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Metabolic pathway engineering for fatty acid ethyl ester production in *Saccharomyces cerevisiae* using stable chromosomal integration

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Abstract Fatty acid ethyl esters are fatty acid derived molecules similar to first generation biodiesel (fatty acid methyl esters; FAMEs) which can be produced in a microbial cell factory. *Saccharomyces cerevisiae* is a suitable candidate for microbial large scale and long term cultivations, which is the typical industrial production setting for biofuels. It is crucial to conserve the metabolic design of the cell factory during industrial cultivation conditions that require extensive propagation. Genetic modifications therefore have to be introduced in a stable manner. Here, several metabolic engineering strategies for improved production of fatty acid ethyl esters in *S. cerevisiae* were combined and the genes were stably expressed from the organisms' chromosomes. A wax ester synthase (*ws2*) was expressed in different yeast strains with an engineered acetyl-CoA and fatty acid metabolism. Thus, we compared expression of *ws2* with and without overexpression of alcohol dehydrogenase (*ADH2*), acetaldehyde dehydrogenase (*ALD6*) and acetyl-CoA synthetase (acs_{SE}^{L641P}) and further evaluated additional overexpression of a mutant version of acetyl-CoA decarboxylase ($ACC1^{S1157A,S659A}$) and the acyl-CoA binding protein (*ACB1*). The combined engineering efforts of the implementation of *ws2, ADH2*, *ALD6* and acs_{SE}^{L641P}, $ACC1^{S1157A,S659A}$ and *ACB1* in a *S. cerevisiae* strain lacking storage lipid formation (*are1Δ, are2Δ, dga1Δ* and *lro1Δ*) and β-oxidation (*pox1Δ*) resulted in a 4.1-fold improvement compared with sole expression of *ws2* in *S. cerevisiae*.

Keywords Yeast · Industrial strain · Chromosomal integration · Homologous recombination · Fatty acid ethyl ester (FAEE) · Metabolic pathway engineering

Introduction

Fatty acid ethyl esters (FAEEs) are compounds with molecular properties similar to crude oil derived diesel and first generation biodiesel (fatty acid methyl esters; FAMEs) and can be produced in a microbial cell factory [4, 13, 26]. Microbial conversion of sugars derived from plant biomass into FAEEs could pave the road towards a production method with a more consistent, scalable and renewable commodity supply which is more cost efficient for biodiesel than the current chemical transesterification of vegetable oils and methanol [4]. During microbial production, the precursors acyl-coenzyme A (acyl-CoA) and ethanol are converted to FAEEs by a transesterification reaction catalyzed by a wax ester synthase [26]. This process could replace the current biodiesel production method for a more sustainable future.

The first metabolic engineering projects for the creation of a microbial cell factory producing FAEEs were

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performed in *Escherichia coli* [12, 13, 30]. Combined efforts resulted in a highly engineered *E. coli* strain with cytosolic expression of thioesterase 'TesA, overexpression of acyl-CoA ligase, inhibition of β-oxidation, introduction of the alcohol synthesis pathway from *Zymomonas mobilis*, and high expression of a heterologous wax ester synthase (*atfA*), which led to an FAEE production of 674 mg/l during cultivation on glucose [29].

FAEEs are meant to become bulk products for the transport industry which implies large scale and long term microbial cultivations in media with low quality. The natural ethanol producer *Saccharomyces cerevisiae* has a long history of being used in industrial large scale fermentations and therefore represents an ideal candidate for this purpose. Due to the requirements for production in large scale, including extensive propagation of the yeast strain, it is important to create microbial strains with a stable genotype. However, most previous modifications for FAEE production in yeast were based on expression of pathway enzymes from self-replicating plasmids, which might cause a metabolic burden on the host cell. In addition, this leads to genetic instability, especially for heterologous genes, mainly caused by segregational and structural instability, which makes these strains non-suitable for industrial fermentations [11, 14, 32].

In yeast, the formation of triacylglycerols (TAGs), steryl esters (both serving as storage lipids) and the β-oxidation pathway compete for acyl-CoA with the production of FAEEs. Therefore, these pathways have previously been inactivated by disruption of *DGA1*, *LRO1* (both encoding diacylglycerol acyltransferases), *ARE1*, *ARE2* (both encoding sterol acyltransferases) and *POX1* (encoding acyl-CoA oxidase) [12, 33]. The knock-out strain was used to express a wax ester synthase from *Marinobacter hydrocarbonoclasticus* (*ws2*) [26] and resulted in a yeast strain with the inability to synthesize storage lipids and an FAEE production of 17.2 mg/l [33]. Furthermore, the combined expression of $Acc1^{S1157A,S659A}$, an acetyl-CoA carboxylase variant, in which posttranslational regulation by phosphorylation is abolished, and *ws2* resulted in an FAEE production of 15.8 mg/l [25]. In another recent attempt to increase the production of FAEEs in yeast, overexpression of native *ACC1* and *FAS1* and *FAS2* (both subunits of fatty acid synthase), in combination with deletion of the β-oxidation pathway and expression of a wax ester synthase from *Acinetobacter baylyi* (AtfA) resulted in an FAEE production of 5.44 mg/l [23]. Following up on the previous work, a FAEE titer of 13.9 mg/l was achieved with an abolished β-oxidation pathway, disruption of acyl-CoA synthetase gene *FAA2* and acyl-CoA transporter gene *PXA2* as well as elimination of acyl-CoA binding protein Acb1 in combination with expression of codon optimized *atfA* under nitrogen limitation cultivation conditions [31]. Finally, one study focused on increasing the expression level of the wax ester synthase, when six copies of *ws2* were randomly integrated into the genome, which resulted in a titer of 34 mg/l [27].

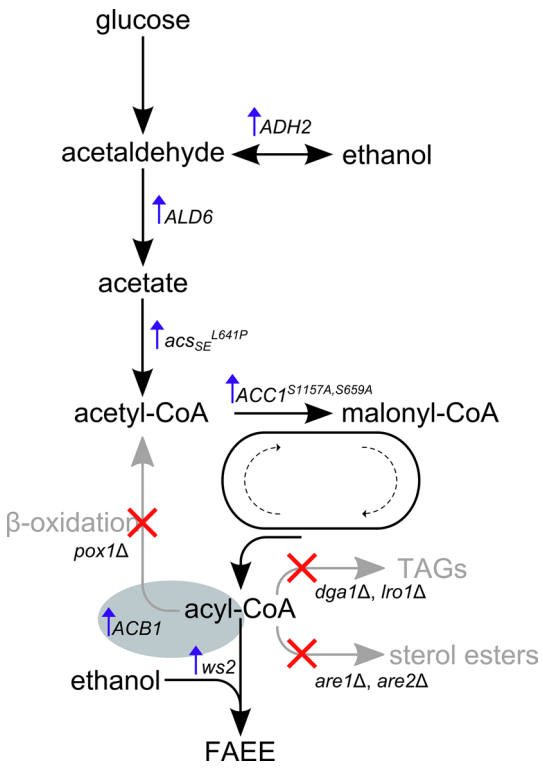

Fig. 1 Overview of metabolic engineering approaches of FAEE in *S. cerevisiae*. *Blue arrow* indicates overexpressing of the gene. This scheme is simplified and does not show co-factors or compartmentalization

Here, a targeted chromosomal integration approach for the combination of multiple FAEE engineering strategies was chosen to stably (over)express and maintain foreign as well as endogenous genes. Specifically, the endogenous alcohol dehydrogenase (encoded by *ADH2*), acetaldehyde dehydrogenase (encoded by *ALD6*) and a heterologous acetyl-CoA synthetase variant from *Salmonella enterica* insensitive to regulation by acetylation (encoded by acs_{SE}^{L641P}) were over-expressed for the improvement of the supply of the precursor acetyl-CoA and cofactor NADPH [5]. This was combined with overexpression of $ACC1^{S1157A,S659A}$ and *ACB1* to increase the acyl-CoA pool [25], which was converted into FAEEs by wax ester synthase Ws2 [26]. These stable chromosomal integrations were introduced in wild type *S. cerevisiae* as well as in a mutant strain lacking acyl-CoA competitive but non-vital pathways (*are1Δ dga1Δ are2Δ lro1Δ pox1Δ*) [33] (Fig. 1).

Table 1 List of strains used in this study

Strain	Genotype or relevant characteristics[a]	Engineered pathways[b]	Source
CEN.PK113-11C	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1*	–	P. Kötter
BdJ10	CEN.PK113-11C *ws2*	WS	This study
BdJ11	CEN.PK113-11C *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P}	WS + EDP	This study
BdJ12	CEN.PK113-11C *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P} $ACC1^{S659A,S1157A}$ *ACB1*↑	WS + EDP + FASP	This study
JV04	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*	–	This study
BdJ13	JV04 *ws2*	WS	This study
BdJ14	JV04 *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P}	WS + EDP	This study
BdJ15	JV04 *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P} $ACC1^{S659A,S1157A}$ *ACB1*↑	WS + EDP + FASP	This study

[a] Native overexpressed genes are market with ↑

[b] Engineered pathways are defined as follows: *WS* wax ester synthesis, *EDP* ethanol degradation pathway, *FASP* fatty acid synthesis pathway

Table 2 List of plasmids used in this study

Plasmid	Description	Reference
pXI-5	specific recognition sites XI-5 up/down; *URA3* selection marker flanked by directed repeats	[19]
pXI-3	specific recognition sites XI-3 up/down; *URA3* selection marker flanked by directed repeats	[19]
pADmGA	2 µm expression plasmid containing $ACC1^{S659A,S1157A}$ and *ACB1*	[25]
pBdJ02	2 µm expression plasmid containing *ADH2*, *ALD6,* acs_{se}^{L641P} and *ws2*	[5]
pBdJ01	2 µm expression plasmid containing *HIS3* selection marker	[5]

Materials and methods

Strains

During this research, yeast strain *S. cerevisiae* CEN.PK 113-11C (*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1*) was used which was kindly provided by P. Kötter, University of Frankfurt, Germany. Additionally, strain JV04, based on strain CEN.PK113-11C, was used which contains five different gene deletions (*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*) to prevent the conversion of free fatty acids to sterol esters, triacylglycerides and their degradation to acetyl-CoA via the β-oxidation pathway. With the exception that JV04 contains an additional *his3-Δ1* mutation, the strain is the same as JV03 (*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*) and was constructed following the same gene deletion and strain crossing procedure [33]. *Escherichia coli* strain DH5α [24] was used for amplification of the plasmids used for pathway engineering.

Yeast pathway construction

Three different strains were constructed based on each of the two background strains, CEN.PK113-11C and JV04. All genes were integrated into the chromosome. The first strain was used as a reference strain and contained *ws2*. The second strain contained *ADH2*, *ALD6* and acs_{SE}^{L641P} in addition to the *ws2* gene. And the third strain contained $ACC1^{S1157A,S659A}$ and *ACB1* in addition to the previously named genes. The strains were constructed with help of linear chromosomal integration cassettes. All strains are listed in Table 1 and the inserts have been sequenced (Eurofins MWG Operon, Ebersberg, Germany).

DNA bricks

To construct the linear chromosomal integration cassettes, it was first necessary to amplify DNA bricks from a given set of plasmids (Table 2) by polymerase chain reaction (PCR). The PCR reaction was performed with Phusion polymerase following the instructions of the manufacturer (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: denaturation at 98 °C for 3 min and then 34 cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 30 s/kb, followed by a 10 min incubation at 72 °C. All DNA-bricks were separated by gel electrophoresis and were purified by a GeneJet Gel extraction kit (Thermo Fisher Scientific). The DNA concentration was determined by a Nanodrop 2,000 spectrophotometer (Thermo Fisher Scientific). The primers used, were specifically designed with overhangs matching the end of the previous or next brick, respectively (Table 3).

Two-step fusion PCR

A two-step fusion PCR [36] was performed to create the linear chromosomal integration modules if necessary. The first PCR

Table 3 List of oligonucleotide primers

Oligonucleotide primers used for construction of the linear integration cassettes		
Primer	Description	Sequence 5′-3′
P1	XI-5 up-F	GCGGAGAAGTCGTTGATAGCA
P2	XI-5 up-R	**GGAATTGCC**TGGTGCACGGAGTTTATGGC
P3	*URA3*-F	**CCGTGCACCA**GGCAATTCCCGGGGATC
P4	*URA3*-R	**ATGAGGTCGCTC**AATTGGGTACCGGGCCC
P5	tADH1-R	**GGTACCCAATT**GAGCGACCTCATGCTATACCTG
P6	*ws2*-F	TGGGAGGTAGAATGAGGCCA
P7	*ws2*-R	CTTACTTTCTAGTACGGGCACGC
P8	*ws2*-F	TAAAACAATGAAGAGATTAGGTACTCTAGAC
P9	*ws2*-R	TCACCACCTGGTCTTGGCA
P10	pTEF1-F	**AATCGAAGGC**GCACACACCATAGCTTCAAAATG
P11	XI-5 down-F	**TGGTGTGTGC**GCCTTCGATTTGACACATCTCTAA
P12	XI-5 down-R	GATCATAGATCCGGCACTTAGAGA
P13	pPGK1-F	AGTTCGTTCGATCGTACTGTTACTC
P14	pPGK1-R	GGATCCTTGTTTTATATTTGTTGTAAA
P15	tADH2-F	ACGTCAAGACGAAAAGTGAAAAAT
P16	*ADH2* -R	GAAGCCGCTATCGAAGCTTC
P17	tADH2-F	**CAAATCGAAGGC**ACGTCAAGACGAAAAGTGAAAAAT
P18	XI-5 down-F	**ATGTCTTTACGAT**GCCTTCGATTTGACACATCTCTAA
P19	XI-3 up-F	AGTTACTTGCTCTATGCGTTTGC
P20	XI-3 up-R	**GTTGTCGACCTGCA**AATCAGACGCACGCTTGG
P21	*HIS3*-F	**CGTGCGTCTGATT**TGCAGGTCGACAACCCTTAAT
P22	*HIS3*-R	**GAAGGTACTTCC**GCCACTAGTGGATCTGATATCACCTA
P23	pPGK1-R	**ATCCACTAGTGGC**GGAAGTACCTTCAAAGAATGGG
P24	$ACC1^{S659A,S1157A}$-F	TGGGGTGGCCATGGC
P25	$ACC1^{S659A,S1157A}$-R	ATGAGCGAAGAAAGCTTATTCG
P26	$ACC1^{S659A,S1157A}$-F	GCCATACCAATTCTGGCACC
P27	$ACC1^{S659A,S1157A}$-R	ACAGATGATTTCTTTATTTCCAACG
P28	tCYC1-F	CTTCGAGCGTCCCAAAACC
P29	*ACB1*-R	GCCTGGGAAAACTTAAAAGGTAA
P30	tCYC1-F	**AATCCACGTAA**CTTCGAGCGTCCCAAAACC
P31	XI-3 down-F	**GGACGCTCGAAG**TTACGTGGATTGAGCCAGCA
P32	XI-3 down-R	TGAGAATCCGGACCAGCAGA
Additional oligonucleotide primers used for verification and sequencing of correct integration insert		
Primer[#]	Description	Sequence 5′-3′
V1	genome XI-5 up-F	CGGTAGTTGTTCCTACACGC
V2	*URA3*-R	TGCGATACGGTAAACGCC
V3	tADH1-F	GAGCGACCTCATGCTATACCT
V4	*ws2*-R	TCAACACGAAGGGCGAAG
V5	*ws2*-F	CAAGACCTTCAATGACATGGC
V6	genome XI-5 down-R	TGCGAATGTGATTTGGAGTG
V7	*ALD6*-F	GGGATAGGTCTTACCGTCTTG
V8	acs_{se}^{L641P}-R	CGAGTATCTTACCTTGCTCGC
V9	*ADH2*-F	CTTAGGTATTGATGGTGGTCCA
V10	genome XI-3 up-F	GTTGGAGTCCGGATCATCC
V11	*HIS3*-R	GAATCCAATTCCCGTGTCTACT
V12	pPGK1-F	CGTGTGACAACAACAGCCTG
V13	$ACC1^{S659A,S1157A}$-R	AGGCAATAGTGGATTCTCGG

Table 3 continued

Additional oligonucleotide primers used for verification and sequencing of correct integration insert		
Primer[#]	Description	Sequence 5′-3′
V14	*ACC1*^{S659A,S1157A}-F	GACCTATTGCTACTCCTTACCCTG
V15	*gapN*-R	CTCCATTGAAGCTATGACTACAGTG
V16	*Acb1*-F	GCAAGCCACTGTAGGTGACAAC
V17	genome XI-3 down-R	GTGGCGATGCTGGTGCA
V18	*ACC1*^{S659A,S1157A}-R	ACTAATGACTTGAATTTATAGGCAGG
V19	acs_{se}^{L641P}-R	GTAGATTGCCACAACATCGC

Overlaps are shown in bold

reaction was performed by mixing 100–300 ng of DNA-bricks in equimolar amounts with Phusion GC buffer, dNTPs, and Phusion polymerase in a total volume of 25 µl. The PCR conditions for amplification were 98 °C for 3 min, 15 cycles of 98 °C for 10 s, 68 °C for 30 s, and 72 °C for 30 s/kb, followed by a 10 min incubation at 72 °C. 2 µl of unpurified PCR product were used as a template for the second PCR-step. Additionally, a forward and reverse primer, dNTPs, Phusion GC buffer and Phusion polymerase were added to the PCR mix in a final volume of 50 µl. The reaction was performed identically to the PCR reaction of the DNA bricks. These fusion PCR-products (modules) were separated on a 1 % agarose gel and purified with a gel purification kit as above. The concentration was determined by a Nanodrop 2,000 spectrophotometer.

Homologous recombination and transformation

The different DNA modules formed the chromosomal integration cassettes and were transformed into *S. cerevisiae* by electroporation at 1.5 kV, 10 µF and 200 Ω using a 0.2 cm gap electroporation cuvette and an Eppendorf Eporator (Eppendorf AG, Hamburg, Germany). 400–800 ng of each of the DNA modules were mixed with the 50 µl of competent cells. Competent cells were prepared following instructions [36]. After electro-transformation the cells were incubated for 3 h in 1 M sorbitol at 30 °C and plated on appropriate dropout plates. Colonies were screened by PCR for correctly integrated cassettes and the PCR fragments spanning the entire integrated constructs were sequenced (Eurofins MWG Operon, Ebersberg, Germany). The additional primers used for the verification are listed in Table 3.

The first chromosomal integration cassette contained only the wax ester synthase gene (*ws2*) controlled by the *TEF1* promotor and the *ADH1* terminator and the resulting strains functioned as reference strains. The second linear integration cassette contained over-expressed genes *ADH2*, *ALD6* and acs_{SE}^{L641P}, which improve the provision of the precursor acetyl-CoA, together with *ws2* [2, 5, 17]. The genes were regulated by constitutively active promoters *HXT7* (truncated; [8]), *PGK1* and *TEF1* and terminators *ADH2*, *CYC1*, and *ADH1,* respectively. Both cassettes (1 and 2) contained the chromosomal recognition sites to be integrated into chromosome XI at integration position 5 characterized in a previous study [19] and contained a *URA3* selection marker. The third linear integration cassette contained genes *ACC1*^{S1157A,S659A} and *ACB1* controlled by, respectively, *PGK1* and *TEF1* promoters and *ADH1* and *CYC1* terminators. This cassette was integrated into chromosome XI at integration position 3 [19] and contained a *HIS3* selection marker. Figure 1 shows the different linear integration fragments, including promoters, terminators, recognition sites and selection marker. It has been demonstrated that both chromosomal integration sites, XI-5 and XI-3, resulted in the highest beta-galactosidase activity during a comparison study for strains with the *lacZ* cassette integrated at 14 integration sites [19].

Media and growth conditions

All described strains of *S. cerevisiae* were cultivated in shake flasks at 30 °C and continuous shaking at 200 rpm in synthetic dextrose (SD) medium containing 20 g l^{-1} glucose, 6.7 g l^{-1} yeast nitrogen base without amino acids (YNB-AA) (Formedium, Hunstanton, UK), and complete supplement mixture (0.750 g l^{-1}; CSM, Formedium) lacking, if necessary, uracil and histidine, respectively.

FAEE quantification

Samples were taken from the cultures after 100 h and handled as previously described [5]. The quantification of fatty acid ethyl esters was performed with a Focus ICQ single quadrupole GC-MS from Thermo Fisher Scientific with a Zebron (ZB-WAX) GC column with 30 m × 25 mm internal diameter and 0.25 µm film thickness (Phenomenex, Macclesfield, UK). The precise GC-MS conditions, compound identification and quantification conditions were described previously [26].

Results

Metabolic pathway integration

Metabolic pathway engineering was performed to implement the FAEE pathway, integrated into the *S. cerevisiae*

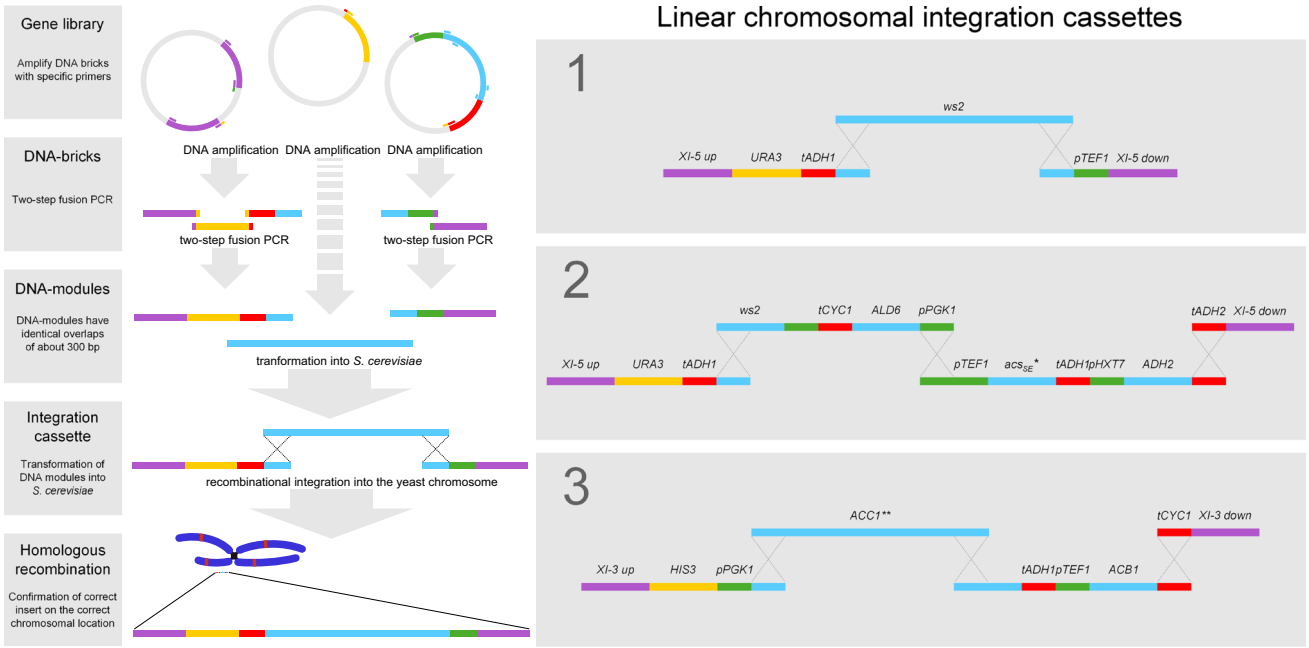

Fig. 2 Integration pipeline and linear chromosomal integration cassettes

chromosomes, in two different background strains: CEN.PK113-11C and JV04. Therefore three different linear chromosomal integration cassettes were constructed containing the genes of interest. Figure 2 shows the integration pipeline and the linear integration cassettes. Chromosomal integration cassette 1, composed of 3 DNA modules and containing i.a. a *TEF1* promotor, *ws2* and the *ADH1* terminator had a total length of 4.915 base pairs (bp) and was integrated successfully into chromosomal position XI-3 of CEN.PK113-11C and JV04. 26 colonies were picked after transformation into both strains and, respectively, 22 (84 %) and 15 (58 %) colonies were confirmed as correct integrations by PCR. For each background strain one colony was picked and confirmed by sequencing. This resulted in strains BdJ10 and BdJ13. Strains BdJ11 and BdJ14, carrying the ethanol degradation pathway (EDP) were constructed by chromosomal integration of the second linear integration cassette containing genes *ADH2*, *ALD6*, acs_{SE}^{L641P} and *ws2* distributed on 4 DNA modules. Both cassettes (1 and 2) contained the chromosomal recognition sites to be integrated into chromosome XI at integration position 5 [19] and contained a *URA3* selection marker. The total length of the integration cassette stretched over 12.033 bp and 63 and 56 %, respectively, of the transformants were confirmed as being correct by PCR. One final colony for each background strain was confirmed by sequencing and subsequently used for the construction of strain BdJ12 and BdJ15, which in addition contained the third integration cassette with genes $ACC1^{S1157A,S659A}$ and *ACB1*. The integration cassette, including the *HIS3* selection marker, consisted of 4 DNA modules and was integrated into chromosome XI at integration position 3 [19]. The total size of cassette 3 was 11.614 bp and the homologous recombination success rate was 80 % and 8 %, respectively, confirmed by PCR for BdJ11 and BdJ14 as background strains. Both newly generated strains, BdJ12 and BdJ15 were confirmed by sequencing.

FAEE production

The sole expression of *ws2* in CEN.PK113-11C resulted in 261 ± 57 μg total FAEEs $gCDW^{-1}$ (strain BdJ10). Additional expression of *ADH2*, *ALD6* and acs_{SE}^{L641P} resulted in an FAEE production of 463 ± 77 μg total FAEEs $gCDW^{-1}$ (strain BdJ11) and further chromosomal integration of $ACC1^{S1157A,S659A}$ and *ACB1* resulted in a strain producing 570 ± 130 μg total FAEEs $gCDW^{-1}$ (strain BdJ12). Compared to the expression of *ws2* alone (BdJ10), strain BdJ11 improved the yield of FAEEs 1.8-fold and the additional expression of $ACC1^{S1157A,S659A}$ and *ACB1* improved the FAEE yield 2.2-fold compared to strain BdJ10 and 1.2-fold compared to strain BdJ11 (Fig. 3a).

In the background strain JV04, the conversion of free fatty acids towards sterol esters, triacylglycerides and acetyl-CoA via the β-oxidation pathway were eliminated and combined with the chromosomal overexpression of *ws2* to create the reference strain, which resulted in 506 ± 58 μg total FAEEs $gCDW^{-1}$ (BdJ13). The additional expression of genes *ADH2*, *ALD6*, acs_{SE}^{L641P}, yielded 797 ± 69 μg total FAEEs $gCDW^{-1}$, which was a 1.6-fold

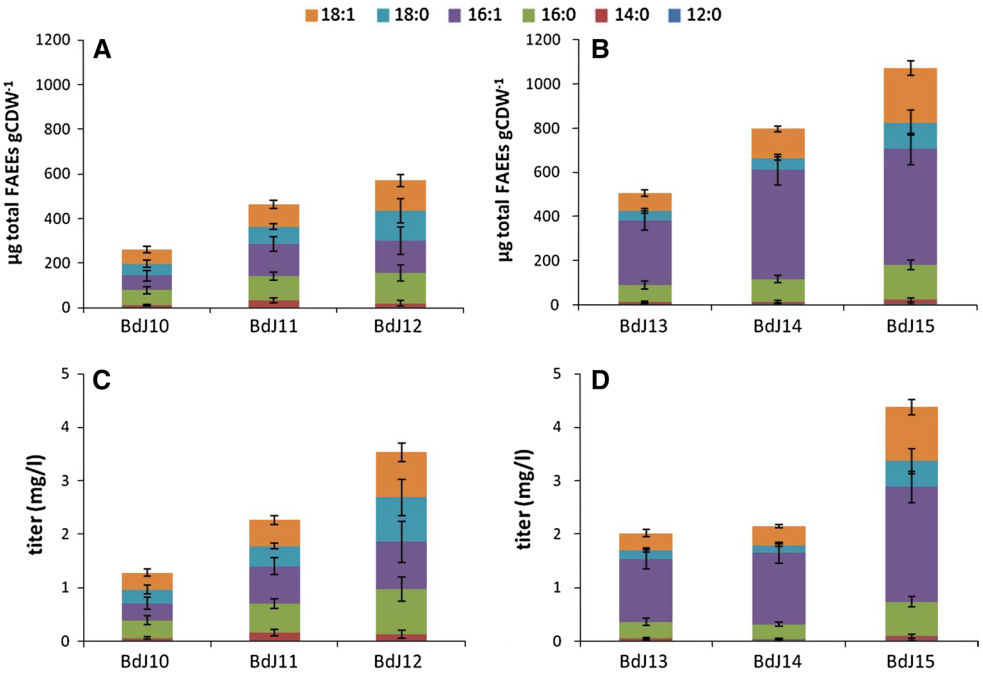

Fig. 3 FAEE yield of engineered strains based on background CEN.PK.113-11C (**a**) and JV04 (**b**) as well as FAEE titers (g/l) of FAEE producing strains based on background CEN.PK113.11C (**c**) and JV04 (**d**). The distribution of acyl moieties in the different FAEE pools is indicated by the *colour code*. Results represent the average ± standard deviation of duplicate performed shake flask experiments with biological triplicates (color figure online)

improvement compared to BdJ13. Strain BdJ15, in which the strategies of expressing *ws2, ADH2*, *ALD6*, acs_{SE}^{L641P}, $ACC1^{S1157A,S659A}$, *ACB1* and eliminating the fatty acid competing pathways were combined, resulted in a yield of 1,072 ± 160 µg total FAEEs $gCDW^{-1}$ which was the highest FAEE yield detected here (Fig. 3b).

The most extensively engineered strain (BdJ15) resulted in a 4.1-fold improvement of the FAEE yield compared with the reference strain, in which *S. cerevisiae* only carried the *ws2* gene (BdJ10). Overall, expression of genes in the JV04 background resulted in an 1.85 ± 0.11-fold improvement in FAEE production compared with the respective counterpart strains based on CEN.PK113-11C (Fig. 3a, b). However, the mutant strains based on JV04 resulted in a lower final biomass concentration after substrate depletion (data not shown). Therefore, while comparing the titers of the different strains with CEN.PK113-11C and JV04 background, the BdJ10, BdJ11 and BdJ12 strains showed titers of, respectively, 1.3 ± 0.3, 2.3 ± 0.4 and 3.5 ± 0.8 mg l^{-1} versus FAEE titers of strains BdJ13, BdJ14 and BdJ15 of 2.0 ± 0.2, 2.2 ± 0.2 and 4.4 ± 0.7 mg l^{-1}, respectively (Fig. 3c, d).

Chain length distribution

All constructed strains contain a wax ester synthase, which catalyzes the transesterification reaction that converts ethanol and acyl-CoA into FAEEs. In *S. cerevisiae*, the predominant fatty acids are sixteen and eighteen carbon atoms long and exist in their saturated form as well as in their mono-unsaturated form [9]. The most obvious change in FAEE chain length distribution occurred for unsaturated 16:1 FAEEs after elimination of the fatty acid competing pathways (Fig. 3). BdJ10-BdJ12 produced 27 ± 3.5 % of unsaturated 16:1 FAEE, whereas BdJ13-BdJ15 produced 56 ± 6.5 % of this FAEE. This increase is almost solely responsible for the improved production of total FAEEs. The fraction of saturated 16:0 FAEE and saturated 18:0 FAEE of strains BdJ10-BdJ12 compared to strain BdJ13-BdJ15 on the other hand were reduced from, respectively, 24 ± 1 to 14 ± 3 % and from 20 ± 1 to 8 ± 2 %. No significant difference for the 18:1 unsaturated FAEE content was detected. Also the implementation of genes *ADH2*, *ALD6* and acs_{SE}^{L641P} or $ACC1^{S1157A,S659A}$ and *ACB1* did not show any significant difference for the distribution of the carbon-chain length.

Discussion

FAEE expression

Expression of genes *ws2, ADH2*, *ALD6* and acs_{SE}^{L641P} in *S. cerevisiae* for improving the production of FAEEs was previously evaluated using multicopy 2 µg plasmids, but resulted in a slightly lower and a more unstable yield of FAEEs than reported here (408 µg total FAEEs $gCDW^{-1}$ compared to 463 µg total FAEEs $gCDW^{-1}$) [5]. The expression of $ACC1^{S1157A,S659A}$ and *ACB1* in *S. cerevisiae* was also earlier reported to have a positive effect on the FAEE production [25, 27]. The combined expression of the above listed genes on two 2 µg plasmids with a total

size of 15 and 18 kb however, decreased the yield of FAEE in *S. cerevisiae* (unpublished results). It was thought that the expression of these multiple genes from high-copy plasmids resulted in a metabolic burden, which caused a (partial) loss of expression resulting in a negative effect on FAEE production. Although it was not explicitly shown here, chromosomal integration has been demonstrated to be a more stable way of expressing genes compared to episomal plasmid expression [11, 18, 34]. We also noticed in this study a much reduced clonal variation of the integrated strains in comparison to the plasmid-based strains used in previous studies (data not shown). Additionally, the most favorable (with regard to growth impairment and gene expression) integration sites investigated by Mikkelsen et al. [19] separated by essential genetic elements, were used in this work and therefore reduced the probability of loss of the integration cassettes. Also, the use of repetitive sequences within the integration cassettes and the introduction of sequence homology in multiple tandem inserts were avoided, because of the risk of homologous recombination and chromosomal rearrangements [11, 18, 34].

In contrast to the observed FAEE decrease in a plasmid-based strain, here we show a clear improvement of the FAEE yield in BdJ15, which contains all the above discussed modifications and this clearly shows the importance of stable expression through genome integration when several metabolic engineering strategies have to be combined and when large genes have to be expressed.

Earlier, several metabolic engineering strategies have been implemented for improving FAEE production by *S. cerevisiae*. These strategies mostly focused on the production of acyl-CoA, its precursors acetyl-CoA [1, 5, 28] and malonyl-CoA [25], the elimination of the production of fatty acid related non-vital byproducts [33] and improved supply of NADPH [5]. Here, the combined implementation of *ws2, ADH2*, *ALD6, acs*$_{SE}^{L641P}$, *ACC1*^{S1157A,S659A} and *ACB1* into a *are1Δ dga1Δ are2Δ lro1Δ pox1Δ S. cerevisiae* strain resulted in a FAEE yield of 1.1 mg total FAEEs gCDW^{-1} and a titer of 4.4 mg l^{-1} during growth on glucose. Despite the clear improvement obtained through the combined metabolic engineering strategies, the chromosomal integration leads to lower FAEE titers than reported in earlier studies. We believe this is primarily due to a limitation of the wax ester synthase, which in all previous studies was expressed in multi-copies, either from multi-copy plasmids or through multi-copy integration. This is consistent with our earlier study which showed that six randomly integrated copies of the gene *ws2* into the chromosomes of *S. cerevisiae* improved the FAEE production compared with a single copy expression of *ws2* [27]. The integration of the above described pathway into the chromosome of the strain with six integrated copies of *ws2*, which is based on an *ura3-52* selection marker, could possibly improve the yield of FAEEs and take away the limiting esterification step of the pathway. However, for large and complex pathway integrations, as presented here, there is a demand of recycling the auxotrophic selection marker of the strain which comes along with several rounds of strain transformation and selection. As reported earlier, Jensen et al. [11] demonstrated that selection marker loop-out for a new round of metabolic engineering resulted in low efficiency for correct integrations, which was thought to be due to the proximity of the integration sites and possible homologous recombination resulting in a loss of pathway genes. We attempted to integrate the above described pathway in two rounds of transformation and marker recycling into the strain with six integrated copies of *ws2*, but a drastic loss of product yield was observed after *URA3* selection marker loop-out, which indicated a loss of pathway genes (data not shown).

Acyl-CoA binding protein (Acb1) plays an important role for facilitating intracellular transport of acyl-CoA esters to the membrane of the endoplasmic reticulum and lipid bodies for phospholipid and TAG biosynthesis [16]. Additionally, it was reported that Acb1 had an attenuating effect on the negative feedback mechanism of acyl-CoAs on fatty acid synthase, acetyl-CoA carboxylase and long chain acyl-CoA synthetase [10, 15, 22]. However, it remains unclear which engineering strategy concerning *ACB1* results in the highest FAEE production. Both the overexpression of the *ACB1* gene [27] to improve the attenuation of the negative feedback loop as well as its deletion to prevent transport of acyl-CoA towards unwanted cellular locations [23] resulted in a positive effect on FAEE production. Here, FAEE production improved with up-regulation of *ACB1* (in combination with *ACC1*^{S1157A,S659A}), but understanding the exact mechanisms will require further investigations.

Distribution acyl moiety chain length

The mechanism for distribution and regulation of the carbon chain length of fatty acids derived products in *S. cerevisiae* is only partly known [6, 9]. Here, we observed a large increase in 16:1 unsaturated FAEE in mutant strains with disrupted *DGA1*, *LRO1* (both encoding diacylglycerol acyltransferases), *ARE1*, *ARE2* (both encoding sterol acyltransferases) and *POX1* (encoding acyl-CoA oxidase) [12, 33]. This increase was surprising, because earlier reports with a yeast strain containing the same gene deletions did not observe any difference in the relative amount of 16:1 free fatty acids [33]. However, in this earlier study a high-copy expression of *ws2* was used, which may affect the fatty acid composition in the produced FAEEs. Another study, in which storage lipid were eliminated (*are1Δ dga1Δ are2Δ lro1Δ*) also showed a much larger ratio of unsaturated fatty acids compared to

saturated fatty acids during different growth conditions [7], whereas a third study only detected a smaller change for the same factor [21]. The disrupted capacity to store fatty acids as TAGs was mentioned to be critical for buffering the regulation of fatty acid composition [21].

The synthesis of the acyl-chain is a major energy- and carbon consuming metabolic step and acyl-CoA also functions as a substrate for vital membrane phospholipids. However, the mechanism regulating the total amount and the specific composition of acyl-CoA and its downstream products, which is under several levels of metabolic and transcriptional control, remains largely unclear [9]. In *S. cerevisiae*, the acyl chain length is partly determined by the fatty acid synthase complex [20] and recently it was found that acetyl-CoA decarboxylation also has a fundamental impact on the relative distribution and quantity of acyl-CoA chains in cellular lipids [9]. In *S. cerevisiae*, Snf1-dependent phosphorylation attenuates the activity of acetyl-CoA carboxylase (Acc1), for maintaining the appropriate acyl-chain length distribution and the ratio of C16 versus C18 fatty acids [35]. It is also thought that the specific molecular composition of acyl-CoA and its downstream products directly regulates genes of the fatty acid synthesis pathway via differential interaction with the repressor Opi1 [9]. In *S. cerevisiae*, a higher content of free fatty acids (and related molecules) was yielded by the elimination of two post-translational sites in acetyl-CoA decarboxylase (*ACC1*) [3, 9, 25].

However, here it was shown that specifically the production of 16:1 unsaturated FAEEs was increased due to the elimination of genes *DGA1*, *LRO1*, *ARE1*, *ARE2* and *POX1*. Most likely eliminating production of TAGs and sterol esters influence the specific regulation of acyl-CoA formation, which subsequently changes the composition of the produced FAEEs. The sensitive balance between acyl-CoA, its downstream products, mainly TAGs in wild type yeast cells, which was interrupted, might cause a change in the carbon chain distribution and influence the energy dependent fatty acid elongation. In addition, it is known that *ws2* prefers longer alcohols and has a low affinity towards short alcohols, like the ethanol used for FAEEs produced by *S. cerevisiae* [26]. Matching the demands of the enzymes which catalyze the downstream reaction with the substrate specificity could likely improve the productivity substantially. Understanding the regulatory mechanisms determining the composition and quantity of downstream acyl-CoA derived intermediates is crucial for future production of designer fatty acid derived products, like FAEEs.

Conclusion

Fatty acid ethyl esters are suitable molecules for substitution of crude oil and current FAME biodiesel. Here, it was demonstrated that chromosomal integration of the metabolic engineering strategy into the chromosomes of *S. cerevisiae* resulted in stable expression and it was therefore possible to quantitatively evaluate complex engineering strategies involving several different genetic modifications. Furthermore, the here presented strains represent a very good starting point for further engineering, e.g. evaluation of the effect of increased expression of the wax ester synthase, which is likely to be limiting production the engineered strains.

Author contribution BWDJ has performed all experimental work, analyzed the data and drafted the manuscript. BWDJ, SS, VS and JN designed and coordinated the work and edited the manuscript. JOV-R provided strain JV04.

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References

1. Chen Y, Daviet L, Schalk M, Siewers V, Nielsen J (2013) Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab Eng 15:48–54
2. Chen Y, Siewers V, Nielsen J (2012) Profiling of cytosolic and peroxisomal acetyl-CoA metabolism in *Saccharomyces cerevisiae*. PLoS One 7:e42475
3. Choi JW, Da Silva NA (2014) Improving polyketide and fatty acid synthesis by engineering of the yeast acetyl-CoA carboxylase. J Biotechnol 187:56–59
4. de Jong B, Siewers V, Nielsen J (2012) Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. Curr Opin Biotechnol 23:624–630
5. de Jong BW, Shi S, Siewers V, Nielsen J (2014) Improved production of fatty acid ethyl esters in *Saccharomyces cerevisiae* through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway. Microb Cell Fact 13(1):39
6. de Kroon AIPM, Rijken PJ, De Smet CH (2013) Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. Prog Lipid Res 52(4):374–394
7. Garbarino J, Padamsee M, Wilcox L, Oelkers PM, D'Ambrosio D, Ruggles KV, Ramsey N, Jabado O, Turkish A, Sturley SL (2009) Sterol and diacylglycerol acyltransferase deficiency triggers fatty acid-mediated cell death. J Biol Chem 284(45):30994–31005
8. Hauf J, Zimmermann FK, Muller S (2000) Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. Enzym Microb Technol 26(9–10):688–698
9. Hofbauer HF, Schopf FH, Schleifer H, Knittelfelder OL, Pieber B, Rechberger GN, Wolinski H, Gaspar ML, Kappe CO, Stadlmann J, Mechtler K, Zenz A, Lohner K, Tehlivets O, Henry SA, Kohlwein SD (2014) Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. Dev Cell 29(6):729–739
10. Hong S-P, Xue Z (2008) Manipulation of acyl-CoA binding protein expression for altered lipid production in microbial hosts. Patent: US20090291479 A1

11. Jensen NB, Strucko T, Kildegaard KR, David F, Maury J, Mortensen UH, Forster J, Nielsen J, Borodina I (2013) EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. FEMS Yeast Res 14(2):238–248
12. Kalscheuer R, Luftmann H, Steinbuchel A (2004) Synthesis of novel lipids in *Saccharomyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. Appl Environ Microbiol 70:7119–7125
13. Kalscheuer R, Stolting T, Steinbuchel A (2006) Microdiesel: *escherichia coli* engineered for fuel production. Microbiology 152:2529–2536
14. Keasling JD (2012) Synthetic biology and the development of tools for metabolic engineering. Metab Eng 14(3):189–195
15. Knudsen J, Faergeman NJ, Skott H, Hummel R, Borsting C, Rose TM, Andersen JS, Hojrup P, Roepstorff P, Kristiansen K (1994) Yeast acyl-CoA-binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size. Biochem J 302:479–485
16. Knudsen J, Jensen MV, Hansen JK, Faergeman NJ, Neergaard TBF, Gaigg B (1999) Role of acylCoA binding protein in acyl-CoA transport, metabolism and cell signaling. Mol Cell Biochem 192(1–2):95–103
17. Kocharin K, Chen Y, Siewers V, Nielsen J (2012) Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in *Saccharomyces cerevisiae*. AMB Express 2:52
18. Lee FWF, DaSilva NA (1997) Improved efficiency and stability of multiple cloned gene insertions at the delta sequences of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 48(3):339–345
19. Mikkelsen MD, Buron LD, Salomonsen B, Olsen CE, Hansen BG, Mortensen UH, Halkier BA (2012) Microbial production of indolylglucosinolate through engineering of a multi-gene pathway in a versatile yeast expression platform. Metab Eng 14(2):104–111
20. Okuyama H, Saito M, Joshi VC, Gunsberg S, Wakil SJ (1979) Regulation by temperature of the chain-lenght of fatty-acids in yeast. J Biol Chem 254(24):2281–2284
21. Petschnigg J, Wolinski H, Kolb D, Zellnig G, Kurat CF, Natter K, Kohlwein SD (2009) Good fat, essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. J Biol Chem 284(45):30981–30993
22. Rasmussen JT, Rosendal J, Knudsen J (1993) Interaction of acyl-CoA-binding protein (Acbp) on processes for which acyl-CoA is a substrate, product or inhibitor. Biochem J 292:907–913
23. Runguphan W, Keasling JD (2014) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. Metab Eng 21:103–113
24. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. In: Argentine J (ed), 3rd ed. Cold Spring Harbor Laboratory Press, New York, ISBN 0-87969-87579-87965
25. Shi S, Chen Y, Siewers V, Nielsen J (2014) Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio 5(3):e01130–e01140
26. Shi S, Valle-Rodriguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. Biotechnol Biofuels 5:7
27. Shi S, Valle-Rodriguez JO, Siewers V, Nielsen J (2014) Engineering of chromosomal wax ester synthase integrated *Saccharomyces cerevisiae* mutants for improved biosynthesis of fatty acid ethyl esters. Biotechnol Bioeng 111(9):1740–1747
28. Shiba Y, Paradise EM, Kirby J, Ro D-K, Keasling JD (2007) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. Metab Eng 9(2):160–168
29. Steen EJ, Kang YS, Bokinsky G, Hu ZH, Schirmer A, McClure A, del Cardayre SB, Keasling JD (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463(7280):559–562
30. Stoveken T, Kalscheuer R, Malkus U, Reichelt R, Steinbuchel A (2005) The wax ester synthase/acyl coenzyme A : diacylglycerol acyltransferase from *Acinetobacter sp* strain ADP1: characterization of a novel type of acyltransferase. J Bacteriol 187(4):1369–1376
31. Thompson RA, Trinh CT (2014) Enhancing fatty acid ethyl ester production in *Saccharomyces cerevisiae* through metabolic engineering and medium optimization. Biotechnol Bioeng 111(11):2200–2208
32. Tyo KEJ, Ajikumar PK, Stephanopoulos G (2009) Stabilized gene duplication enables long-term selection-free heterologous pathway expression. Nature Biotechnol 27(8):760–765
33. Valle-Rodriguez JO, Shi S, Siewers V, Nielsen J (2014) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways. Appl Energy 115:226–232
34. Wang XH, Wang ZJ, DaSilva NA (1996) G418 selection and stability of cloned genes integrated at chromosomal delta sequences of *Saccharomyces cerevisiae*. Biotechnol Bioeng 49(1):45–51
35. Woods A, Munday MR, Scott J, Yang XL, Carlson M, Carling D (1994) Yeast snf1 is functionally related to mammalian AMP-activated protein-kinase and regulates acetyl-CoA carboxylase in-vivo. J Biol Chem 269(30):19509–19515
36. Zhou YJJ, Gao W, Rong QX, Jin GJ, Chu HY, Liu WJ, Yang W, Zhu ZW, Li GH, Zhu GF, Huang LQ, Zhao ZBK (2012) Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc 134(6):3234–3241