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Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways

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HIGHLIGHTS

- We present a novel alternative for producing fatty acid ethyl esters utilizing yeast.
- Metabolic engineering of the cell was performed in order to increase the FAEE titer.
- A 3-fold increase in FAEE production was achieved in the ultimate mutant strain.

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ABSTRACT

Microbial production of fatty acid derived chemicals and fuels is currently of great interest due to the limited resources and increasing prices of petroleum and petroleum-based products. The development of *Saccharomyces cerevisiae* as a fatty acid ethyl ester (FAEE) cell factory would represent an opportunity for biodiesel production due to its successful history in the biotechnology area. However, fatty acid (FA) biosynthesis is highly regulated and usually not high enough for developing an efficient production process. In *S. cerevisiae*, FAs are degraded by β-oxidation and a large fraction is utilized to synthesize steryl esters (SEs) and triacylglycerols (TAGs), which are not essential for the cell. Here, by eliminating non-essential FA utilization pathways, we developed a metabolic engineering strategy resulting in a *S. cerevisiae* strain that can overproduce FAs and in turn use these for producing FAEEs (biodiesel). Compared to the wild-type, there is an about 3-fold increase in free FA content in a strain devoid of both TAG and SE formation, a 4-fold increase in free FA content in a strain that is incapable of β-oxidation, and a 5-fold increase of free FAs in a strain lacking all of these non-essential FA utilization pathways. It is also demonstrated that there are similar positive effects on FAEE production in these deletion strains. The highest production of FAEEs is 17.2 mg/l in the strain in which all these pathways were blocked. The results of this study serve as a basis for further strategies to improve the production of FA derivatives in *S. cerevisiae*.

1. Introduction

Petrochemistry has shaped modern industrial and societal developments. However, the inevitable depletion of finite petroleum resources and environmental concerns caused by the use of fossil fuel have led to efforts in developing sustainable fuels to substitute fossil fuels. Microbial production of FA derived chemicals or fuels is currently of great interest [1–11].

Biodiesel, the most commonly known and commercialized biomass-derived diesel fuel, is a specific class of diesel composed of mono alkyl esters of long chain fatty acids. Currently, it has been considered to take center stage as a sustainable and environmentally friendly energy source for the future. Unfortunately, biodiesel is traditionally produced from plant oils by chemical transesterification, which has become a rising problem for large-scale commercial viability of its production in terms of cost and availability of feedstock [12–17]; and an excess of alcohol is required to drive the reaction close to completion, but increases costs [18]. In response to this challenge, using tools of systems and synthetic biol-

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Nomenclature

μ_{max}	maximum specific cell growth rate (h^{-1})	Y_{se}	yield of ethanol on glucose (g_{eth}/g_{glc})
Y_{sx}	yield of biomass on glucose (g_{cell}/g_{glc})	FAEEs	concentration of fatty acid ethyl esters (mg/l)

ogy, considerable attention has been put into developing microorganisms (e.g. *Escherichia coli* and *Saccharomyces cerevisiae*) as "green" alternatives to bypass chemical transesterification to produce advanced biodiesel, fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs), directly *in vivo* [1,2,5,7–10,14]. Several enzymes, e.g. wax ester synthase (WS) and fatty acid methyltransferase (FAMT), have been reported for direct intracellular synthesis of biodiesel molecules, and FAs are used as common precursors for producing microbial biodiesel (Fig. 1).

The first step in generating high yields of microbial biodiesel is to supply enough FAs. The metabolism and production of FAs is already an active field of research both in *E. coli* and *S. cerevisiae* [3,4,10,11,19–22], which makes them ideal candidates to become microbial biodiesel factories. It has been demonstrated that, in *E. coli*, a large increase in FAEE production could be obtained by up-regulating the FA biosynthetic pathway through a number of conceivable strategies [8,10].

S. cerevisiae is also a valuable microbial platform for producing FAEEs. It has a long, successful history in the biotechnology area and has been proved feasible to be a FAEE producer [1,7,9]. Like in *E. coli*, the production of FAEEs is usually limited by the insufficient availability of free FAs. In *S. cerevisiae*, FAs are used to synthesize phospholipids and storage neutral lipids. Phospholipids constitute the cell membrane, which is essential to the cell; storage neutral lipids, such as triacylglycerols (TAGs) and steryl esters (SEs), are the main FA reserves [23], can constitute up to 97% of the storage lipid content of the cell [24] and are not essential to the cell [20]. The syntheses of TAGs and SEs are widespread among eukaryotic organisms, whereas they do not occur in *E. coli*. In *S. cerevisiae*, TAGs can be synthesized from FAs through two different enzymes, acyl-CoA:diacylglycerol acyltransferase (DGAT, encoded by *DGA1*) or lecithin:cholesterol acyltransferase (LCAT, encoded by *LRO1*); SEs are formed from FAs through the action of an acyl-CoA:sterol acyltransferase (ASAT, encoded by *ARE1* and *ARE2*) (Fig. 2). Syntheses of TAGs and SEs would compete with a biodiesel forming pathway for utilizing the same substrate, free FAs. It is supposed that deleting these genes (i.e. *DGA1*, *LRO1*, *ARE1* and *ARE2*) to block the storage neutral lipid forming pathways would be beneficial for the accumulation of FAs and in turn increase the production of biodiesel [1]. Previous studies have shown that storage neutral lipid synthesis is non-essential in yeast and the quadruple mutant, in which *DGA1*, *LRO1*, *ARE1* and *ARE2* were disrupted, showed a 2.5-fold increase in the intracellular level of free FAs [21].

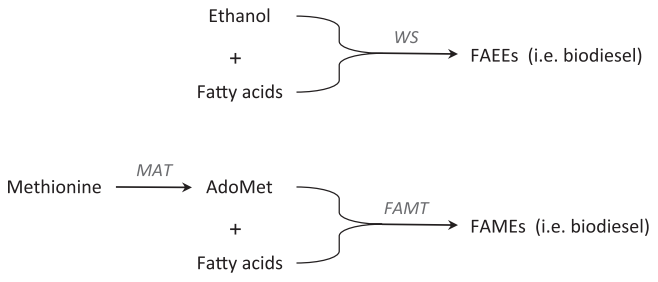

Fig. 1. Schematic representation of the metabolic pathways for the production of biodiesel molecules. FAEEs and FAMEs are both grouped into the biodiesel category. Abbreviations: WS, wax ester synthase; MAT, methionine adenosyltransferase; FAMT, fatty acid methyltransferase; FAMEs, fatty acid methyl esters; FAEEs, fatty acid ethyl esters.

FAs can be oxidized to generate energy by β-oxidation. In *S. cerevisiae*, there is only one peroxisomal acyl-CoA oxidase (encoded by the *POX1* gene) that determines the flux through β-oxidation (Fig. 2) [25,26]. A mutant strain of *Yarrowia lipolytica* with an inactivated β-oxidation had more free FAs available that could be used for the synthesis of fatty acid-derived products [27].

Here, we aimed to increase the supply of free FAs for efficient biodiesel synthesis by eliminating these non-essential FA utilization pathways (Fig. 2). This approach included: (a) deletion of *DGA1*, *LRO1*, *ARE1* and *ARE2* to block storage neutral lipid formation; (b) deletion of *POX1* to avoid FA degradation; (c) a combination of both strategies by deleting all five genes. In a prior work, we demonstrated a metabolic engineering strategy for producing biodiesel using a wax ester synthase (WS) from *Marinobacter hydrocarbonoclasticus* DSM 8798 [7]. This previously reported biodiesel producing pathway was introduced to these mutants, and abilities for biodiesel production were evaluated.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae strains used in this work are listed in Table 1. All deoxyribonucleic acid (DNA) manipulations were carried out in *E. coli* DH5α as described by Sambrook and Russell [28].

ARE1 and *DGA1* were deleted in *S. cerevisiae* CEN.PK 113-5D, while *ARE2* and *LRO1* were deleted in CEN.PK 110-10C. After these gene deletions, these haploid strains were mated, sporulated, asci were dissected and strains with the desired genotype were selected. Then, *POX1* was deleted in CEN.PK 113-5D and JV01 (Table 1). The detailed procedures are described in the following.

Gene deletions were carried out by replacement with a *kanMX* resistance cassette using a bipartite strategy as described previously [29]. For the deletion of *ARE1*, the 5′ and 3′ ends of the *ARE1* open reading frame used for homologous sequence targeting were individually amplified from genomic DNA of CEN.PK 113–5D by PCR, using the following oligonucleotide primers: ARE1-UP-f and ARE1-UP-r for the upstream section and ARE1-DW-f and ARE1-DW-r for the downstream section (Table 2). The *kanMX* expression module was amplified in two overlapping parts from the plasmid pUG6 [33] using the following oligonucleotides: kanMX-1-f and kanMX-1-r for the upstream section; and kanMX-2-f and kanMX-2-r for the downstream section. Two *loxP* sites (ATAACTTCGTATAATGTATGCTATACGAAGTTAT) flanking the *kanMX* marker allowed for its recovery. The selectable marker was recovered and reused for further deletions. *kanMX* was looped out as described previously [33] with help of the Cre recombinase expression plasmid pSH47 [30] and subsequently, strains that had lost the plasmid containing the *URA3* auxotrophy marker gene were selected utilizing 5-fluoroorotic acid [31,32].

The same approach was used for the other deletions in this study. All oligonucleotide primers used to perform gene deletions are listed in Table 2. The deletion of each gene was verified by diagnostic polymerase chain reaction (PCR) (results are presented in Supplementary file). These PCRs were performed by using the genomic DNA from each strain as template, and for each gene

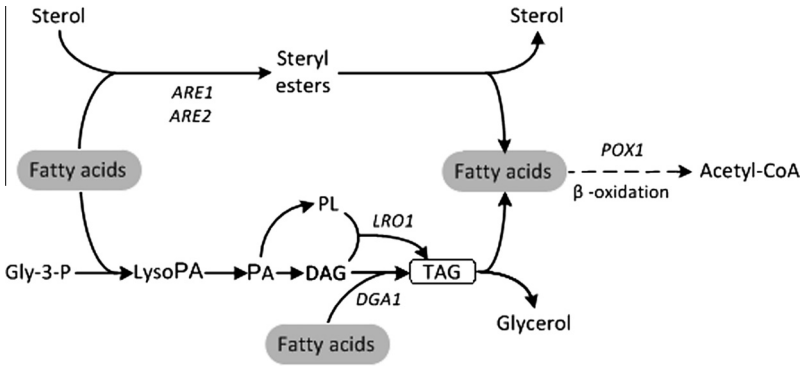

Fig. 2. Schematic representation of the pathways involved in FA storage and degradation. Genes that were deleted in the course of this work are stated. Abbreviations: Gly-3-P, glycerol-3-phosphate; LysoPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PL, phospholipids.

Table 1
Strains used in this study.

Strain	Genotype	Plasmid	Source
E. coli strain			
E. coli DH5α	*supE*44 *lacU*169 (φ80*lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1		
S. cerevisiae strains			
CEN.PK 113-5D	*MAT*a *MAL2-8*^{c} *SUC20 ura3-52*		P. Kötter, University of Frankfurt, Germany
CEN.PK 110-10C	*MAT*α *MAL2-8*^{c} *SUC2 his3*		P. Kötter, University of Frankfurt, Germany
CB0	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52*	pSP-GM2	Shi et al., 2012
CB2	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52*	pSP-B2	Shi et al., 2012
JV01	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 HIS3 are1Δ dga1Δ are2Δ lro1Δ*		This study
JV03	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 HIS3 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*		This study
JV05	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 HIS3 pox1Δ*		This study
JV01:WS	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 HIS3 are1Δ dga1Δ are2Δ lro1Δ*	pSP-B2	This study
JV03:WS	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 HIS3 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*	pSP-B2	This study
JV05:WS	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 HIS3 pox1Δ*	pSP-B2	This study

WS: wax ester synthase.

two pairs of primers were used, one binding out of locus (e.g., ARE1-Ch-UP and ARE1-Ch-DW for *ARE1*) and one with one of the primers binding in the targeted locus (e.g., ARE1-Ch-IN and ARE1-Ch-DW for *ARE1*). All primers used for strain confirmation are listed in a table contained in Supplementary file. A final confirmation was realized by sequencing of PCR products (Eurofins MWG Operon, Ebersberg, Germany).

The mating process was carried out mixing the haploid mutants CEN.PK 113-5D *MAT*a *are1Δ dga1*Δ and CEN.PK 110-10C *MAT*α *are2Δ lro1*Δ on YPD agar plates at 30 °C for 3 days. Then, sporulation and dissection were performed following the protocol described by Sherman et al. [34]. Diploid cells were sporulated at 20 °C for 4 days on a sporulation plate containing 1% potassium acetate, 0.1% bacto-yeast extract, 0.05% dextrose and 2% agar. The cell wall lysis of asci was realized using Zymolyase T100 (ICN, Costa Mesa, CA, USA). The dissection of asci was carried out utilizing a LEICA DM2000 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

Genetic analysis of tetrads was effectuated by replica plating on plates with or without uracil and/or histidine and by confirmation of gene deletions and mating type by colony PCR. The strain with the desired properties was selected.

For biodiesel production in *S. cerevisiae*, plasmid pSP-B2 (2-micron plasmid pSP-GM2 carrying *ws2* gene from *M. hydrocarbonoclasticus* DSM 8798, under the *TEF1* promoter) was adopted from a previous work [7].

PCRs of DNA constructs of upstream and downstream regions, deletion bipartite cassettes and fragments dedicated to sequence analysis were performed employing Phusion® High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). PCRs for DNA constructs of deletion verification were realized employing DreamTaq™ DNA Polymerase (Finnzymes). Protocols stated in manuals were followed.

2.2. Yeast transformation

Yeast transformations for the gene knock-outs construction were performed with a lithium acetate/single-stranded carrier DNA/polyethylene glycol method [35] following a slight modification of cultivation for 6 h under non-selective conditions before plating on antibiotic containing plates, while transformations for the introduction of plasmids were realized following a similar method (pESC Yeast Epitope Tagging Vectors, Agilent Technologies, Santa Clara, CA, USA). Transformants were picked from agar plates containing the G418 antibiotic or lacking the corresponding nutrient (uracil or histidine).

2.3. Growth conditions

E. coli recombinant cells were grown in Luria–Bertani (LB) medium in the presence of ampicillin (100 mg/l) at 37 °C. *S. cerevisiae* strains containing the *kanMX* cassette were selected on YPD plates containing 10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, 20 g/l agar and 200 mg/l G418 (Formedium LTD, Hunstanton, UK).

Mutant strains of *S. cerevisiae* not carrying the plasmid pSP-B2 were cultured in 500 ml baffled shake flasks containing 100 ml SD-CSM (synthetic dextrose – complete supplement mixture containing 20 g/l D-glucose (VWR International AB, Stockholm, Sweden), 6.9 g/l yeast nitrogen base without amino acids (Formedium LTD), 0.79 g/l complete supplement mixture (Formedium LTD)) medium at 30 °C with orbital agitation at 120 rpm,

Table 2
Oligonucleotide primers used to perform gene deletions in this study.

Name	Sequence (5′ → 3′)	Fragment
ARE1-UP-f	TGTGTTTCCGTACCGCAC	Upstream section of *ARE1*
ARE1-UP-r	CAGCGTACGAAGCTTCAGCTGCGGAATTGAGTCTGC	
ARE1-DW-f	GTGATATCAGATCCACTAGGCAACACCAAGTTTCTACGG	Downstream section of *ARE1*
ARE1-DW-r	ATTTTTGTCACCTGCAAACTC	
ARE2-UP-f	CTCGTCGGTTTATCTGCC	Upstream section of *ARE2*
ARE2-UP-r	CAGCGTACGAAGCTTCAG CGTTGAGCTTTTGGATGC	
ARE2-DW-f	GTGATATCAGATCCACTAG GCTCGGTATCTGCATGGG	Downstream section of *ARE2*
ARE2-DW-r	GCACGATATGAATAGCAGTGG	
DGA1-UP-f	CGTTATTGTAACTGGTAATCAGAG	Upstream section of *DGA1*
DGA1-UP-r	CAGCGTACGAAGCTTCAG CCTTTCGGTAATACCGGC	
DGA1-DW-f	GTGATATCAGATCCACTAG AATGTTGTTGTTGGAAGGC	Downstream section of *DGA1*
DGA1-DW-r	GCTTTCCTAAACTTACATTCAAA	
LRO1-UP-f	CTCCTTTGTACTTCTTTGTTCC	Upstream section of *LRO1*
LRO1-UP-r	CAGCGTACGAAGCTTCAG CCTGTTGATGATGAATGTGG	
LRO1-DW-f	GTGATATCAGATCCACTAG CAAGCGGTAATGGCGATC	Downstream section of *LRO1*
LRO1-DW-r	CGGTTGTTTTTCCTCTATGC	
POX1-UP-f	GCCCTATATTTACGGTATTAGTTG	Upstream section of *POX1*
POX1-UP-r	CAGCGTACGAAGCTTCAGGGGATTAATAGTAGTACGTCTCGT	
POX1-DW-f	GTGATATCAGATCCACTAG CAGATGGGGCAGGGAAG	Downstream section of *POX1*
POX1-DW-r	GTAGTCATGTCATTGATTCGTCA	
kanMX-1-f	CTGAAGCTTCGTACGCTG	First section of *kanMX*
kanMX-1-r	TCACCATGAGTGACGACTGA	
kanMX-2-f	TTCCAACATGGATGCTGAT	Second section of *kanMX*
kanMX-2-r	CTAGTGGATCTGATATCAC	

which were inoculated to an optical density at 600 nm of 0.02 from pre-cultures.

Recombinant strains of *S. cerevisiae* carrying the plasmid pSP-B2 were grown in 500 ml baffled shake flasks containing 100 ml SD-CSM medium lacking uracil (containing 20 g/l D-glucose (VWR International AB), 6.9 g/l yeast nitrogen base without amino acids (Formedium LTD), 0.77 g/l complete supplement mixture without uracil (Formedium LTD)) at 30 °C with orbital agitation at 120 rpm, which were inoculated to an optical density at 600 nm of 0.02 from pre-cultures.

2.4. Analytical methods

The growth was followed by measuring optical density at 600 nm utilizing a GENESYS™ 20 spectrophotometer (Thermo Electron Corporation, Madison, WI, USA) and samples were taken every 2 h for growth kinetics and in the early stationary phase for lipid and FAEE analyses. The dry cell weight was determined by filtering 5 ml of the cell culture through a 0.45 μm pore-size nitrocellulose filter (Sartorius Stedim, Göttingen, Germany). The filters with the biomass were washed with water, dried for 15 min in a microwave oven at 150 W, and the increased weight of the dry filter was measured. The exponential growth rate was calculated by log-linear regression analysis of the biomass versus cultivation time. The concentrations of residual glucose and external metabolites were analyzed using a Dionex Ultimate® 3000 Nano high-pressure liquid chromatography (HPLC) system (Dionex Softron GmbH, Germering, Germany) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65 °C using 5 mM H_2SO_4 as mobile phase at 0.6 ml/min and measured with a Shodex® RI-101 refraction index detector (Shoko Scientific Co., Ltd., Yokohama, Japan). Yields on substrate and specific rates were calculated according to Nielsen et al. [36].

2.4.1. Lipid extraction and analysis

Cells were harvested from a volume of 50 ml, washed twice with distilled water and centrifuged at 3000 RCF for 5 min at 4 °C. These samples were then frozen at −80 °C and freeze-dried for about three days or until they appeared dry.

To determine the total lipid content, FAMEs were prepared from around 10 mg of dried cells as described in a previous report [37].

To determine the content of the different lipid classes, lipids were extracted from 10 mg of lyophilized cell pellets using a previously reported method [7]; 25 μg of heptadecanoic acid, glyceryl tri-heptadecanoate, cholesteryl heptadecanoate, and heptadecanoic acid ethyl ester were used as the internal standards for free FAs, TAGs, SEs, and FAEEs, respectively. The lipids were separated and purified by preparative TLC using TLC Silica gel 60 F254 plates (Merck, Darmstadt, Germany) and a solvent system consisting of heptane, 2-propanol and acetic acid in the ratio 95:5:1 (v/v/v). The lipid areas were located by being sprayed with 0.05% 2,7-dichlorofluoresceine in ethanol and identified by means of appropriate standards. Spots corresponding to FAs, TAGs, SEs and FAEEs, were excised from plates using a razor-blade and transferred to 12-ml Teflon-lined screw-capped tubes. FAEEs were extracted and detected by GC–MS using the previously described method [7]. FAs, TAGs and SEs were converted to FAMEs using a microwave-assisted derivatization method [37].

2.4.2. Gas chromatography–mass spectrometry (GC–MS) analysis of FAMEs

FAMEs were separated and quantified using a Trace GC DSQII single quadrupole GC–MS (Thermo Scientific, Oberhausen, Germany). Separation was performed with an Omegawax 250 column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA). Helium was used as carrier gas. After injection at 50 °C, the oven temperature was raised to 180 °C at a rate of 20 °C min^{-1}, the temperature was kept for 1 min at 180 °C, then it was raised to 210 °C at a rate of 3 °C min^{-1}, held at 210 °C for 5 min, raised to 215 °C at 1 °C min^{-1}, held constant for 3 min, raised to 221 °C at 1 °C min^{-1}, held constant for 5 min, raised to 230 °C at 3 °C min^{-1}, held constant for 5 min, raised to 250 °C at 3 °C min^{-1}, held constant for 2 min, and finally it was raised to 270 °C at 4 °C min^{-1}, thereafter being held constant for 2 min. Mass transfer line and ion source were held at 250 °C and 200 °C, respectively. FAME peaks were identified using their spectrum patterns compared to an authentic standard mix consisting of methyl esters of myristic, palmitic, palmitoleic, heptadecanoic (internal standard), stearic, oleic and arachidic acids (Sigma–Al-

drich, St. Louis, USA) and also by searching against the NIST library. The serial dilutions in hexane of FAME mix standards were injected in the same analysis to generate standard curves for FA quantification performed with help of the QuanBrowser function in the Xcalibur software version 2.0 (Thermo Scientific).

3. Results and discussion

3.1. Construction of strains devoid of TAGs, SEs and β-oxidation

FAs can be used as precursors for a number of chemicals or biofuels. In *S. cerevisiae*, FAs are mainly stored as TAGs and SEs, called storage neutral lipids [20], and they are catabolized by β-oxidation [25]. By assessing deletion of storage neutral lipids and β-oxidation, we wanted to determine whether the free FA content could be increased by eliminating these non-essential FA utilization pathways and whether this effect could increase the production of FA derived fuels, e.g. FAEEs. To this end, four genes, *DGA1*, *LRO1*, *ARE1* and *ARE2*, were deleted in *S. cerevisiae* to generate quadruple disruption strain JV01, which is devoid of both TAGs and SEs. *POX1* was deleted in CEN.PK 113-5D to generate JV05, which is incapable of β-oxidation, and in JV01 to generate quintuple disruption strain JV03, in which all non-essential FA utilization pathways are eliminated. The deletion of each gene was verified by diagnostic PCR (list of primers in Supplementary file 1 and results in Supplementary file 2).

We found that all deletion strains are viable and their main physiological parameters are shown in Table 3. Compared to the wild-type, the maximum specific growth rates (μ_{max}) of JV01 and JV03 are reduced by 17% and 22%, respectively, very likely due to blocking the biochemical pathways of FA utilization, while in JV05, no significant reduction in μ_{max} was found. Similarly, the biomass yield (Y_{sx}) of the quintuple disrupted strain JV03 in the glucose consumption phase is only 67% of the one determined for the wild-type. The effect is less pronounced in strain JV01 with fewer genes disrupted. It has been reported that strains deficient in TAG or SE synthesis were not able to tolerate high amounts of FAs [38]. The decrease in Y_{sx} of JV01 and JV03 may be due to the toxicity caused by accumulated FAs *in vivo*, as revealed later. During growth on glucose, ethanol is accumulated, and as expected, there are no FAEEs detected in these strains. The storage neutral lipid free strain constructed in this study has a similar physiological performance compared to that one obtained in a previous report [20]. There is no previous report on the physiological consequences of *POX1* deletion in *S. cerevisiae*.

3.2. Effect of host strain genetic background on total lipid content and FA composition

Since lipids accumulate mainly during late exponential and stationary phases [20], we proceeded to analyze the total lipid content of each strain during early stationary phase. Fig. 3A shows the total lipid content of these four strains: CEN.PK 113-5D, JV05, JV01 and JV03. We found that the total lipid content is strongly influenced in the multiple deletion strains. Because TAGs and SEs constitute a major part of the lipids in stationary phase cells, JV01 and JV03, which are devoid of TAGs and SEs, both have a significant reduction in their total lipid content, which is about 50% of wild-type. Results are similar to a previous report on a strain unable to produce TAGs and SEs [20]. There was no significant effect on the total lipid content in JV05, which is incapable of β-oxidation. This is in contrast to *Y. lipolytica*, where a 50% increase in the total lipid content was observed in a *gut2*Δ strain upon elimination of β-oxidation [27].

The major FAs that accumulate in yeast are palmitic, palmitoleic, stearic, and oleic acids with C_{16} or C_{18} straight carbon chains. The natural chain length of FAs is also reflected in the composition of FA derivatives produced in the microorganism, such as found in the case of alkane production in *E. coli* [6]. To see whether the gene deletions would affect the balance between different FAs, we proceeded to analyze the FA composition in the different strains (Fig. 3B). There was no significant difference in the FA composition between CEN.PK 113-5D and JV05. In contrast, the fraction of FAs with a carbon chain length of 18 in the storage neutral lipid free strain (JV01) was 36%, which is higher than the value of 22% in CEN.PK 113-5D. The reason for this is not clear, and we speculate that it could be caused by the alteration of host cell physiology and changes in elongase I activity (encoded by *ELO1*) of converting C_{16} to C_{18}. However, it is clearly shown that the FA composition in all strains is still dominated by carbon chain lengths of C_{16} and C_{18}, which are appropriate for producing FA derived biodiesel [7].

3.3. Effect of host strain genetic background on accumulation of FAs, TAGs and SEs

Concentrations of FAs as well as TAGs and SEs for each strain are shown in Fig. 4. The gene deletions successfully enhanced the accumulation of free FAs in the cells. Compared to CEN.PK 113-5D, there was a 3-fold increase of free FAs in the strain JV01, in which the syntheses of TAGs and SEs were blocked (*DGA1*, *LRO1*, *ARE1* and *ARE2* deleted). Similar results have been found earlier, i.e. that elimination of TAGs and SEs syntheses resulted in a yeast strain with a 2.5-fold increase in the FA content of *S. cerevisiae* [20]. The abolishment of the pathway of β-oxidation (*POX1* deletion, strain JV05), led to a 4-fold increase in free FA accumulation. By combining the disruption of these three pathways of β-oxidation and syntheses of TAGs and SEs (strain JV03), FA production was enhanced 5-fold. Thus, inhibition of β-oxidation in strain JV01 led to an additional increase in free FA availability of 73%. This indicates the important role of β-oxidation for accumulation of FAs and their derivatives in *S. cerevisiae*. In addition, it has also been observed that abolishing the β-oxidation pathway leads to a positive effect on FA accumulation in *E. coli* [4,8] and in *Y. lipolytica* [27].

3.4. Effect of host strain genetic background on FAEE production

The above results have shown that by eliminating non-essential FA utilization pathways it is possible to increase the accumulation of intracellular free FAs, which would provide more precursors for the production of FA derivatives, such as FAEEs. To test this hypothesis, WS2 from *M. hydrocarbonoclasticus* DSM 8798, which was previously shown to have the highest activity among five different enzymes for FAEE production, was chosen and a codon optimized version of the gene was expressed [7].

The enzyme was introduced into the different deletion strains to investigate the effects on FAEE production. The physiological parameters and biodiesel production abilities of these strains are listed in Table 3. Compared to the strains without the pSP-B2 plasmid, the introduction of this plasmid harboring the WS gene resulted in no significant affectation (ANOVA test, P-value > 0.32) of their physiological parameters, except that significant amounts of biodiesel were detected in strains containing the plasmid. As expected, strains with higher FA supply could produce higher amounts of biodiesel. Abolishing FA degradation by β-oxidation (JV05 strain), the FAEE production increased by 48%, while through removing the storage neutral lipid synthesis (JV01 strain), FAEE production was improved by 68%. The quintuple disrupted strain JV03 was shown to have the highest free FA content, and here (Table 3), we show that it in turn results in the highest FAEE production of 17.2 mg/l. This concentration represents a 3-fold in-

Table 3
Physiological parameters and FAEE production of the reference strain CEN.PK 113-5D and the deletion strains without or with plasmid harboring the wax ester synthase (WS) gene.

Strain	μ_{max} (h^{-1})	Y_{sx} (g/g)	Y_{se} (g/g)	FAEEs (mg/l)
Strains without plasmid[a]				
CEN.PK 113-5D	0.407 ± 0.032	0.097 ± 0.007	0.341 ± 0.007	N.D.
JV01	0.339 ± 0.017*	0.074 ± 0.006*	0.242 ± 0.012*	N.D.
JV03	0.316 ± 0.021*	0.065 ± 0.006*	0.249 ± 0.01*	N.D.
JV05	0.390 ± 0.033	0.090 ± 0.008	0.310 ± 0.01*	N.D.
Strains with plasmid harboring WS gene[b]				
CEN.PK 113-5D:WS	0.397 ± 0.038	0.101 ± 0.004	0.353 ± 0.003	6.0 ± 1.18
JV01:WS	0.355 ± 0.035	0.085 ± 0.004*	0.251 ± 0.005*	10.1 ± 1.2*
JV03:WS	0.326 ± 0.031*	0.070 ± 0.005*	0.262 ± 0.002*	17.2 ± 2.0*
JV05:WS	0.385 ± 0.037	0.091 ± 0.004	0.318 ± 0.006*	8.9 ± 1.2

Y_{sx} yield of biomass on substrate (glucose), Y_{se} yield of ethanol on substrate (glucose), N.D. not detected. The reported results are the average of three replicate experiments ± standard deviation.

[a] The values reported are calculated for the exponential phase during aerobic batch cultivations on SD-CSM (complete supplement mixture) medium with 2% (w/v) D-glucose at 30 °C with orbital shaking at 120 rpm for strains of *S. cerevisiae* that do not carry any plasmid.

[b] The values reported are calculated for the exponential phase during aerobic batch cultivations on SD medium lacking uracil with 2% (w/v) D-glucose at 30 °C with orbital shaking at 120 rpm for strains of *S. cerevisiae* that carry the WS plasmid pSP-B2.

* Values significantly different compared to the wild-type strains as controls (Dunnett's test).

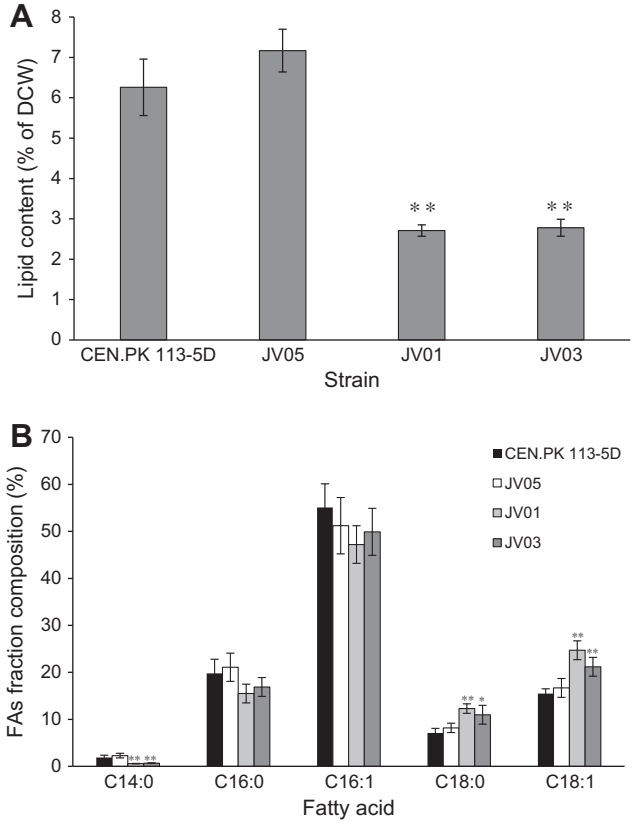

Fig. 3. Total lipid analysis (A) and FA composition analysis (B) of strains. The total lipid contents are expressed as percentage of biomass dry weight. The FA compositions are expressed as percentage of total FAs in cells. Strains were incubated in SD-CSM medium until early stationary phase. Abbreviations: DCW, dry cell weight; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C20:0, arachidic acid. The reported results are the average of three replicate experiments and error bars show the standard deviation. Asterisks (*) indicate significant difference (Fisher's LSD test) compared to CEN.PK 113-5D strain as control; *P*-values are: * <0.05 and ** <0.01. No statistically differences between strains were found in the FA fractions of C16:0 and C16:1 (ANOVA test).

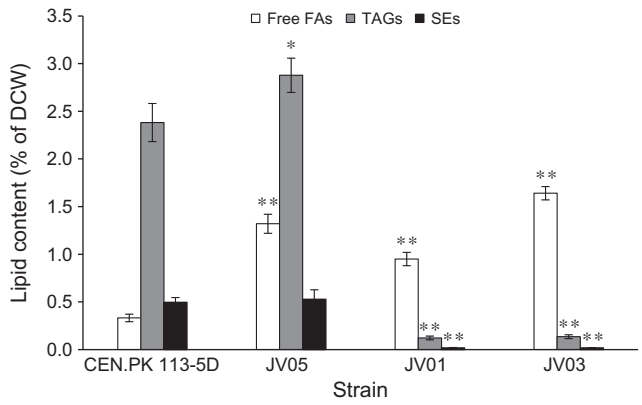

Fig. 4. Fraction of FAs, TAGs and SEs as percentage of total lipids for each deletion strain and the wild-type as control strain. Cells were cultivated in SD-CSM medium in shake flasks and harvested in early stationary phase. DCW: dry cell weight. The reported results are the average of three replicate experiments and error bars show the standard deviation. Asterisks (*) indicate significant difference (Fisher's LSD test) compared to CEN.PK 113-5D strain as control; *P*-values are: * <0.05 and ** <0.01.

crease compared to the wild-type strain, which indicates that JV03 is an excellent host strain for production of FA derivatives. Additionally, no TAGs or SEs were detected in strain JV01:WS nor in JV03:WS (data not shown). WSs can have DGAT activity as found in *Acinetobacter calcoaceticus* [39]. However, this WS (WS2) from *M. hydrocarbonoclasticus* DSM 8798 does not have DGAT activity that re-catalyzes FAs to form TAGs. This result is in accordance with the previous report indicating that there was no DGAT activity in this same WS [40], avoiding direction of fatty acyls towards TAG biosynthesis.

4. Conclusions

In *S. cerevisiae* free FAs are, besides being used for phospholipid biosynthesis, converted to and stored as TAGs and SEs [21] and they can also be degraded by β-oxidation [25]. Here, we showed how the amount and proportion of lipids changed and stepwise elimination of these non-essential fatty acid utilization pathways enhanced the free FA accumulation. Meanwhile, we also introduced a WS to convert these mutant strains into FAEE producers and a substantial increase in FAEE production was obtained in the strain without the ability to synthesize storage neutral lipids nor to perform β-oxidation. We also found a slightly higher proportion of C_{18} fatty acids (stearic and oleic acids) and a slightly lower

proportion of C_{14} and C_{16} fatty acids (myristic, palmitic and palmitoleic acids) in the storage neutral lipid devoid strains.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apenergy.2013.10.003.

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