Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals

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ABSTRACT

As the serious effects of global climate change become apparent and access to fossil fuels becomes more limited, metabolic engineers and synthetic biologists are looking towards greener sources for transportation fuels. In recent years, microbial production of high-energy fuels by economically efficient bioprocesses has emerged as an attractive alternative to the traditional production of transportation fuels. Here, we engineered the budding yeast *Saccharomyces cerevisiae* to produce fatty acid-derived biofuels and chemicals from simple sugars. Specifically, we overexpressed all three fatty acid biosynthesis genes, namely acetyl-CoA carboxylase (*ACC*1), fatty acid synthase 1 (*FAS*1) and fatty acid synthase 2 (*FAS*2), in *S. cerevisiae*. When coupled to triacylglycerol (TAG) production, the engineered strain accumulated lipid to more than 17% of its dry cell weight, a four-fold improvement over the control strain. Understanding that TAG cannot be used directly as fuels, we also engineered *S. cerevisiae* to produce drop-in fuels and chemicals. Altering the terminal "converting enzyme" in the engineered strain led to the production of free fatty acids at a titer of approximately 400 mg/L, fatty alcohols at approximately 100 mg/L and fatty acid ethyl esters (biodiesel) at approximately 5 mg/L directly from simple sugars. We envision that our approach will provide a scalable, controllable and economic route to this important class of chemicals.

1. Introduction

Unsustainable demands, rising oil costs and concerns over climate change have inspired interest in renewable fuels and chemicals (Fortman et al., 2008). Microbial production of high-energy fuels via economically efficient and environmentally sustainable bioprocesses has recently emerged as a viable alternative to the conventional production of transportation fuels (Lynd et al., 2005). Fatty acids, sometimes touted as nature's 'petroleum', are long-chain carboxylic acids that cells use for both chemical and energy storage functions. These energy-rich molecules are currently derived from plant oils and animal fats. However, increasing food prices worldwide have rekindled debate over the competition of agricultural resources between the energy sector and the food industry. Therefore, alternatives to agricultural crops are urgently needed for the production of sustainable and economical biofuels. Namely, producing fatty acid-derived biofuels directly from abundant and cost-effective renewable resources by microbial fermentation is an attractive alternative biofuel production method.

In the phospholipid form, fatty acids are a major component of cell membranes in all organisms. Certain species of yeasts and microalgae can accumulate fatty acids in the neutral form as triacylglycerols (TAG) at up to 30–70% of dry cell weight (Beopoulos et al., 2009). While naturally possessing a lower lipid content (between 3.5% and 10.7% of DCW) (Johnson et al., 1972), *Saccharomyces cerevisiae* offers several advantages over oleaginous yeasts and microalgae as a production host for fatty acids and derivatives (Tang et al., 2013). Namely, *S. cerevisiae* is more genetically tractable than oleaginous yeasts and microalgae; thus, genetic tools for metabolic pathway manipulation are more abundant. Second, the generation, isolation, and analysis of *S. cerevisiae* mutant strains can be performed with relative ease, and deletion strains for most coding genes are commercially available. Third, *S. cerevisiae* has a proven track record in various industrial applications, and the fermentation of *S. cerevisiae* has been previously manipulated to produce numerous heterologous metabolites. Finally, *S. cerevisiae* is easily cultivated in chemically defined medium and exhibits fast growth rates, thus facilitating scaling-up processes.

Because fatty acids are integral parts of all living organisms, their biosynthesis and regulation have been comprehensively

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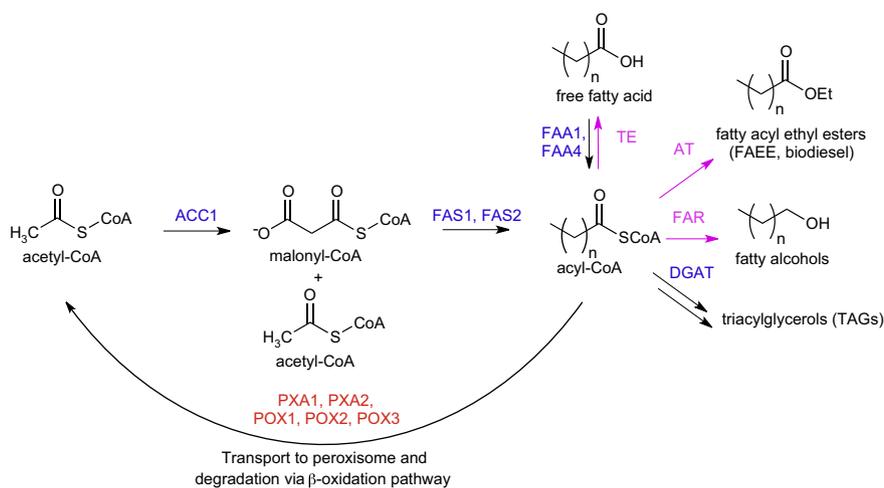 *E-mail address:* keasling@berkeley.edu (J.D. Keasling).

Fig. 1. Engineered pathways for the production of fatty acid-derived molecules from simple sugars in *S. cerevisiae*. Flux through the native fatty acid pathway (black lines) can be increased to improve production of acyl-CoAs by overexpressing acetyl-CoA carboxylase (ACC1) and fatty acid synthases (FAS1 and FAS2), and by eliminating a portion of the β-oxidation pathway (peroxisomal transporters PXA1 and PXA2, and β-oxidation enzymes POX1, POX2 and POX3). Various products can be produced from non-native pathways (magenta lines) including free fatty acids, fatty alcohols and fatty acid ethyl esters (FAEEs or biodiesel). Free fatty acids can be produced directly from acyl-CoAs by overexpressing an acyl-CoA thioesterase (TE); fatty alcohols can be produced by overexpressing a fatty acyl-CoA reductase (FAR); and biodiesels can be produced by expressing a wax-ester synthase/acyltransferase (WS/AT). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

studied in both prokaryotes and eukaryotes (Magnuson et al., 1993; Tehlivets et al., 2007). *De novo* fatty acid biosynthesis in *S. cerevisiae* requires acetyl-CoA carboxylase (ACC; encoded by the *ACC*1 gene) and the fatty acid synthase complex (FAS; encoded by *FAS*1 and *FAS*2) (Al-Feel et al., 1992) (Fig. 1). ACC converts acetyl-CoA into malonyl-CoA. Subsequently, the FAS complex condenses one equivalent of acetyl-CoA and 7–8 equivalents of malonyl-CoA into C16–C18 fatty acyl-CoAs. Yeast FAS complex is a 2.6-MDa protein consisting of two non-identical, multifunctional subunits, α and β, organized as a hexamer ($\alpha 6\beta 6$) (Schweizer and Hofmann, 2004). As they emerge from the FAS complex, newly synthesized fatty-acyl CoAs are bound to acyl-CoA binding protein (ACBP; encoded by the *ACBP*1 gene), which facilitates intracellular transport of acyl-CoA to the endoplasmic reticulum and lipid bodies for phospholipids and TAG biosynthesis (Knudsen et al., 1999). Notably, all of *S. cerevisiae* C16–C18 fatty acid biosynthesis enzymes are encoded by merely two genes (*FAS*1 and *FAS*2), as opposed to ten separate genes (*FabA, FabB, FabD, FabF, FabG, FabH, FabI, FabZ, Acp* and *TesA*) as is the case for *E. coli*. This distinction allows us to overexpress the entire pathway in a more straightforward manner.

Because fatty acids serve multiple cellular functions in yeast, their biosynthesis—from the conversion of acetyl-CoA to malonyl-CoA by ACC to the subsequent production of fatty acyl-CoA by the FAS complex—is tightly regulated at multiple levels (Tehlivets et al., 2007). Moreover, fatty acid biosynthesis is feedback inhibited by long chain acyl-CoA. ACC is inhibited by extremely low concentrations of long-chain acyl-CoA ($K_i = 1$–5 nM) (Ogiwara et al., 1978). Altogether, these mechanisms ensure that the cell does not accumulate excess quantities of this energy-rich metabolite. In order to overproduce fatty acid-derived biofuels in *S. cerevisiae*, these regulatory elements must be mitigated. A common strategy to relieve feedback inhibition by acyl-CoA is the overexpression of either the endogenous or heterologous acyl–acyl carrier protein (ACP) or acyl-CoA thioesterase to produce free fatty acids (Fig. 1).

While TAGs and free fatty acids are valuable, they cannot be used directly as fuels and must first be chemically processed prior to utilization. Therefore, renewable fuels that are directly compatible with existing infrastructure are in great demand. Over 1 billion gallons of biodiesel, a renewable alternative to diesel fuel, are produced each year in the US alone (U.E.P. Agency, 2012). Composed of fatty acid methyl and ethyl esters (FAMEs and FAEEs,

respectively), biodiesel is traditionally derived from the chemical transesterification of plant oils and animal fats (Hill et al., 2006). Fatty alcohols are also important oleochemicals and find many industrial applications ranging from lubricants to cosmetics. Traditionally, fatty alcohols are produced in two chemical steps from plant oils and animal fats: (1) transesterification/hydrolysis of plant oils and animal fats to methyl esters and fatty acids and (2) hydrogenation of methyl esters and fatty acids to fatty alcohols.

Our lab and others have recently engineered *E. coli* to produce free fatty acids, FAEEs, fatty alkanes/alkenes and fatty alcohols directly from glucose at titers of up to several grams per liter in the case of free fatty acids (Dellomonaco et al., 2011; Howard et al., 2013; Steen, 2010; Xu et al., 2013). A disadvantage of using *E. coli* as a host for FAEE and fatty alcohol production is that the direct product of fatty acid synthase enzymes is in the form of fatty acyl-ACP. This enzyme-linked product needs to be hydrolyzed by a thioesterase to free fatty acid and subsequently activated to fatty acyl-CoA by a ligase before any converting enzymes (acyltransferase to produce FAEEs or fatty acyl-CoA reductase to produce fatty alcohols) can act on them (Steen, 2010). Because the product of *S. cerevisiae* fatty acid synthase is already in the form of fatty acyl-CoA—the correct form for the "converting enzymes"—the yeast system is a more direct way to produce FAEEs and fatty alcohols. Moreover, *E. coli* is susceptible to phage attacks, which could hamper production at industrial levels (Los et al., 2004). Here, we demonstrate that engineering fatty acid biosynthesis by overexpressing key fatty acid and TAG biosynthesis enzymes augmented TAG accumulation (Fig. 1). Additionally, we developed a fatty acid-overproducer strain by replacing the native promoters of all fatty acid biosynthesis genes with a strong constitutive promoter (TEF1 promoter (P_{TEF1})). Depending on the choice of terminal "converting enzyme"—a thioesterase, fatty acyl-CoA reductase or wax ester synthase—this overproducer could produce and secrete free fatty acids, fatty alcohols and FAEEs into the culture medium.

2. Materials and methods

2.1. Yeast strain, media and transformation

The yeast strains used in this study were constructed from BY4742 (derivative of S288C, (Mat α; his3Δ1; leu2Δ0; lys2Δ0;

Table 1
Strains used in this study.

Strain name	Genotype	Description	Reference
BY4742	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0	None	
BY4742 ΔPOX1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pox1Δ	BY4742 in which POX1 has been deleted	Saccharomyces Genome Deletion Project
BY4742 ΔPXA2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pxa2Δ	BY4742 in which PXA2 has been deleted	Saccharomyces Genome Deletion Project
BY4742 ΔFAA1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ	BY4742 in which FAA1 has been deleted	This study
BY4742 ΔFAA4	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa4Δ	BY4742 in which FAA4 has been deleted	This study
BY4742 ΔFAA1 ΔFAA4	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; faa4Δ	BY4742 in which FAA1 and FAA4 have been deleted	This study
BY4742 P$_{TEF1}$-ACC1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; acc1::P$_{TEF1}$-ACC1	BY4742 in which the promoter of ACC1 has been changed to TEF1 promoter	This study
BY4742 ΔPOX1 P$_{TEF1}$-ACC1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pox1 Δ; acc1::P$_{TEF1}$-ACC1	BY4742 in which POX1 has been deleted and the promoter of ACC1 has been changed to TEF1 promoter	This study
BY4742 ΔPXA2 P$_{TEF1}$-ACC1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pxa2 Δ; acc1::P$_{TEF1}$-ACC1	BY4742 in which PXA2 has been deleted and the promoter of ACC1 has been changed to TEF1 promoter	This study
BY4742 P$_{TEF1}$-FAS1-FAS2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	BY4742 in which the promoters of FAS1 and FAS2 have been changed to TEF1 promoter	This study
BY4742 ΔPOX1 P$_{TEF1}$-FAS1-FAS2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pox1 Δ; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	BY4742 in which POX1 has been deleted and the promoters of FAS1 and FAS2 have been changed to TEF1 promoter	This study
BY4742 ΔPXA2 P$_{TEF1}$-FAS1-FAS2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pxa2 Δ; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	BY4742 in which PXA2 has been deleted and the promoters of FAS1 and FAS2 have been changed to TEF1 promoter	This study
WRY1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	BY4742 in which the promoters of ACC1, FAS1 and FAS2 have been changed to TEF1 promoter	This study
BY4742 P$_{TEF1}$-DGA1	Mat α; ; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; dga1::P$_{TEF1}$-DGA1	BY4742 in which the promoter of DGA1 has been changed to TEF1 promoter	This study
BY4742 ΔPOX1 P$_{TEF1}$-DGA1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pox1 Δ; dga1::P$_{TEF1}$-DGA1	BY4742 in which POX1 has been deleted and the promoter of DGA1 has been changed to TEF1 promoter	This study
BY4742 ΔPXA2 P$_{TEF1}$-DGA1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pxa2 Δ; dga1::P$_{TEF1}$-DGA1	BY4742 in which PXA2 has been deleted and the promoter of DGA1 has been changed to TEF1 promoter	This study
WRY1 ΔPOX1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pox1Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which POX1 has been deleted	This study
WRY1 ΔPXA2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; pxa2Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which PXA2 has been deleted	This study
WRY1 ΔFAA1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA1 has been deleted	This study
WRY1 ΔFAA1 ΔPOX1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; pox1Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA1 and POX1 have been deleted	This study
WRY1 ΔFAA1 ΔPXA2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; pxa2Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA1 and PXA2 have been deleted	This study
WRY1 ΔFAA4	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa4Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA4 has been deleted	This study
WRY1 ΔFAA4 ΔPOX1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa4Δ; pox1Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA4 and POX1 have been deleted	This study
WRY1 ΔFAA4 ΔPXA2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa4Δ; pxa2Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA4 and PXA2 have been deleted	This study
WRY1 ΔFAA1 ΔFAA4	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; faa4Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA1 and FAA4 have been deleted	This study
WRY1 ΔFAA1 ΔFAA4 ΔPOX1	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; faa4Δ; pox1Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA1, FAA4 and POX1 have been deleted	This study
WRY1 ΔFAA1 ΔFAA4 ΔPXA2	Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; faa1Δ; faa4Δ; pxa2Δ; acc1::P$_{TEF1}$-ACC1; fas1::P$_{TEF1}$-FAS1; fas2::P$_{TEF1}$-FAS2	WRY1 in which FAA1, FAA4 and PXA2 have been deleted	This study

Table 2
Plasmids used in this study.

Plasmid name	Description	Reference
pESC-His3-FAS1-FAS2	pESC-His3-P_{GAL1}-FAS1/P_{GAL10}-FAS2	This study
pESC-Ura3-ACC1	pESC-Ura3-P_{GAL10}-ACC1	This study
pESC-Leu2d-DGA1	pESC-Leu2d-P_{GAL1}-DGA1	This study
pESC-Leu2d-'TesA	pESC-Leu2d-P_{GAL10}-'TesA	This study
pESC-Leu2d-mFAR1	pESC-Leu2d-P_{GAL1}-mFAR1	This study
pESC-Leu2d-m FAR1-MaME	pESC-Leu2d-P_{GAL1}-m FAR1/P_{GAL10}-MaME	This study
pESC-Leu2d-Atfa	pESC-Leu2d-P_{GAL1}-Atfa	This study
p416Tef1-URA3	p416-loxP-URA3-loxP-P_{TEF1}	This study

ura3Δ0)) (Table 1). The yeast ΔPOX1 and ΔPXA2 knockout strains were purchased from ATCC. The other yeast knockout strains were generated using a previously reported gene disruption cassette for repeated use in *S. cerevisiae* (Gueldener et al., 2002). The plasmids used in this study, which are listed in Table 2, were generated from the pESC vectors (Agilent Technology). These episomal plasmids contain the yeast 2 μ origin of replication, which allows autonomous replication of the plasmids and results in transformants with a high plasmid copy number (10–40 copies per cell) (Schneider and Guarente, 1991). The pESC-Leu2d vector, which contains the *leu2d* allele of a leucine biosynthetic gene LEU2, results in transformants with an even higher plasmid copy number (more than 100 copies per cell).

Yeast and bacterial strains were stored in 25% glycerol at −80 °C. *E. coli* was grown in Luria-Bertani medium. Carbenicillin at 100 μg/mL was added to the medium when required. Yeast strain BY4742 without plasmid was cultivated in YPD medium (10 g/L yeast extract, 20 g/L Bacto Peptone and 20 g/L glucose). Selection of yeast transformants with either HIS3, URA3 or LEU2 was done on a yeast minimal medium (6.7 g/L of Yeast Nitrogen Base (Difco), 20 g/L glucose, and a mixture of appropriate nucleotide bases and amino acids with dropouts (CSM-HIS, CSM-URA, CSM-LEU, CSM-HIS-URA or CSM-LEU-URA)). Yeast cells were cultivated at 30 °C in Erlenmeyer flasks closed with metal caps and shaken at 200 rpm.

Gene knockouts were generated using a previously reported gene disruption cassette for repeated use in *S. cerevisiae* (Gueldener et al., 2002). Gene disruption cassettes containing the URA3 selectable marker flanked by loxP sites (obtained by PCR of the pUG72 plasmid) were produced with 42 base pairs of homology on either side of each target integration site. Chromosomal replacement of native yeast promoters with P_{TEF} was performed as previously described (Nevoigt et al., 2006). Oligonucleotide primers used for PCR, cloning, knockouts, and promoter replacement in this study are included in the Supplementary information. Yeast cells were transformed using the Li/Ac/PEG method as previously described (Gietz and Schiestl, 2007a, 2007b). Following yeast transformations, colonies were selected on minimal medium lacking uracil and confirmed via PCR. The marker gene (URA3) was removed by overexpressing the Cre recombinase to excise the selection marker between the loxP sites in the disruption cassette. This enables subsequent rounds of genomic integrations. Cre recombinase was expressed using the inducible GAL1 promoter on plasmid pSH62 (Hegemann and Heick, 2011). The strain harboring pSH62 was grown in SD medium plus 1 g/L 5-fluoroorotic acid to encourage loss of the URA3 (Boeke et al., 1984). To verify the genetic stability of the engineered strains, their genomic DNA was isolated (Promega Wizard Genomic DNA Purification kit) and then subjected to a diagnostic PCR amplification that amplified regions both upstream and downstream of the integration/deletion sites (see Supplementary information for primer sequences). PCR products were purified (Qiagen PCR Purification kit) and then sequenced.

2.2. Plasmid construction

Plasmid pESC-His3-FAS1-FAS2: FAS2 was amplified from *S. cerevisiae* genomic DNA using primers S1 and S2. (See Supplementary information for primer sequences.) The FAS2 amplicon was ligated to the SpeI site of pESC-His to yield pESC-His-FAS2. FAS1 was amplified from *S. cerevisiae* genomic DNA in two fragments using primers S3 and S4, and S5 and S6. The two fragments were joined together via overlap extension PCR using primers S3 and S6. The FAS1 amplicon was ligated to the BamHI/XhoI site of pESC-His-FAS2 to yield pESC-His-FAS1-FAS2.

Plasmid pESC-Ura3-ACC1: ACC1 was amplified from *S. cerevisiae* genomic DNA using primers S7 and S8. The amplicon was ligated to the NotI site of pESC-Ura.

Plasmid pESC-Leu2d-Dga1: Dga1 was amplified from *S. cerevisiae* genomic DNA using primers S9 and S10. The Kozak sequence AAACA was added 5′ of the start codon to enhance expression. The amplicon was ligated to the BamHI/SalI site of pESC-Leu2d.

Plasmid pESC-Leu2d-'TesA: 'TesA was amplified from *E. coli* genomic DNA using primers S11 and S12. The Kozak sequence AAACA was added 5′ of the start codon to enhance expression. The amplicon was ligated to the BglII/SpeI site of pESC-Leu2d.

Plasmid pESC-Leu2d-mFAR1: mFAR1 was amplified from pmFAR1 (Steen, 2010) using primers S13 and S14. The Kozak sequence AAACA was added 5′ of the start codon to enhance expression. The amplicon was ligated to the BamHI/SalI site of pESC-Leu2d.

Plasmid pESC-Leu2d-mFAR1-MaME: The malic enzyme from *Mortierella alpina* codon-optimized for *S. cerevisiae* expression was synthesized by GenScript and was provided in the pUC57 vector. The gene was amplified from pUC57-MaME using primers S15 and S16. The Kozak sequence AAACA was added 5′ of the start codon to enhance expression. The amplicon was ligated to the BglII/SpeI site of pESC-Leu2d-mFAR1 to yield pESC-Leu2d-mFAR1-MaME.

Plasmid pESC-Leu2d-atfA: The wax ester synthase (atfA), codon-optimized for *S. cerevisiae* expression, was synthesized by IDT-DNA as three gBLOCKS gene fragments. The gene was stitched together using the primer-extension PCR method with primers S17 and S18. The Kozak sequence AAACA was added 5′ of the start codon to enhance expression. The amplicon was ligated to the BamHI/SalI site of pESC-Leu2d to yield pESC-Leu2d-atfA.

Plasmid p416Tef1-URA3: The plasmid for PCR amplification of the promoter replacement cassette with the URA3 selectable marker was constructed by amplifying the loxp-URA3-loxp region from pUG72 (Gueldener et al., 2002) with primers S19 and S20. The amplicon was placed 5′ of the translation elongation factor-1a (TEF) promoter region in p416Tef using homologous recombination in yeast.

2.3. Determination of lipid content in engineered strains

Quantification of the lipid content of engineered strains was performed as previously described with some modifications (Kamisaka et al., 2006). Strains were grown in nitrogen-limited (1 g/L ammonium sulfate) minimal medium to enhance lipid production. Strains were pre-cultured in 5-mL aliquots in minimal medium (1× yeast nitrogen base without ammonium sulfate, 1 g/L ammonium sulfate, 2% glucose, complete supplement mixture (CSM) with appropriate amino acid dropouts) overnight and used to inoculate 50 mL minimal medium (1× yeast nitrogen base without ammonium sulfate, 1 g/L ammonium sulfate, 0.2% glucose, 1.8% galactose, and CSM with appropriate amino acid dropouts) in 250-mL flask cultures to achieve an initial OD_{600} of 0.05. After 72 and 168 h, the OD_{600} was measured, and 10-mL aliquots of yeast cultures were collected and centrifuged at 3000g for 5 min. Cell pellets were then washed once with 10 mL of distilled water and lyophilized

at $-45\,^{\circ}C$ for 2 days. Lyophilized cells were then weighed to obtain the dry cell weight (DCW). To transesterify total fatty acids, 1 mL of 3 N HCl in methanol (Sigma) and 0.1 mL chloroform were added to lyophilized cells. After incubation at $70\,^{\circ}C$ for 3 h, the reaction mixture was cooled to room temperature, and 2 mL of saturated NaCl solution was added followed by 15 s of vortex. Two milliliters of hexane were added, and the reaction was agitated by vortex for 15 s. After a brief centrifugation step at $3000g$ for 1 min, the hexane (top) layer containing fatty acid methyl esters (FAME) was then analyzed on gas chromatography–mass spectrometry (GC–MS) using an HP 6890 Series GC with an Agilent 5973 Network MSD equipped with a DB5 column (Thermo). The GC program was as follows: The initial temperature of $40\,^{\circ}C$ was maintained for 3 min, then ramped to $250\,^{\circ}C$ at a rate of $20\,^{\circ}C/min$ and held there for 5 min. The lipid content (%) is calculated as total fatty acid amount (mg) per dry cell weight (mg) $\times$ 100.

2.4. GC–MS analysis of free fatty acids, fatty alcohols and FAEEs

For free fatty acid production, strains were pre-cultured in 5-mL aliquots in minimal medium ($1\times$ yeast nitrogen base, 2% glucose, complete supplement mixture (CSM) with appropriate amino acid dropouts) overnight and used to inoculate 50 mL minimal medium ($1\times$ yeast nitrogen base, 0.2% glucose, 1.8% galactose, and CSM with appropriate amino acid dropouts) in 250-mL flask cultures to achieve an initial OD_{600} of 0.05. After 96 h, 100 μL of yeast culture were spiked with 1.5 μL of pentadecanoic acid standard (6 mg/mL) and then mixed with 10 μL of 40% v/v tetrabutylammonium hydroxide (TBAH) solution (Sigma). Then, 100 μL of dichloromethane (DCM)/iodomethane (MeI) was added to the sample, and the mixture was agitated by vortex for 10 s. The organic (bottom) layer was transferred to a GC–MS vial and the solvent was allowed to evaporate completely. Then, 100 uL of fresh DCM was added to the extract, and the samples were run using a previously described method (Steen, 2010) with some differences. The GC program was as follows: an initial temperature of $40\,^{\circ}C$ was maintained for 3 min, followed by ramping to $250\,^{\circ}C$ at a rate of $20\,^{\circ}C/min$ where the temperature was held for 5 min.

In cultures where white precipitates were visible, the cultures were filtered through the Nalgene Rapid-Flow filtration unit. Precipitates were collected and dissolved in 100 μL of dichloromethane (DCM)/iodomethane (MeI). Then, 1.5 μL of the pentadecanoic acid standard (6 mg/mL), 10 μL of 40% v/v tetrabutylammonium hydroxide (TBAH) solution and 100 μL of double-distilled water were added to the mixture. The mixture was agitated by vortex for 10 s. The organic (bottom) layer was transferred to a GC–MS vial and the solvent was allowed to evaporate completely. Then, 100 uL of fresh DCM was added to the extract, and the samples were run using the GC program as described above.

For fatty alcohol production, strains were pre-cultured in 5-mL aliquots in minimal medium ($1\times$ yeast nitrogen base, 2% glucose, complete supplement mixture (CSM) with appropriate amino acid dropouts) overnight and used to inoculate 50 mL of minimal medium ($1\times$ yeast nitrogen base, 0.2% glucose, 1.8% galactose, and CSM with appropriate amino acid dropouts) in 250-mL flask cultures to achieve an initial OD_{600} of 0.05. Yeast cultures were overlaid with 10% dodecane to reduce evaporation of the fatty alcohols. The amount of fatty alcohols dissolved in the dodecane layer was determined using GC–MS. Briefly, 10 μL of dodecane from the cultures was mixed with 990 μL of ethyl acetate and analyzed on GC–MS. Samples were run using a previously described method (Steen, 2010) with some differences. The GC program was as follows: an initial temperature of $40\,^{\circ}C$ was maintained for 3 min, followed by ramping to $250\,^{\circ}C$ at a rate of $20\,^{\circ}C/min$ where the temperature was held for 5 min.

For FAEE production, strains were pre-cultured in 5-mL aliquots in minimal medium ($1\times$ yeast nitrogen base, 2% glucose, complete supplement mixture (CSM) with appropriate amino acid dropouts) overnight and used to inoculate 50 mL of minimal medium ($1\times$ yeast nitrogen base, 0.2% glucose, 1.8% galactose, and CSM with appropriate amino acid dropouts) in 250 mL flask cultures to achieve an initial OD_{600} of 0.05. Yeast cultures were overlaid with 10% dodecane to reduce evaporation of the FAEEs. The amount of FAEEs dissolved in the dodecane layer was determined using gas chromatography–mass spectrometry (GC–MS). Briefly, 100 μL of dodecane from the cultures was mixed with 900 μL of ethyl acetate and analyzed on GC–MS. Samples were run using a previously described method (Steen, 2010) with some differences. The GC program was as follows: an initial temperature of $40\,^{\circ}C$ was maintained for 3 min, followed by ramping to $250\,^{\circ}C$ at a rate of $20\,^{\circ}C/min$ where the temperature was held for 5 min.

2.5. HPLC analysis of ethanol accumulation in engineered strains

Engineered strains overproducing FAEEs were pre-cultured in 5-mL aliquots in minimal medium ($1\times$ yeast nitrogen base, 2% glucose, complete supplement mixture (CSM) with appropriate amino acid dropouts) overnight and used to inoculate 50 mL minimal medium ($1\times$ yeast nitrogen base, 0.2% glucose, 1.8% galactose, and CSM with appropriate amino acid dropouts) in 250-mL flask cultures to achieve an initial OD_{600} of 0.05. After 72 and 168 h, 1 mL of culture was centrifuged at $18,000g$ for 5 min and the supernatant was applied to an Agilent 1100 series HPLC equipped with an Aminex HPX-87H ion exchange column (Biorad). The LC program was performed using 4 mM H_2SO_4 as the solvent at a flow rate of 0.6 mL/min. The column was maintained at $50\,^{\circ}C$. All metabolites were detected with an Agilent 1200 series DAD and RID detectors.

2.6. RNA isolation and transcript quantification

Strains were pre-cultured in 5-mL aliquots in minimal medium ($1\times$ yeast nitrogen base, 2% glucose, complete supplement mixture (CSM) with appropriate amino acid dropouts) overnight and used to inoculate 50 mL of minimal medium ($1\times$ yeast nitrogen base, 0.2% glucose, 1.8% galactose, and CSM with appropriate amino acid dropouts) in 250-mL flask cultures to achieve an initial OD_{600} of 0.05. After 72 h, a 5-mL aliquot of each culture was collected and centrifuged for 5 min at $3000g$. The pellets were washed with 5 mL of distilled water. Total RNA was extracted using the QIAgen RNeasy Kit under the manufacturer's protocol. Contaminating genomic DNA was removed from the RNA samples by DNaseI (NEB) digestion using the manufacturer's protocol. The RNA quantity was analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and samples were stored at $-80\,^{\circ}C$ until qRT-PCR analysis. Relative expression levels of FAS1, FAS2, ACC1, FAA1, FAA4 and DGA1 were quantified using iScript One-step RT-PCR Kit with SYBR (Biorad) on StepOnePlus Real-time PCR Systems (Applied Biosystems). TAF10, a gene that encodes a subunit of transcription factor II D (TFIID), was used to normalize the amount of the total mRNA in all samples.

3. Results and discussion

3.1. Overexpression of single fatty acid biosynthesis enzyme/enzyme complex in S. cerevisiae improved lipid accumulation

3.1.1. Overexpression of acetyl-CoA carboxylase (ACC)

Prior studies have highlighted the conversion of acetyl-CoA to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) as the rate-limiting step in fatty acid biosynthesis in many organisms including yeast (Tehlivets et al., 2007). Several research groups

Table 3
Lipid content, yield and dry cell weight for reference and engineered *S. cerevisiae* strains.

Strain	Plasmid(s)	Lipid content (%)	Total fatty acids (mg/L)	Dry cell weight (g/L)
BY4742	pESC-Leu2d	4.3 ± 0.1	42.7 ± 1.0	0.99 ± 0.02
BY4742	pESC-His3-FAS1-FAS2	5.6 ± 0.6	70.6 ± 5.4	1.26 ± 0.09
BY4742	pESC-Ura3-ACC1	6.8 ± 0.2	63.2 ± 1.2	0.93 ± 0.02
BY4742	pESC-Leu2d-DGA1	10.9 ± 0.4	168.3 ± 4.1	1.54 ± 0.04
BY4742	pESC-His3-FAS1-FAS2+pESC-Leu2d-DGA1	12.0 ± 1.1	147.1 ± 11.2	1.23 ± 0.07
BY4742	pESC-Ura3-ACC1+pESC-Leu2d-DGA1	11.6 ± 0.2	153.8 ± 0.0	1.32 ± 0.03
BY4742 P_{TEF1}-DGA1	None	10.4 ± 0.4	143.3 ± 5.2	1.38 ± 0.03
BY4742 P_{TEF1}-FAS1-FAS2	pESC-Leu2d-DGA1	13.4 ± 1.0	166.4 ± 9.4	1.24 ± 0.06
BY4742 P_{TEF1}-ACC1	pESC-Leu2d-DGA1	14.2 ± 1.7	155.0 ± 17.6	1.09 ± 0.04
WRY1	pESC-Leu2d-DGA1	17.3 ± 1.1	171.5 ± 10.0	0.99 ± 0.03
BY4742 ΔPOX1	pESC-Leu2d-DGA1	11.4 ± 0.6	149.9 ± 7.6	1.32 ± 0.02
BY4742 ΔPOX1 P_{TEF1}-ACC1	pESC-Leu2d-DGA1	13.0 ± 0.8	141.4 ± 8.8	1.08 ± 0.01
BY4742 ΔPXA2	pESC-Leu2d-DGA1	11.6 ± 0.4	155.3 ± 5.5	1.34 ± 0.00
BY4742 ΔPXA2 P_{TEF1}-ACC1	pESC-Leu2d-DGA1	12.5 ± 1.9	143.5 ± 21.7	1.15 ± 0.04
BY4742 ΔPXA2 P_{TEF1}-FAS1-FAS2	pESC-Leu2d-DGA1	14.0 ± 1.7	190.3 ± 22.0	1.36 ± 0.06
BY4742 ΔPOX1 P_{TEF1}-DGA1	None	9.8 ± 1.2	183.1 ± 19.5	1.87 ± 0.10
BY4742 ΔPXA2 P_{TEF1}-DGA1	None	10.1 ± 0.8	170.4 ± 12.4	1.69 ± 0.06
BY4742 ΔPOX1 P_{TEF1}-FAS1-FAS2	pESC-Leu2d-DGA1	8.0 ± 1.2	79.4 ± 11.0	0.99 ± 0.06
WRY1 ΔPOX1	pESC-Leu2d-DGA1	16.6 ± 1.7	131.0 ± 13.5	0.79 ± 0.00
WRY1 ΔPXA2	pESC-Leu2d-DGA1	16.2 ± 1.7	140.0 ± 14.0	0.86 ± 0.03

Lipid content is expressed as total fatty acids (mg)/dry cell weight (mg) × 100. Total fatty acid is given as means ± S.D. ($n=3$) after 72 h.

have targeted this enzyme in their metabolic engineering strategies to improve production of fatty acids and lipid content (Davis et al., 2000; Liu et al., 2010). Overexpression of ACC in the oleaginous yeast *Yarrowia lypolytica* increased the lipid content 2-fold over the control, or from a lipid content of 8.8% to 17.9% (Tai and Stephanopoulos, 2013). Similarly, overexpression of the four endogenous ACC genes in *E. coli* led to a six-fold increase in the rate of fatty acid biosynthesis (Davis et al., 2000; Liu et al., 2010). In this study, we overexpressed endogenous ACC, encoded by the *ACC*1 gene, in *S. cerevisiae* to increase the pool of malonyl-CoA and subsequently the pool of fatty acyl-CoA. Plasmid-based overexpression of endogenous *ACC*1 led to a lipid content of 6.8%, a 58% increase from a lipid content of 4.3% in the background strain BY4742 (Table 3). Notably, total fatty acid production also increased from 42.7 mg/L to 63.2 mg/L.

This improvement of total lipid is considerably lower than those observed under similar strategies in *E. coli* (Liu et al., 2010). Indeed, our results are consistent with other works done in eukaryotic organisms, where overexpression of ACC generally leads to a small improvement of lipid production. Cumulatively, these results underscore the strict level of metabolic and regulatory control over this enzyme.

3.1.2. Overexpression of fatty acid synthase complex (FAS complex)

Based on our results and previous work, we speculate that overexpression of *ACC*1 led to higher levels of malonyl-CoA, the primary substrate for fatty acid biosynthesis. We directed this pool of malonyl-CoA into fatty acid production by overexpressing the fatty acid synthase (FAS) complex. Plasmid-based overexpression of both *FAS*1 and *FAS*2 led to a 30% increase in lipid content (from 4.3% to 5.6%) (Table 3). Total fatty acid production also increased from 42.7 mg/L to 70.6 mg/L.

3.1.3. Overexpression of diacylglycerol acyltransferase (DGAT)

The final step of triacylglycerol (TAG) biosynthesis—the acylation of diacylglycerol using acyl-CoA as the acyl donor—is catalyzed by the enzyme diacylglycerol-acyltransferase (DGAT). This step has been postulated to be a rate-limiting step in yeast lipid biosynthesis (Bouvier-Nave et al., 2000; Dahlqvist et al., 2000). Therefore, DGAT, which is encoded by *DGA*1 in *S. cerevisiae*, serves as an attractive engineering target to overproduce lipids. In

previous studies, overexpression of DGAT led to increases in TAG production in plants and several strains of yeast, including *S. cerevisiae* and the oleaginous yeast *Y. lypolitica* (Bouvier-Nave et al., 2000; Hobbs et al., 1999; Jako et al., 2001; Kamisaka et al., 2007; Tai and Stephanopoulos, 2013). In one example, overexpression of *DGA*1 in *Y. lypolitica* led to a 4-fold increase in lipid content over the control (from 8.8% to 33.8%) (Tai and Stephanopoulos, 2013). Similarly, overexpression of *DGA*1 in a Δ*Snf*2 *S. cerevisiae* mutant led to a 2.7-fold increase in lipid content over the control (from 11.6% to 27%) (Kamisaka et al., 2007). While we were initially intrigued by this finding and had hoped to use the Δ*Snf*2 *S. cerevisiae* mutant as the starting strain in our metabolic engineering efforts, further investigation reveals that this mutant strain shows severe growth defects on galactose, thereby rendering it incompatible with the *GAL*1/*GAL*10 expression system, one of the most commonly used inducible expression systems in yeast. Given these considerations, we decided against using the Δ*Snf*2 *S. cerevisiae* mutant and opted instead to use the "wild-type" strain, *S. cerevisiae* BY4742. Plasmid-based overexpression of *DGA*1 led to a 150% increase in lipid content (from 4.3% to 10.9%), which corresponds to an increase in total fatty acid production from 42.7 mg/L to 168.3 mg/L (Table 3). Real-time reverse-transcription PCR (qRT-PCR) analysis of this strain confirmed high expression level of *DGA*1 over the level observed in the control strain.

3.2. Overexpression of multiple fatty acid biosynthesis enzymes in S. cerevisiae improved lipid accumulation

While single-gene transformants had higher lipid contents than the empty vector controls, we speculated that simultaneously overexpressing all fatty acid biosynthesis genes would further enhance lipid production by enhancing the metabolic flux through the entire pathway. This strategy has been successfully employed in the high-titer production of amorphadiene, an isoprenoid precursor to the antimalarial drug artemisinin (Westfall et al., 2012). The overexpression of every mevalonate pathway gene up to *ERG*20, coupled to the heterologous expression of amorphadiene synthase (*ADS*), in *S. cerevisiae* CEN.PK2 led to an amorphadiene titer of over 1.2 g/L, which is more than a five-fold increase in the production level observed in a strain where only selected genes were overexpressed.

When considering how to overexpress all of the fatty acid biosynthesis genes, we chose to chromosomally replace the native promoters of fatty acid biosynthesis genes with a strong constitutive promoter (Nevoigt et al., 2006). We did not use plasmid-based overexpression of fatty acid biosynthetic genes due to the relatively large sizes of these genes (ACC1, 6702 bps; FAS1, 6156 bps; and FAS2, 5664 bps). Moreover, modifications to the host chromosome ensure genetic stability of the host strain and eliminate selection requirements. To overexpress fatty acid biosynthetic enzymes in yeast, we successively replaced the native promoter of ACC1, FAS1 and FAS2 with the TEF1 promoter (P$_{TEF1}$), a strong constitutive promoter. This strain, called WRY1, was used as a host strain for other "converting enzymes" to generate free fatty acids, fatty alcohols and FAEE that are non-native to yeast.

In parallel, we replaced the native promoter of DGA1 with P$_{TEF1}$. This led to a 142% increase in the lipid content (from 4.3% to 10.4%), which corresponds to an increase in total fatty acid production from 42.7 mg/L to 143.3 mg/L (Table 3). Since replacement of the DGA1 promoter with P$_{TEF1}$ led to a lower increase in lipid content than plasmid-based overexpression of DGA1 (10.4% vs. 10.9%, respectively), we decided to overexpress DGA1 using a plasmid with a high-copy number (2μ origin of replication). Replacement of the ACC1 promoter with P$_{TEF1}$ coupled with plasmid-based overexpression of DGA1 led to a 230% increase in lipid content (from 4.3% to 14.2%), which corresponds to an increase in total fatty acid production from 42.7 mg/L to 155.0 mg/L. Similarly, replacement of both FAS1 and FAS2 promoters with P$_{TEF1}$ coupled to the plasmid-based overexpression of DGA1 led to a 205% increase in lipid content (from 4.3% to 13.4%), which corresponds to an increase in total fatty acid production from 42.7 mg/L to 166.4 mg/L. Finally, replacement of ACC1, FAS1 and FAS2 promoters with P$_{TEF1}$ (i.e., WRY1 strain) coupled to the plasmid-based overexpression of DGA1 led to a 302% increase in lipid content (from 4.3% to 17.3%), the highest lipid content out of all of our engineered strains. This corresponds to an increase in total fatty acid production from 42.7 mg/L to 171.5 mg/L. The distribution of fatty acids was as follows: C12:0, 3.5%; C14:0, 6.9%; C16:0, 38.3%; C16:1, 32.8%; C18:0, 6.8%; and C18:1, 11.8%. Diagnostic PCR amplification of the genomic DNA confirmed the genetic stability of the engineered strains (Supplementary Fig. S6). Real-time reverse-transcription PCR (qRT-PCR) analysis of the engineered strains confirmed high expression levels (7–16 fold for native promoter replacement with P$_{TEF1}$ and 22–250 fold for plasmid-based overexpression) of ACC1, FAS1, FAS2 and DGA1 over levels observed in the control strain (Supplementary Fig. S5). Our results demonstrate that overexpressing multiple genes gave rise to higher lipid contents than single-gene overexpression. Interestingly, while the overall lipid content was higher in these engineered strains, the biomass (dry cell weight) generated was lower than control strains (Table 3). Our results suggest that overexpression of ACC1, FAS1, FAS2, and DGA1 may divert cellular resources away from biomass production towards lipid biosynthesis. This phenomenon was also observed in previous work (Tai and Stephanopoulos, 2013).

3.3. Deletion of genes in the β-oxidation pathway did not improve lipid accumulation further

To further increase TAG production we aimed to down-regulate the β-oxidation pathway, which breaks down fatty acyl-CoAs to acetyl-CoA, a key carbon building block for many metabolic pathways. In S. cerevisiae, β-oxidation of fatty acids occurs solely in the peroxisome. The import of fatty acids in the acyl-CoA form into the peroxisomes requires the transporters PXA1 and PXA2 (Shani and Valle, 1996). Once inside the peroxisomes, acyl-CoA is oxidized to

trans-2-enoyl-CoA by acyl-CoA oxidase, which is encoded by the POX1 gene (Dmochowska et al., 1990). Trans-2-enoyl-CoA is subsequently hydrated and oxidized to 3-ketoacyl-CoA by a bifunctional protein encoded by the POX2 gene (Hiltunen et al., 1992). The final cleavage of the ketoacyl to yield acetyl-CoA and the shortened acyl-CoA is catalyzed by a thiolase, which is encoded by the FOX3 gene (Einerhand et al., 1991). Our lab and others have successfully exploited the analogous β-oxidation pathway to improve fatty acid productions in E. coli (Liu et al., 2010; Lu et al., 2008; Steen et al., 2010). Deletion of FadE, the E. coli homolog of POX1, led to three- to four-fold increase in the fatty acid titer.

We chose to delete PXA2, which encodes a key component of the peroxisomal transporter, and POX1, which encodes the first enzyme in the oxidation pathway. Replacement of the ACC1, FAS1 and FAS2 promoters with P$_{TEF1}$ (i.e., WRY1 strain) coupled to the deletion of PXA2 and the plasmid-based overexpression of DGA1 led to a 277% increase in lipid content (from 4.3% to 16.2%) (Table 3). This corresponds to an increase in total fatty acid production from 42.7 mg/L to 140.0 mg/L. Similarly, replacement of ACC1, FAS1 and FAS2 promoters with P$_{TEF1}$ coupled to the deletion of POX1 and the plasmid-based overexpression of DGA1 led to a 286% increase in lipid content (from 4.3% to 16.6%). This corresponds to an increase in total fatty acid production from 42.7 mg/L to 131.0 mg/L. Notably, the lipid content and biomass (dry cell weight) are higher in the original strains, where the β-oxidation pathway genes are present. This suggests that, unlike in E. coli, the β-oxidation pathway may not be an ideal engineering target for further improving lipid production in S. cerevisiae.

3.4. Production of free fatty acids in S. cerevisiae

Our mixed success in improving lipid accumulation in S. cerevisiae underscores the intricate and strict regulation of fatty acid and TAG biosynthesis in S. cerevisiae. Indeed, the extent to which S. cerevisiae and other yeast species ensure proper lipid homeostasis is well documented (Tehlivets et al., 2007). For example, both ACC and the FAS complex are regulated at the transcriptional, translational and post-translational levels. This level of regulation may hamper our ability to engineer a lipid overproducer that surpasses oleaginous yeast strains (a lipid content of over 20%). Therefore, we contend that, by choosing an alternative target that is non-native to yeast or one that is subjected to a lower level of regulation, we may be able to improve the production yield more freely. To this end, we turn our attention to free fatty acids. While there have been significant efforts to increase TAG production in S. cerevisiae and other yeasts, fewer efforts have been focused on engineering S. cerevisiae overproducers of free fatty acids.

3.4.1. Overexpression of the E. coli acyl-ACP thioesterase ('TesA) in S. cerevisiae led to production of free fatty acids

In S. cerevisiae fatty acid biosynthesis, after the last round of chain elongation, the fatty acyl-enzyme intermediate is released from the FAS synthase complex in the fatty acyl-CoA form (Tehlivets et al., 2007). Fatty acids can be produced by expressing an enzyme with acyl-CoA thioesterase activity. E. coli TesA exhibits both acyl-ACP and acyl-CoA thioesterase activities in vivo (Steen, 2010). Gratifyingly, plasmid-based overexpression of 'TesA (TesA lacking the membrane signal peptide at the amino-terminal end) in S. cerevisiae BY4742 led to the production of 5 mg/L of free fatty acids (Fig. 2). This is eight times the production level observed in the background strain BY4742 (0.6 mg/L). We then explored whether we could improve the production of free fatty acids further by overexpressing all the fatty acid biosynthesis enzymes. Plasmid-based overexpression of 'TesA in the WRY1 strain, which
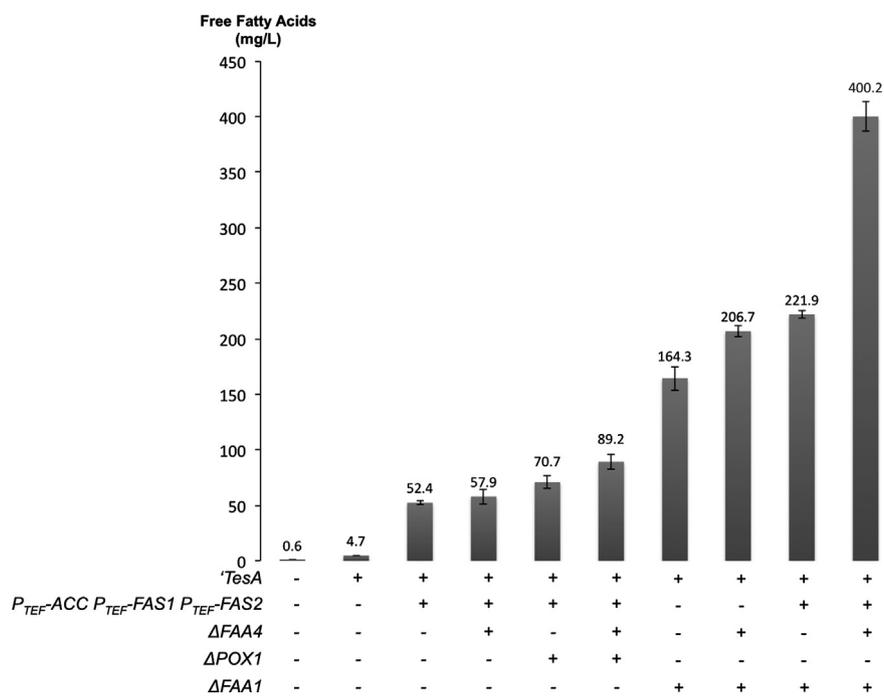
Fig. 2. Free fatty acid production in the *E. coli* acyl-ACP thioesterase-expressing and control strains. All strains were cultured in minimal medium lacking the appropriate amino acid and/or nucleotide and containing a mixed carbon source (0.2% glucose and 1.8% galactose). Values are the mean of three biological replicates $\pm$ standard deviation ($n=3$) after 96 h.

has all fatty acid biosynthesis genes driven by the strong constitutive promoter P_{TEF1}, improved production levels to 52 mg/L.

3.4.2. Deletion of acyl-CoA synthetase genes in S. cerevisiae improved production of free fatty acids

Encouraged by the above results, we turned our attention to *S. cerevisiae* endogenous fatty-acyl-CoA synthetases to further improve the yield of free fatty acids. We suspect that a portion of free fatty acids produced by 'TesA are converted back to fatty acyl-CoAs by the fatty-acyl-CoA synthetases FAA1 and FAA4. Yeast has an inherent ability to break down fatty acids into acetyl-CoAs, a building block in many anabolic processes (Trotter, 2001). Notably, yeast is able to survive on medium- and long-chain fatty acids as the sole carbon source. The process of breaking down fatty acids begins with the transportation of fatty acids into yeast cells. Once inside the cells, these fatty acids first need to be activated to the CoA form before they can undergo degradation through the β-oxidation pathway. To convert fatty acids into fatty acyl-CoAs, yeast expresses acyl-CoA synthetases that attach the CoA moiety to free fatty acids. *S. cerevisiae* contains five acyl-CoA synthetases (FAA1-4 and FAT1), but FAA1 and FAA4 are responsible for the majority of this activity (Trotter, 2001). Therefore, fatty acyl-CoA synthetases appear to be viable engineering targets to improve production of free fatty acids. Indeed, previous engineering efforts in *E. coli* demonstrated that deletion of *fadD*, the *E. coli* homolog of acyl-CoA synthetase, increased the free fatty acid production by more than two-fold (Steen, 2010).

Deletion of the acyl-CoA synthetase *FAA*1 in *S. cerevisiae* BY4742 coupled to the plasmid-based overexpression of 'TesA led to production levels of 164 mg/L of free fatty acids (Fig. 2). Deletion of both *FAA*1 and *FAA*4 coupled to the plasmid-based overexpression of 'TesA led to production levels of 207 mg/L of free fatty acids. To further improve fatty acid yields, we deleted both *FAA*1 and *FAA*4 in the strain WRY1, which has all fatty acid biosynthesis genes driven by the strong constitutive promoter P_{TEF1}. Overexpression of 'TesA in the resulting strain increased free fatty yields

further to 400 mg/L, a 670-fold improvement over the level observed in the reference strain (empty vector control). To the best of our knowledge, this is the highest production level of free fatty acids reported in *S. cerevisiae*. The distribution of fatty acids was as follows: C12:0, 2.7%; C14:0, 9.4%; C16:0, 47.0%; C16:1, 19.3%; C18:0, 10.4%; and C18:1, 10.7%. Remarkably, at this production level, free fatty acids that have been secreted out of the cells precipitated out of the solution (see supplementary Fig. S1). Microscopic analysis of cell cultures and GC–MS analysis of dissolved precipitates confirmed the precipitation of free fatty acids (supplementary Fig. S2 and S3). Real-time reverse-transcription PCR (qRT-PCR) analysis of this strain confirmed high expression levels (2–3 fold higher) of *ACC*1, *FAS*1 and *FAS*2 over levels observed in the control strain and the absence of *FAA*1 and *FAA*4 transcripts (supplementary Fig. S4).

Notably, our best free fatty acid-producing strain produced roughly two-fold higher total fatty acids than our best TAG-producing strain (Table 3 and Fig. 2). Our results suggest that production of free fatty acids may be subjected to a less stringent level of regulation compared to the production of TAGs. Moreover, the demonstrated ability of *S. cerevisiae* to secrete some of the free fatty acids into the medium could potentially provide a driving force towards higher fatty acid production.

3.5. Production of fatty alcohols and fatty acid ethyl esters (FAEEs) in S. cerevisiae

While free fatty acids and TAGs are valuable, choosing TAGs and free fatty acids as end fuel targets suffers from a practical standpoint. Specifically, TAGs and free fatty acids cannot be used directly as fuels and must first be converted to fatty acid alkyl esters, fatty acid-derived alkanes, alkenes or alcohols. Thus, a more direct strategy to produce fatty acid-derived biofuels via a microbial platform is to bypass TAG production altogether and convert fatty acids (in the fatty acyl-CoA form) directly to the desired fuels in vivo. To this end, we engineered *S. cerevisiae* to produce fatty alcohols and fatty acid ethyl esters (FAEEs, biodiesels) directly from simple sugars.
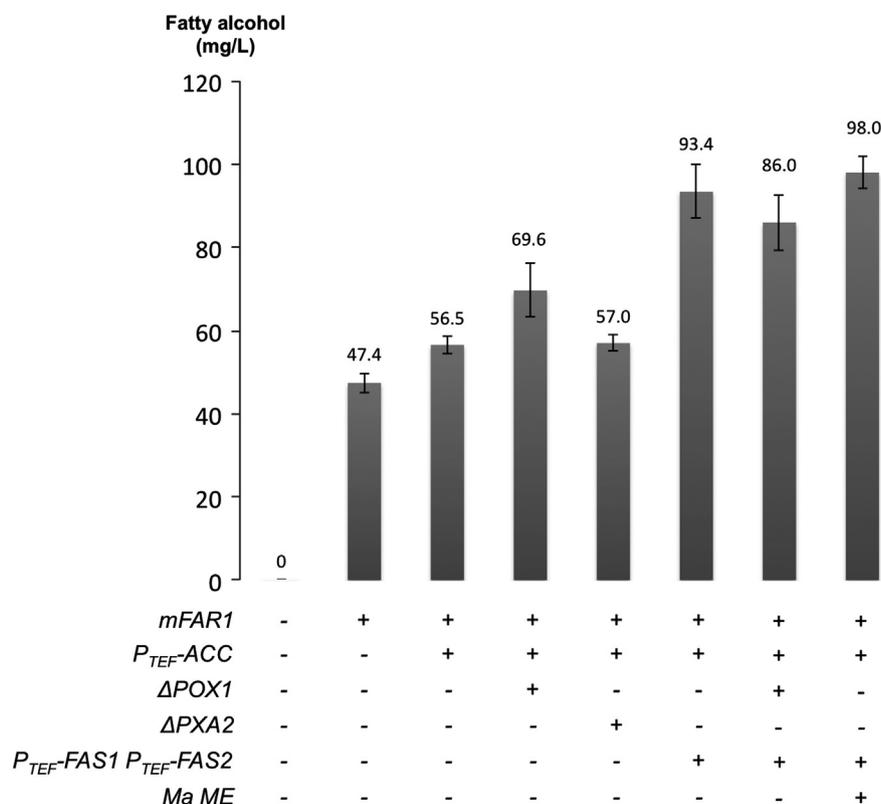
Fig. 3. Fatty alcohol production in the mouse fatty acyl-CoA reductase-expressing and control strains. All strains were cultured in minimal medium lacking the appropriate amino acid and/or nucleotide and containing a mixed carbon source (0.2% glucose and 1.8% galactose). Values are the mean of three biological replicates $\pm$ standard deviation ($n=3$) after 168 h.

3.5.1. Overexpression of the mouse fatty acyl-CoA reductase (mFAR1) in S. cerevisiae led to production of fatty alcohols

There is an increasingly large market for fatty alcohols, which are used in a wide range of products from surfactants (the foaming agents used in many consumer products including detergent and shampoo) to cosmetics. The global market for fatty alcohols reached $1.87 billion in 2002 and has been growing ever since (Gupta, 2004). Fatty alcohols can be produced from fatty acyl-CoAs using an NAD(P)H-dependent fatty acyl-CoA reductase (FAR). Several FARs have been characterized and heterologously expressed in *E. coli* and yeast, endowing them with the ability to produce fatty alcohols (Cheng and Russell, 2004; Doan et al., 2009; Metz et al., 2000; Steen, 2010; Vioque and Kolattukudy, 1997). For example, our lab has previously expressed either *acr*1, an NADPH-dependent fatty acyl-CoA reductase from *Acinetobacter calcoaceticus* BD413, or *mFAR*1, an NADPH-dependent fatty acyl-CoA reductase from *Mus musculus* (mouse), in *E. coli* and showed that the engineered *E. coli* strain produced fatty alcohols up to 60 mg/L (Steen, 2010).

In this study, we overexpressed *mFAR*1 in *S. cerevisiae*. Gratifyingly, our engineered strains produced and exported fatty alcohols into the medium, as demonstrated by the presence of fatty alcohols in the dodecane overlay (10% v/v). The empty vector control did not produce detectable levels of fatty alcohols. Plasmid-based overexpression of *mFAR*1 in BY4742 led to a fatty alcohol production of 47.4 mg/L (Fig. 3). Replacement of the native *ACC*1 promoter with P_{Tef1} coupled to the plasmid-based overexpression of *mFAR*1 improved production levels to 56.5 mg/L. Finally, plasmid-based overexpression of *mFAR*1 in the WRY1 strain, which has all fatty acid biosynthesis genes driven by the strong constitutive promoter P_{TEF1}, improved production levels to 93.4 mg/L. Deletion of *POX*1, the first gene

in the β-oxidation pathway, did not improve fatty alcohol production titer.

Given that the reduction of one molecule of fatty acyl-CoA to fatty alcohol requires one molecule of NADPH, we explored whether increasing the pool of cytosolic NADPH would lead to an increase in fatty alcohol yield. A common strategy to achieve this is by over-expressing an NADP-dependent malic enzyme (Moreira dos Santos et al., 2004; Wynn et al., 1999; Zhang et al., 2007). This oxidor-eductase converts malate and NADP+ to pyruvate and NADPH, releasing one molecule of carbon dioxide in the process. We overexpressed the malic enzyme from the oleaginous fungus *M. alpina* in our top fatty alcohol producer strain. This led to a small increase in the final fatty alcohol titer to 98.0 mg/L. To the best of our knowledge, this is the highest production level of fatty alcohols reported in *S. cerevisiae*. The distribution of fatty alcohols was as follows: C16:0, 91.1% and C18:0, 8.9%. This distribution is consistent with the reported mFAR1 substrate preference for C16 and C18 fatty acyl-CoAs (Cheng and Russell, 2004).

3.5.2. Overexpression of the wax-ester synthase from A. calcoacericus ADP1 (atfA) in S. cerevisiae led to production of fatty acid ethyl esters (FAEEs, biodiesels)

Encouraged by our results in overproducing free fatty acids and fatty alcohols, we next turned our attention to fatty acid ethyl esters (biodiesels). Conversion of fatty acyl-CoAs into FAEEs requires an acyl-CoA:alcohol transferase (wax ester synthase, WS) that can accept ethanol, the most abundant short-chain alcohol in *S. cerevisiae*, as the alcohol substrate. Such an enzyme was recently identified from *A. calcoaceticus* ADP1 (Stoveken et al., 2005). The enzyme, encoded by *atfA*, exhibits fatty acyl-CoA: alcohol acyltransferase activity towards a broad range of alcohol
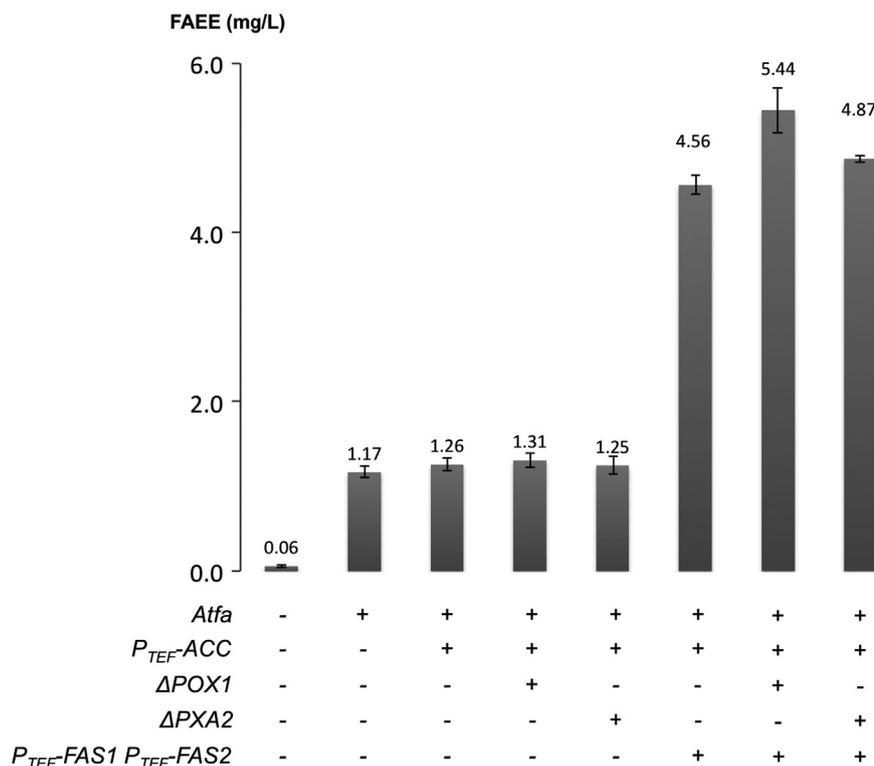
FAEE (mg/L)

Fig. 4. Fatty acid ethyl ester (FAEEs) production in the wax-ester synthase-expressing and control strains. All strains were cultured in minimal medium lacking the appropriate amino acid and/or nucleotide and containing a mixed carbon source (0.2% glucose and 1.8% galactose). Values are the mean of three biological replicates $\pm$ standard deviation ($n=3$) after 168 h.

substrates including ethanol. Heterologously expressing this enzyme in *E. coli* (Steen, 2010), and more recently in *S. cerevisiae* CEN.PK, led to the production of FAEEs (Kalscheuer et al., 2004; Shi et al., 2012; Yu et al., 2012).

In this study, overexpression of the codon-optimized *atfA* led to the production of FAEEs, which were detected in the dodecane layer, suggesting that the compounds were produced and secreted into the medium. Plasmid-based expression of *atfA* in BY4742 led to FAEE production at 1.2 mg/L (Fig. 4). Replacement of the native *ACC*1 promoter with P_{TEF1} coupled to the plasmid-based overexpression of *atfA* improved production levels to 1.3 mg/L. Finally, plasmid-based overexpression of *atfA* in the WRY1 strain, which has all fatty acid biosynthesis genes driven by the strong constitutive promoter P_{TEF1}, improved production levels to 4.6 mg/L. The production levels further improved slightly to 4.9 and 5.4 mg/L after the deletion of *PXA*2 and *POX*1, respectively. The latter is a 90-fold improvement over the level observed in the reference strain (empty vector control). The distribution of FAEEs was as follows: C12:0, 26.3%; C14:0, 14.9%; C16:0, 44.5%; and C18:0, 14.4%.

Intriguingly, the yield of FAEEs in our best producer is at least one order of magnitude lower than the yields of TAGs, free fatty acids and fatty alcohols in the corresponding best producers ($\sim$5 mg/L of FAEEs compared to $\sim$400 mg/L of free fatty acids). We quantified ethanol levels in the culture medium to verify that ethanol production is not limiting. This was indeed the case as high levels of ethanol were observed in the culture medium (3.6 g/L after 72 h and 4.1 g/L after 168 h). Notably, the expression levels and in vivo activities of these enzymes in *S. cerevisiae*, the inherent toxicity of these fuel molecules, the ability of yeast to excrete these molecules into the medium could each contribute to differences in production titers. Understanding which of these factors plays the largest role in determining the yields of biofuel production will lead to further insights for metabolic engineering efforts.

4. Conclusions

Fatty acid-derived biofuels and chemicals are in great demand. Given the grave concerns over global climate change and the increasingly difficult access to fossil fuels, development of new microbial platforms for biofuel production is essential. Here, we engineered the budding yeast *S. cerevisiae* to produce fatty acid-derived biofuels and chemicals from simple sugars. Specifically, we overexpressed all three primary genes involved in fatty acid biosynthesis, namely *ACC*1, *FAS*1 and *FAS*2. Combining this metabolic engineering strategy with terminal "converting enzymes" (diacylglycerol-acyltransferase, fatty acyl-CoA thioesterase, fatty acyl-CoA reductase, and wax ester synthase for TAG, fatty acid, fatty alcohol and FAEE production, respectively) improved the production levels of all biofuel molecules and chemicals. In short, we demonstrated that *S. cerevisiae* provides a compelling platform for a scalable, controllable and economic route to this important class of chemicals.

Disclosure Statement:

Acknowledgments

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.2013.07.003.

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