-hexadecanol production (Fig. 2). Interestingly, removing or down-regulating the β-oxidation pathway cannot improve the fatty alcohol production and even had adverse effects, as we found in this study. Such observation emphasized the pivotal role played by the metabolic regulation in controlling the fate of yeast lipid metabolism. Indeed, when we selectively knocked out the negative regulators of yeast lipid metabolism, we achieved an additional improvement of 1-hexadecanol production based on the manipulation of structural genes in lipid pathways. Therefore, it is worth noting that the tight regulation of yeast lipid metabolism could be another rate-limiting step in yeast metabolic engineering for producing fatty acid-derived chemicals.

The fatty acid synthesis pathway connects with the central carbon metabolic pathways in the cytoplasm through the hub metabolite, acetyl-CoA. Acetyl-CoA metabolism in yeast is quite complex, as the molecule is generated and consumed in different compartments and cannot be freely transported among these compartments (Chen et al., 2013). Here in this study, we found that enhancing the supply of cytosolic acetyl-CoA via diverse approaches could universally improve the 1-hexadecanol production, indicating that the limited availability of precursors stands as another rate-limiting step in fatty alcohol production. The critical effects of increasing the supply of cytosolic acetyl-CoA have been reported in many studies, improving the production of a series of chemicals, including α-santalene (Chen et al., 2013), polyhydroxybutyrate (PHB) (Kocharin et al., 2013), 3-hydroxypropionic acid (Chen et al., 2014), and *n*-butanol (Krivoruchko et al., 2013; Lian et al., 2014). In our study, we have found that the expression of ACL from *Y. lipolytica* led to a higher fatty alcohol titer than that from *A. thaliana*, which was hypothesized to be due to better protein solubility. To prove the hypothesis, we have fused EGFP with the ACL proteins from *Y. lipolytica* and *A. thaliana*, respectively, and compared their fluorescence intensity. In this way, we could easily identify significant differences in protein solubility. As expected (Fig. S2), the fusion protein of YlACL1-EGFP (i.e., linking ACL1 from *Y. lipolytica* and EGFP) had much stronger signals (~1.6 fold) than that of AtACL1-EGFP (i.e., linking ACL1 from *A. thaliana* and EGFP). Similarly, the fluorescence intensity of fusion protein YlACL2-EGFP was also much stronger (~2.7 fold) than that of AtACL2-EGFP. Such results were consistent with our hypothesis that the ACL proteins from *Y. lipolytica* were more soluble when expressed in yeast and hence led to better improvement in supplying cytosolic acetyl-CoA. Another pathway using a codon-optimized ACS from a facultative anaerobic bacterium (i.e., *Salmonella enterica*) also led to a dramatic increase of 1-hexadecanol production (~69%) in this study. It has been reported that the activity of ACL increased with the supply of oxygen (Chávez-Cabrera et al., 2010), while the activity of some bacterial ACS enzymes was found to be sensitive to oxygen (Jetten et al., 1989; Seravalli et al., 2002). Since fatty acid biosynthesis in yeast requires oxygen (Fornairon-Bonnefond et al., 2002), we have chosen the aerobic growth condition in this study to produce 1-hexadecanol. This could be the reason that the bacterial ACS used in this study did not lead to a similarly high increase of 1-hexadecanol production as that from ACL, as its maximal activity might not be achieved under the aerobic condition.

The 1-hexadecanol was produced at a titer over 1.1 g/L. However, the yield of 1-hexadecanol (~0.03 g/g) was still far away from the theoretical yield (~0.34 g/g), indicating the presence of other rate-limiting steps for fatty alcohol production. One of the bottlenecks we hypothesized is the limited supply of NADPH as required in fatty acid synthesis and fatty alcohol production. However, previous endeavors to enhance the NADPH supply in the cytoplasm by expressing a $NADP^+$ dependent malic enzyme from *Mortierella alpina* failed to increase fatty alcohol production (Runguphan and Keasling, 2013). Recently, it was found that when coupled with the enhanced precursor supply, the over-production of NADPH could lead to over five-fold increase of 3-hydroxypropionic acid production (Chen et al., 2014). Therefore, we are currently trying to engineering the cytosolic NADPH supply, together with our established strategies of expressing ACL, over-expressing the *ACC1* gene and knocking out the *RPD3* gene, to further increase the 1-hexadecanol production.

5. Conclusions

S. cerevisiae was engineered to produce 1-hexadecanol from glucose in minimal medium at the highest reported titer (> 1100 mg/L) in fed-batch fermentation with resting cells. To achieve this result, a bird FAR was first heterologously expressed, followed by over-expressing the *ACC1* gene, deleting a negative regulator in phospholipid synthesis, RPD3, and enhancing the supply of cytosolic acetyl-CoA via expression of ACL from *Y. lipolytica*. As illustrated in this study, a panel of regulators was recognized as the potential targets for engineering yeast lipid metabolism, while the cytosolic acetyl-CoA supply was identified as one of the rate-limiting steps when engineering yeast for producing fatty acid-derived biofuels and chemicals.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.10.001.

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