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Overexpression of delta-12 desaturase in the yeast *Schwanniomyces occidentalis* enhances the production of linoleic acid

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ABSTRACT

The oleaginous yeast *Schwanniomyces occidentalis* was previously isolated because of its excellent suitability to convert lignocellulosic hydrolysates into triacyl glycerides: it is able to use a broad range of sugars and is able to tolerate high concentrations of lignocellulosic hydrolysate inhibitors. Compared to other oleaginous yeasts *S. occidentalis* however produces a low content of unsaturated fatty acids. We show here that the linoleic acid content can be significantly improved by (over)expression Δ12-desaturases derived from *S. occidentalis* and *Fusarium moniliforme*. Expression was stable for the homologous expression but decreased during heterologous expression. Both homologous and heterologous expression of mCherry-Δ12-desaturase led to a 4-fold increase in linoleic acid from 0.02 g/g biomass to 0.08 g/g biomass resulting in the production of 2.23 g/L and 2.05 g/L of linoleic acid.

1. Introduction

Polyunsaturated fatty acids, such as linoleic acid and linolenic acid, are widely used for the production of lubricants, resins, plastics and alkyd paints (Joseph et al., 2004; Köckritz and Martin, 2008; Nasrollahi et al., 2018; Orellana-Coca et al., 2005; van Gorkum and Bouwman, 2005). Furthermore, polyunsaturated fatty acids containing lipids have a good nutritional value, are utilized for health applications and have been associated with reduced atherosclerosis, inflammation and carcinogenesis (Bellou et al., 2016a; Calder, 2010; Saini and Keum, 2018). Due to the inability of humans and other higher animals to synthesize certain polyunsaturated fatty acids, such as linoleic acid, they depend on acquiring them via dietary uptake from common sources, such as soy oil and flaxseed oil (Saini and Keum, 2018).

Polyunsaturated fatty acids (PUFAs) are formed from stearic acid, which is converted by a Δ9-desaturase (Ole1) to oleic acid. Oleic acid can subsequently be converted to linoleic acid by a Δ12-desaturase (FAD2) and to α-linolenic acid by a Δ15-desaturase (FAD3), or to γ-linoleic acid by a Δ6-desaturase. Subsequent elongation and desaturation lead to the formation of longer chain PUFAs such as arachidonic acid and eicosapentaenoic acid (Hao et al., 2016; Leonard et al., 2004).

Oleaginous yeasts are capable of accumulating lipids, in the range of 20%–76% of their biomass, depending on culture conditions and species, and can often be genetically modified to further increase lipid productivity and yield (Liang and Jiang, 2013; Qiao et al., 2015; Ratledge, 2004; Zhang et al., 2016). Overexpression of an FAD2 from the oleaginous fungus *Mortierella alpina* in *Saccharomyces cerevisiae,* which is able produce the monounsaturated fatty acid oleic acid but is incapable of producing PUFAs, lead to an increase of linoleic acid content to 25% of total fatty acids produced (Huang et al., 1999). However, the overall quantity of linoleic acid accumulated was only 1.24 mg/L, rendering it irrelevant for the production of substantial quantities of linoleic acid. Utilization of oleaginous yeasts to produce linoleic acid could overcome the low fatty acid yield associated with using a non-oleaginous yeast such as *S. cerevisiae*. Previously, linoleic acid has been produced using genetically modified oleaginous microorganisms by overexpressing FAD2, leading to increased linoleic acid contents of up to 46.4% but with greatly varying titers, from 0.07 g/L to 1.3 g/L (Sakamoto et al., 2017; Wang et al., 2016; Zhang et al., 2017). Furthermore, the *F. moniliforme* FAD2 was overexpressed in *Yarrowia lipolytica* leading to an increased linoleic acid content from 39.6% up to 65.2% (Damude et al., 2006).

The oleaginous yeast *Schwanniomyces occidentalis* is able to produce fatty acids up to 42% of its biomass and utilize a broad range of carbon

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sources such as xylose, glycerol, lactose, inulin and cellobiose (Lamers et al., 2016). Furthermore, it has the ability to tolerate growth inhibitors that are associated with the hydrolysis of lignocellulosic material, such as furfural to concentrations up to 1.0 g/L, HMF to concentrations up to 2.0 g/L and acetic acid to concentrations 2.5 g/L (Sitepu et al., 2014). The ability to grow in the presence of these inhibitors, thereby removing the need for detoxification and/or metabolic engineering that is applied to other oleaginous yeasts, makes *S. occidentalis* a promising strain for the production of fatty acids (Chen et al., 2009; Jönsson and Martín, 2016; Tsigie et al., 2011). However, the level of linoleic acid produced is only ± 4.8% of total fatty acid content, whereas oleic acid reaches ± 69% of total fatty acid.

Here, we report the cloning and expression of FAD2 from *S. occidentalis* and *F. moniliforme* in *S. occidentalis* to increase linoleic acid production.

2. Materials and methods

2.1. Sequence analysis

The genome of *S. occidentalis* has been sequenced and annotated (data not shown) and, by performing a blast search using the functionally annotated FAD2 sequences of *Kluyveromyces lactis* (Q6CKY7) and *Lachancea kluyveri* (Q765N3), a putative FAD2 sequence was identified (MN065153). The FAD2 sequences of *Kluyveromyces lactis* (Q6CKY7), *Lachancea kluyveri* (Q765N3) and *Fusarium moniliforme* (Q27ZJ7) (De Angelis et al., 2016; Watanabe et al., 2004) were used to perform protein blast searches and to construct alignments with the putative *S. occidentalis* FAD2. Protein blast was performed using the NCBI protein blast suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), multiple sequence alignments were performed using the Clustal Omega server (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the prediction of the transmembrane domains was performed using the TMHMM server V2.0 (http://www.cbs.dtu.dk/services/TMHMM).

2.2. Culture conditions

S. occidentalis and transformants were cultured in YPD medium containing 10 g/L yeast extract (Gistex LS, DSM), 20 g/L peptone and 20 g/L glucose. When required, hygromycin was added to the culture to a final concentration of 100 mg/l.

For fermentation, *S. occidentalis* and transformants were precultured at 30 °C and 150 rpm in 500 ml C/N 5 medium containing 9.7 g/L NH_4Cl, 1.5 g/L yeast extract (Gistex LS, DSM containing 10% N), 3.2 g/L KH_2PO_4, 1.0 g/L $MgSO_4.7H_2O$, 33 g/L D-(+)-glucose monohydrate. Glucose was sterilized separately. Biotin was filter sterilized and added to a final concentration of 0.02 mg/l. The preculture was used to inoculate a 2-l fermenter (Bioflo/Celligen 115, New Brunswick) containing 1.25 l of C/N 90 batch medium at 5% (v/v), which contains 1.8 g/L yeast extract (Gistex LS, DSM containing 10% N), 3.2 g/L KH_2PO_4, 1.0 g/L $MgSO_4.7H_2O$, 33 g/L D-(+) glucose monohydrate. Upon depletion of the glucose in the batch phase, a feed was started at 8.5 g feed medium/hour. Feed medium contained 550 g/L glucose. The feed was stopped once the glucose concentration reached 90 g/L to prevent adverse effects. Total feed time was ± 40 h leading to a total amount of ± 150 g glucose added. Fermentations were performed in triplicate at a fixed agitation of 500 rpm. pH was maintained at pH 6.0 using 6 M NaOH and 6 M H_3PO_4. Samples were taken using an automatic sampler (Gilson art. No. F203B). The dry weight was determined gravimetrically by weighing known amounts of samples that were centrifuged and washed with sterile milli-Q followed by freeze drying until the weight was stable. Lipid free biomass was calculated by subtracting the lipid content from the dry weight. The glucose concentration was measured using the Horiba ABX Pentra Glucose HK CP reagent (art. no. A11A01667) on a Gallery Plus auto analyser (ThermoFisher Scientific art. no. 98620001).

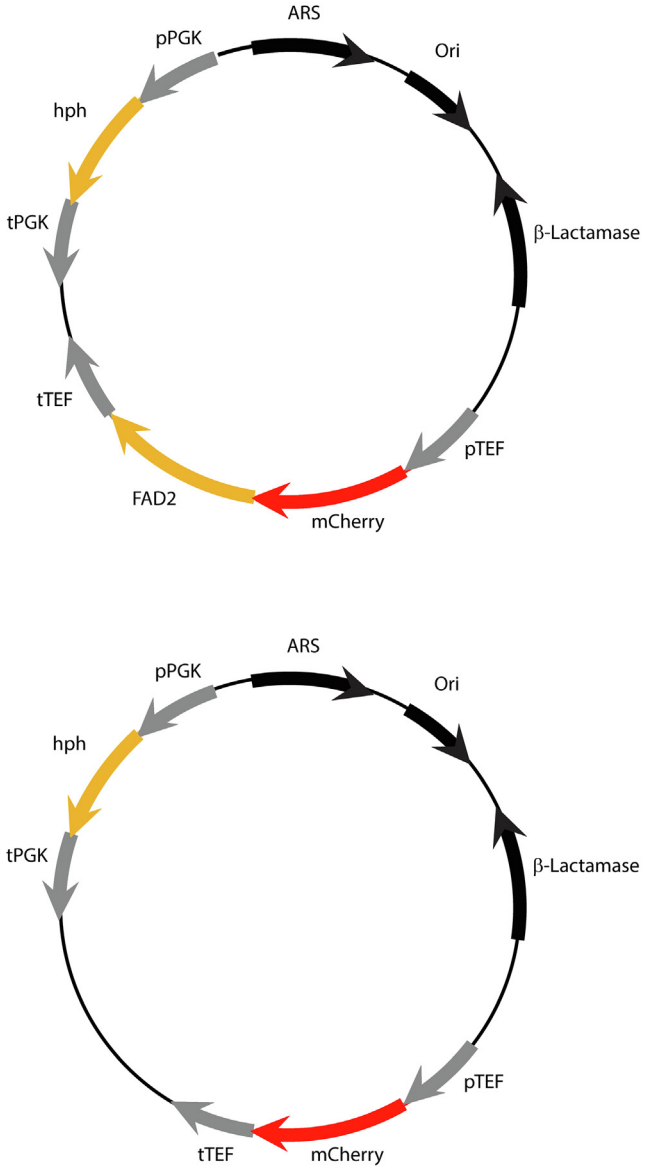

Fig. 1. Schematic overview of the expression plasmids used. ARS autonomous replication site from *S. occidentalis*, *pPGK* phosphoglycerate kinase promoter, *tPGK* phosphoglycerate kinase terminator, *hph* hygromycin-B phosphotransferase, *pTEF* translation elongation factor EF1-α promoter, *tTEF* translation elongation factor EF1- α terminator, mCherry red fluorescent protein, FAD2 Δ12-desaturase (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

2.3. Construction of plasmids

The expression plasmids used are based on the pBR322 plasmid and contain an ARS sequence which was amplified from the His4 region of *S. occidentalis* (Dohmen et al., 1989). The hygromycin resistance gene (*hph*), optimized for codon usage in CTG-clade strains, was synthesized by Invitrogen. The hygromycin gene was fused to the *S. occidentalis* PGK promoter and PGK terminator via Golden Gate cloning. The *F. moniliforme* FAD2 sequence was codon optimized for expression in CTG-clade strains and generated with mCherry fused in frame at the 5′-end using the *S. occidentalis Tef* promoter and terminator from Baseclear. Likewise, the homologous FAD2 and a plasmid containing only mCherry utilizing the *Tef* promoter and terminator was designed. All expression cassettes were synthesized by Baseclear and subcloned into

the ARS containing plasmid generating FM-mCherry-FAD2 and SO-mCherry-FAD2. See Fig. 1 for a schematic overview of the plasmids used.

2.4. Transformation of S. occidentalis

S. occidentalis was grown on YPD plates overnight at 30 °C. The cells were harvested by applying 5 ml of a 0.9% NaCl solution to the plate, and gently removing the cells using a spatula. The suspension was collected in a 15 ml tube followed by centrifugation at 1250 g at 4 °C for 5 min. The supernatant was discarded, and the pellet was resuspended in 5 ml of sterile demi water. Lithium acetate (final concentration 100 mM) and DTT (final concentration 10 mM) were added, followed by incubation at 20 °C in an overhead rotor for 1 h. The suspension was centrifuged at 1250 g at 4 °C for 5 min, followed by two washing steps using 5 ml of ice-cold sterile demi water and one washing step using 5 ml of ice cold 1 M sorbitol in demi water. The cell pellet was re-suspended in 500 µl of ice cold 1 M sorbitol in demi water and kept on ice. The cell suspension was homogenized and 1 µg of plasmid DNA was added to 100 µl of cell suspension followed by mixing and transfer to an 0.2 µm electroporation cuvette. The cuvette was immediately pulsed at 1500 V, 25 µF and 200 Ω for 5 ms using a Gene Pulser Xcell (Bio Rad).

The cells were removed from the electroporation cuvette and 1 ml of YPD containing 1 M sorbitol was added followed by incubation in a shaker at 30 °C at 150 rpm for 1 h. The cells were plated out on YPD plates containing 100 µg/ml hygromycin B and incubated for 48 h at 30 °C, or until growth was observed.

Colonies were grown in YPD containing 100 µg/ml hygromycin B and screened for mCherry expression using fluorescence microscopy. Several mCherry positive clones were selected and the presence of intact plasmid DNA in *S. occidentalis* was confirmed by isolating plasmid DNA and transforming it to *E. coli* (Singh and Weil, 2002). Colonies were grown and plasmid DNA was isolated followed by restriction digestion and gel analysis for confirmation.

2.5. Fatty acid analysis

The total fatty acid content and composition was measured according to the method previously described (Lamers et al., 2016). Briefly, 30 mg of freeze-dried cells were treated with 1 ml BF_3/methanol and 1 ml of heptane. After overnight incubation at 70 °C, 5 ml of demi water was added. Following centrifugation, 1 µl of the upper layer was analyzed on a Nexis GC-2030 (Shimadzu) using an SH-Famewax column (Shimadzu). The injection temperature was set at 250 °C, detector temperature was set at 275 °C, oven temperature was set at 195 °C and was increased to 240 °C at a rate of 5 °C/min and kept at 240 °C for 1 min.

2.6. RT-qPCR analysis

Total RNA was isolated from *S. occidentalis* strains using the Machery Nagel Nucleospin RNA isolation kit according to manufacturer's protocol. cDNA synthesis was performed using the iScript cDNA synthesis Kit (Bio-Rad) using 140 ng of RNA as input and amplification was performed using the iQ SYBR Green Supermix (Bio-Rad). The reaction consisted of 6.3 µl of iQ SYBR Green Supermix, 3.5 µl of milliQ, 0.13 µl of forward primer (25 µM), 0.13 µl of reverse primer (25 µM) and 2.5 µl of cDNA. Non-RT reactions were tested to confirm the absence of residual DNA. Primer sequences used for SO-FAD2 amplification were Fw-ACAACACACTGACCCAACGA and Rv-ATGTGCTG GCCAATAAAACC for FM-FAD2 amplification Fw-TCCCATCAACCCCA GCTAGA and Rv-AGCACCATGACCACATTCGT for GAPDH amplification Fw-TTCGGGCGTATTGGTCGTTT and Rv-GTAGGCGGCATAATCG GGTG. The expression of the GAPDH gene was used as the internal reference, relative expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Fluorescence microscopy

Neutral lipids were stained with Bodipy 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, ThermoFisher Scientific) by adding 1 µl of 0.1 mg/ml Bodipy 505/515 in DMSO to 100 µl of cell suspension. After 3 min incubation at room temperature 1 µl was transferred to a microscopic slide. Images were taken with a ZEISS Axio Imager M2 with a Colibri.2 light source using the Zen 2 Pro software. The percentage of positive fluorescent cells per strain was determined by counting single cells using the fluorescence microscope at a 200-fold magnification, scoring for fluorescence and dividing the amount by the total number of cells counted. An average of 330 cells was counted per timepoint.

3. Results and discussion

3.1. Identification of a putative FAD2 in S. occidentalis

We identified a putative Δ12-desaturase sequence in the genome of *S. occidentalis* based on homology with the functionally annotated Δ12-desaturases of *Kluyveromyces lactis* and *Lachancea kluyveri* (De Angelis et al., 2016; Watanabe et al., 2004). The length of the sequence was 1272 bp and encoded a protein of 423 amino acids that shared a 65.7% and 64.1% identity with the FAD2 of *Kluyveromyces lactis* and *Lachancea kluyveri* respectively. The *F. moniliforme* FAD2, which was previously expressed in *Rhodosporidium toruloides* to increase linoleic acid content, shared an identity of 49.4% with our putative sequence. In addition, there was 50.1% identity between *S. occidentalis* FAD2 and *K. lactis* FAD2 and 51.1% identity with *L. kluyveri* FAD2 (Wang et al., 2016). Δ12-fatty acid desaturases are structurally conserved, composed of several membrane spanning regions and three conserved histidine motifs that are essential for the desaturase activity (Avelange-Macherel et al., 1995; Meesapyodsuk et al., 2007; Shanklin et al., 1994). Consensus between the amino acid sequences, the presence of the transmembrane regions and the histidine boxes was observed between *S. occidentalis* and other FAD2 proteins, indicating similar activities (Fig. 2). Both the *S. occidentalis* FAD2 and *F. moniliforme* FAD2 were selected for overexpression in *S. occidentalis*. Fusion of mCherry to FAD2 allows for easy screening for positive transformants. Furthermore, it allows for direct visualization of FAD2 expression, following expression in time and observing expression differences within a cell population which cannot be observed using RT-qPCR. As a control, an mCherry overexpression strain was used (Fig. 1). After plasmid construction the plasmid DNA was transformed into *S. occidentalis* and transformants were grown on selective media. Transformants were screened for mCherry fluorescence using fluorescence microscopy. To confirm the presence of intact plasmid DNA a DNA isolation was performed on several mCherry positive clones, the isolated DNA was transformed into *Eschericha coli* and incubated under selective conditions. Subsequently, the plasmid DNA was isolated followed by a digestion to confirm the presence of the proper plasmid (data no shown). Based on this outcome a single positive colony was selected and cultivated for fermentation.

3.2. Fermentation profiles

Growth and lipid production of the two mCherry-FAD2 strains was compared to the mCherry control strain in a 1.25 l fed-batch fermentation for 156 h. Cell size increased during this period and Bodipy staining for neutral lipids showed an increase in lipid accumulation, concentrated to a single large lipid droplet in the cells throughout the duration of the fermentation. Overall growth patterns of all fermentations were similar; the homologous and heterologous expressing strains of mCherry-FAD2 generated comparable biomass and total fatty acids to the mCherry control strain. Biomass and fatty acid content were determined every 24 h and were still increasing, reaching maximum

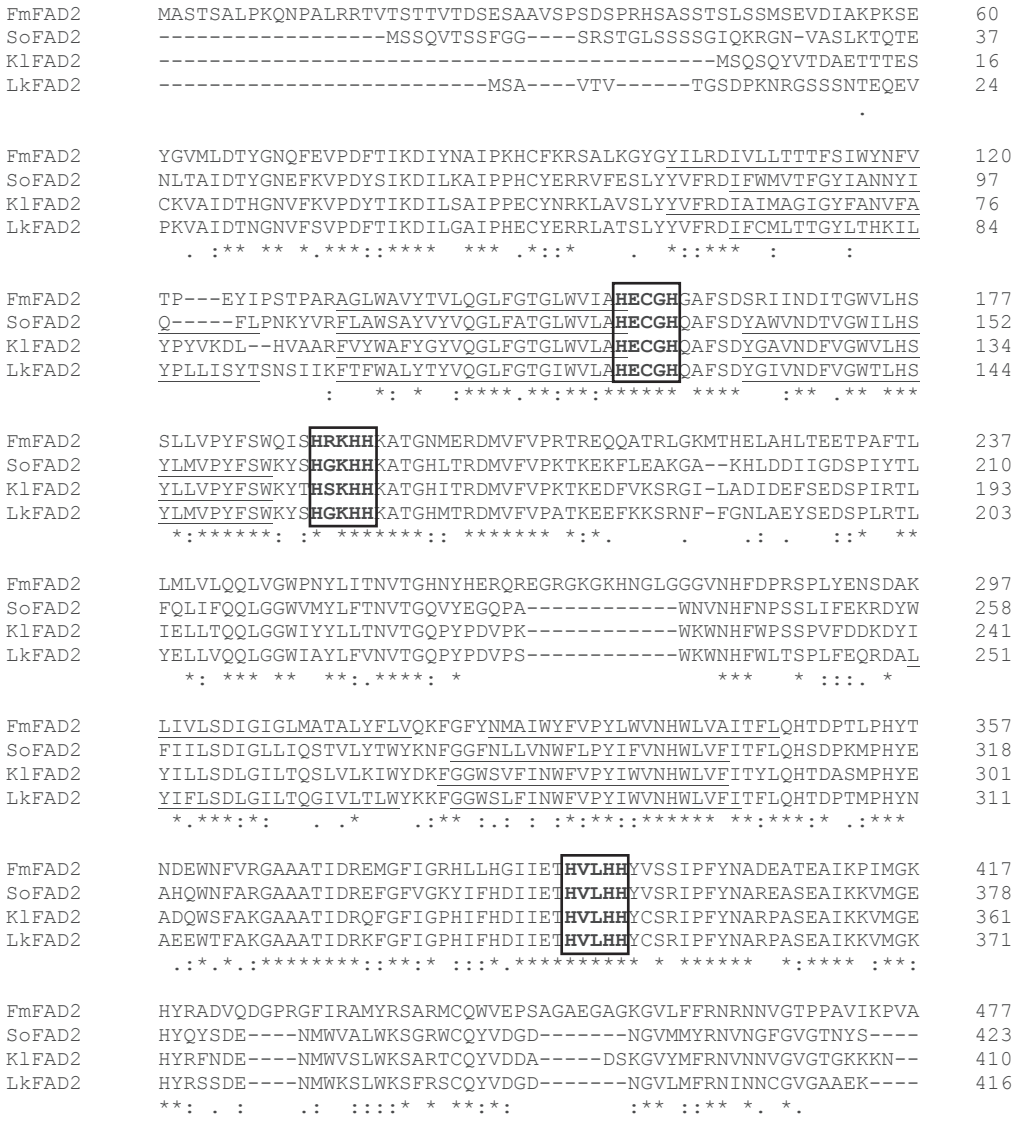

Fig. 2. Amino acid sequence alignments of FAD2 from *F. moniliforme* (FmFAD2), *S. occidentalis* (SoFAD2), *K. lactis* (KlFAD2) and *L. kluyveri* (LkFAD2). Transmembrane domains are underlined, histidine boxes are boxed in and depicted in bold. Conserved amino acids are noted by*, amino acids sharing strongly similar properties are noted by: and notes amino acids sharing weakly similar properties.

biomass and fatty acid content at the end of the fermentation, at comparable levels for all strains at ± 26 g/L and ± 9 g/L, respectively (Fig. 3). Lipid-free biomass increased up to 48 h before stabilizing until the end of the fermentation reaching 17.1 g/L for the SO-mCherry-FAD2, 17.5 g/L for the FM-mCherry-FAD2 and 16.3 g/L for the mCherry control strain. Similar biomass, fatty acid and lipid free biomass patterns were previously reported in the fermentation of wild type *Yarrowia lipolytica* (Qiao et al., 2015).

As a consequence, the lipid yield on glucose and lipid productivity were comparable for all strains with 0.07 g/g glucose and 0.06 g lipid/l/h, respectively (Table 1). These yields are comparable to wild-type *Y. lipolytica* and *Rhodosporidium toruloides* which reached yields of 0.07 ± 0.01 g lipid/g glucose and 0.09 ± 0.01 g/g, whereas productivities in these studies were slightly higher with 0.11 g/L/h and 0.13 g/L/h compared to our study (Friedlander et al., 2016; Zhang et al., 2016). After extensive genetic modification, the lipid yields were increased to 0.2 g/g in *Y. lipolytica* and 0.22 g/g in *R. toruloides*. Genetic modifications in other studies led to an increase in total fatty acid content from 12.8 g/L to 84.5 g/L by focusing on increased TAG formation by overexpression of key components of fatty acid synthesis, such as DGA1 and ACC1, increased NADPH regeneration by overexpression of ME and G6PD and preventing TAG mobilization by decreasing TGL3 and TGL4 lipase activity indicating that lipid yield in *S. occidentalis* can potentially be further increased by applying a similar methodology as the theoretical maximum yield that can be reached is 0.36 g/g (Bellou et al., 2016b; Chen et al., 2013; Friedlander et al., 2016; Ratledge, 2014; Silverman et al., 2016). Furthermore, productivity and yield of fatty acids can be further increased by optimizing medium and/or fermentation conditions.

3.3. Expression of FAD2

Relative expression levels of mCherry-FAD2 were analyzed using RT-qPCR during the fermentation after 24, 96 and 156 h (Fig. 4). FAD2 expression in the SO-mCherry-FAD2 strain, relative to FAD2 expression in the mCherry-control strain, was increased 2.6-fold and decreased to 0.4-fold whereas FM-mCherry-FAD2 expression was increased 10.5-fold and decreased until 5.3-fold. The 4-fold difference in relative expression

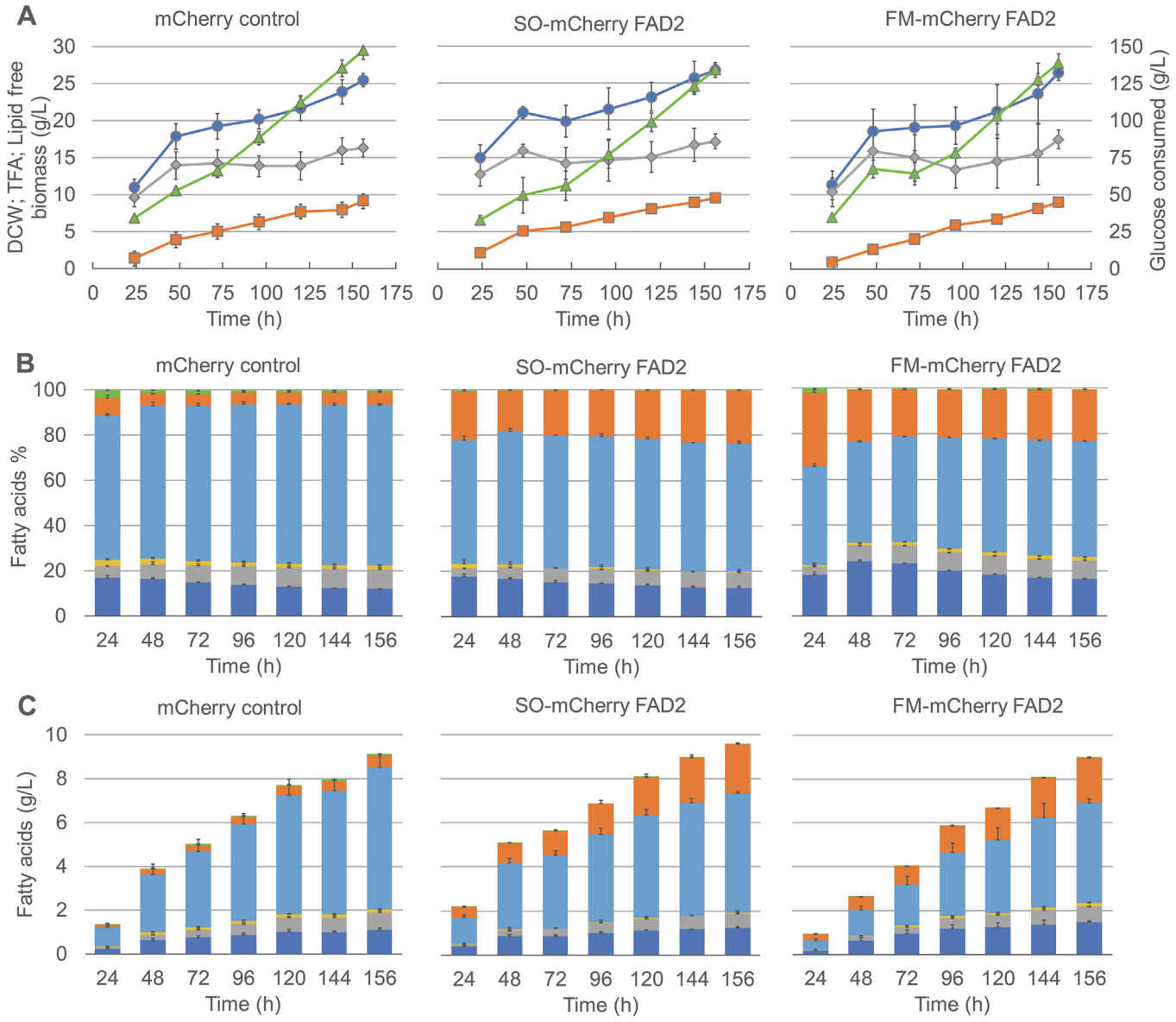

Fig. 3. Fermentation profiles (a) showing dry cell weight (blue circle), total fatty acids (orange square), lipid free biomass (grey diamond) and consumed glucose (green triangle) in g/L of strains cultivated for 156 h. Fatty acid composition (%) during fermentation (b) and fatty acid composition in g/L (c) during fermentation. C16 (dark blue), C16:1 (grey), C18 (yellow), C18:1 (light blue), C18:2 (orange) and C18:3 (green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

between SO-mCherry-FAD2 and FM-mCherry-FAD2 could potentially be caused by the regulation of unsaturated fatty acid synthesis by controlling the transcription and mRNA stability as has been observed for OLE1 (Gonzalez and Martin, 1996; Martin et al., 2007). The lack of regulation of mRNA stability for the heterologous expressed FM-mCherry-FAD2 might lead to the increased expression levels observed.

Fluorescence of the SO-mCherry-FAD2, FM-mCherry-FAD2 and mCherry control overexpressing strains was monitored during the fermentations using fluorescence microscopy. The number of fluorescent cells was determined per fermentation and the mCherry signal in the mCherry control and SO-mCherry-FAD2 fermentations was stable in all the cells throughout the fermentation with 99 ± 2% and 97 ± 1% after 156 h, indicating continued mCherry-FAD2 expression and/or stability under nitrogen depleted conditions. The intensity of the mCherry signal in the control expression strain was higher at the start of the fermentation compared to the FAD2 fusion proteins. Similar biomass content was reached in all strains, suggesting that the increased production of the fluorescence marker during the fermentation did not

Table 1
Cell growth, lipid content, lipid free biomass, yield and productivity of *S. occidentalis* strains cultivated for 156 h.

Strain	DCW (g/L)	Lipid content %	Lipid (g/L)	Lipid free biomass (g/L)	Lipid yield (g/g glucose)	Productivity (g lipid/L/h)
mCherry control	25.5 ± 0.9	35.9 ± 2.0	9.1 ± 0.8	16.3 ± 1.2	0.06 ± 0.01	0.06 ± 0.01
SO-mCherry-FAD2	26.7 ± 1.0	35.9 ± 1.4	9.6 ± 0.1	17.1 ± 1.0	0.07 ± 0.00	0.06 ± 0.00
FM-mCherry-FAD2	26.5 ± 1.1	34.1 ± 1.1	9.0 ± 0.7	17.5 ± 1.3	0.07 ± 0.01	0.06 ± 0.00

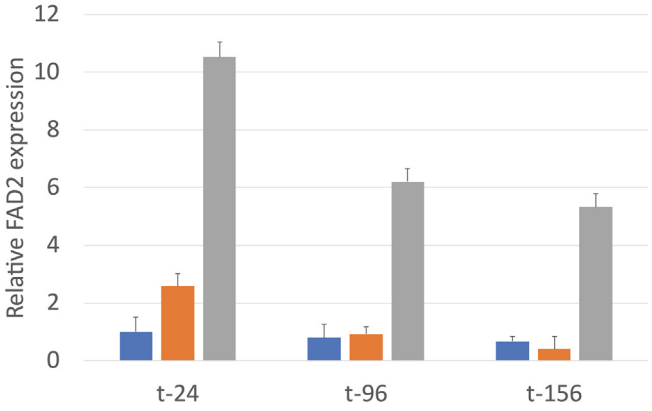

Fig. 4. Relative FAD2 expression levels at 24, 96 and 156 h during fermentation. mCherry control (blue), SO-mCherry-FAD2 (orange), FM-mCherry-FAD2 (gray) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

lead to toxicity. This is in correspondence with a study in which GFP was fused N- or C-terminally to the Δ6-desaturase of *Ribes nigrum* followed by overexpression in *S. cerevisiae* (Song et al., 2010). The fatty acid profile of the non GFP-fusion and the N- and C-terminally fused desaturase was compared, and it was observed that only the C-terminally fused GFP lead to a decrease in GLA production, whereas no difference in GLA production could be observed between the non GFP and the N-terminally fused Δ6-desaturase.

The fluorescence of FM-mCherry-FAD2 decreased from 100% to 66 ± 2% of the total cell population over time, indicating a loss of protein expression/stability or instability of the expression vector. The observation of this decrease combined with the initial 10.5-fold increased mCherry-FAD2 expression could indicate that metabolic burden plays a role in the loss of fluorescence. Other studies have demonstrated that metabolic burden associated with strong overexpression could lead to loss of plasmid(Gorgens et al., 2001; Nevoigt, 2008; Silva et al., 2012).

Other reports show that instability and partial degradation of fluorescent protein fusion products is dependent on the fluorescent protein used and the protein it is fused to (Snaith et al., 2010). Loss of expression plasmid has been reported to be plasmid specific and could occur at a rate of up to 10% per generation but this seems unlikely in this study, as both the mCherry-control and the homologous expressed *S. occidentalis* FAD2 show no signs of loss of plasmid (Hensing et al., 1995; Romanos et al., 1992). The observed loss of fluorescence in our study has no negative effect on biomass and lipid content as similar levels are obtained compared to the SO-mCHerry-FAD2 and the mCherry control but could influence fatty acid composition.

3.4. Fatty acid composition

The fatty acid composition of the two FAD2 overexpressing *S. occidentalis* strains was compared to the control strain expressing mCherry. Linoleic acid content increased 4-fold from 5.6 ± 0.2% in the mCherry-control to 23.3 ± 0.6% in the SO-mCherry-FAD2 expressing strain confirming that the putative *S. occidentalis* FAD2 sequence indeed codes for an Δ12-desaturase. (Fig. 3). It increased to 22.7 ± 0.1% in the FM-mCherry-FAD2 expressing strain. The 4-fold increase reached in this study is higher in comparison to other studies, however the total percentage of linoleic acid reached is lower. Homologous overexpression of a FAD2 in *Y. lipolytica* led to an increase in linoleic acid form 19.4 ± 1.2% to 38.7 ± 1.1% (Chuang et al., 2010). Identification and overexpression of a *Lipomyces starkeyi* FAD2 in *S. cerevisiae* led to a linoleic acid content of 9.1 ± 0.3% and, when expressed in *L. starkeyi,* to a 2.2-fold increase in linoleic acid content from 15.4 ± 2.6% to 34.6 ± 2.7% (Matsuzawa et al., 2018). Overexpression of *F. moniliforme* FAD2 in *Y. lipolytica* led to a 1.6-fold increase in linoleic acid content from 39.6 ± 4.0% to 65.2 ± 2.4% (Damude et al., 2006).

The observed decrease in oleic acid content, in favor of linoleic acid content, is comparable with studies in which overexpression of FAD2 leads to a decrease of up to 23% of oleic acid (Passorn et al., 1999; Sakamoto et al., 2017). Initial linoleic acid levels at the start of the fermentation were higher in the overexpressing strains, at ± 30% for the SO-mCherry-FAD2 and ± 32% for the FM-mCherry-FAD2 compared to just ± 9% linoleic acid in the mCherry control strain.

Expression of FM-mCherry-FAD2 also resulted in an increase of palmitic acid from 12% in the control strain to 16%. This increase in palmitic acid is not observed in other FAD2 overexpression studies albeit that the initial palmitic acid content in these strains was higher than in our control strain (Wang et al., 2016; Zhang et al., 2017).

The percentual increase in linoleic acid content does not have to reflect a significant increase in lipid titer. Therefore, the volumetric linoleic acid production allows for a better comparison of different linoleic acid producing strains. Unfortunately, in general the aforementioned studies only report on the linoleic acid content as a percentage of total fatty acids leaving no possibility to discuss the significance of these overexpression studies. Initial volumetric linoleic acid production was higher in the FAD2 overexpressing strains with 0.5 g/L and 0.3 g/L for the SO-mCherry-FAD2 and FM-mCherry, respectively, compared to 0.1 g/L in the mCherry control. Volumetric production of linoleic acid increased 4-fold during the lipogenic phase from 0.5 g/L in the control strain to 2.2 g/L in the SO-mCherry-FAD2 and 2.1 g/L in the FM-FAD2 expressing strains (Fig. 4). Overexpression of *F. moniliforme* FAD2 in *R. toruloides* by Wang et al (2016) showed a higher increase in linoleic acid of 7 fold, but produces just 1.3 g/L of linoleic acid, which is substantially lower compared to the production levels of *S. occidentalis* in this study (Wang et al., 2016). Likewise, a substantially lower linoleic acid content of 1.4 g/L was reached, compared to this study, when genetically modifying FAD2 expression aimed at producing linoleic and linolenic acid in *Mortierella alpina* (Sakamoto et al., 2017).

Although the fluorescent signal of the FM-mCherry-FAD2 expressing strain decreased by ± 35% during the fermentation, the total linoleic acid content did not differ substantially from the SO-mCherry-FAD2 strain that showed stable expression. Other studies have shown that overexpression of FM-FAD2 leads to a further increased linoleic acid content compared to homologous expression which is not observed in our study (Damude et al., 2006; Wang et al., 2016). A possible explanation for this is that the decreasing FM-FAD2 stability negates this increase. Alternatively, the loss of fluorescence could be attributed to degradation of the mCherry leaving the FM-FAD2 functionally active meaning that both the SO-FAD2 and FM-FAD2 are able to desaturate similar levels of oleic acid under these conditions.

For the commercial production of linoleic acid in *S. occidentalis* the yield and productivity need to be increased via further genetic modifications. Previous research has indicated that for the economically feasible production of most products from oleaginous yeasts improvements on genetic accessibility, fermentation conditions, oil extraction and utilization of by-products need to be performed to lower the costs (Ochsenreither et al., 2016; Shi and Zhao, 2017; Vasconcelos et al., 2019).

4. Conclusions

Based on sequence analysis a putative Δ12-desaturase gene was identified in the *S. occidentalis* genome. Overexpression of either homologous FAD2 or heterologous *F. moniliforme* FAD2 in *S. occidentalis* led to an increase in linoleic acid content from 5.6% to 23.3% and 22.7%, respectively, confirming the gene indeed encodes a Δ12-desaturase. In this study the second highest fold increase in linoleic acid has been achieved under non-optimized fermentation conditions. Although a higher linoleic acid percentage has been reached in other studies the

relevance of our study is not only dependent on the linoleic acid percentage and yield achieved but strain specific traits of *S. occidentalis* should also be considered. The ability to utilize biotechnologically relevant carbon sources combined with the ability to grow in the presence of inhibitors commonly found in lignocellulosic hydrolysates are of importance in the production of fatty acids on biorenewable resources. In this study linoleic acid production increased from 0.02 g/g biomass to 0.08 g/g biomass whereas volumetric production of increased from 0.51 g/L to 2.23 g/L and 2.05 g/L, respectively, after a 156-hour fermentation, and showed signs that this was still increasing. Heterologous FAD2 expression showed a decrease in mCherry positive cells indicating loss of expression or stability whereas homologous and control expression remained stable.

Researched aimed at increased yield and productivity needs to be performed to produce economically feasible quantities of linoleic acid.

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