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Metabolic engineering of *Saccharomyces cerevisiae* for overproduction of triacylglycerols

Raphael Ferreiraa,b, Paulo Gonçalves Teixeiraa,b, Michael Gossinga,b, Florian Davida,b, Verena Siewersa,b, Jens Nielsena,b,c

**A B S T R A C T**

Triacylglycerols (TAGs) are valuable versatile compounds that can be used as metabolites for nutrition and health, as well as feedstocks for biofuel production. Although *Saccharomyces cerevisiae* is the favored microbial cell factory for industrial production of biochemicals, it does not produce large amounts of lipids and TAGs comprise only ~1% of its cell dry weight. Here, we engineered *S. cerevisiae* to reorient its metabolism for overproduction of TAGs, by regulating lipid droplet associated-proteins involved in TAG synthesis and hydrolysis. We implemented a push-and-pull strategy by overexpressing genes encoding a deregulated acetyl-CoA carboxylase, ACC1S659A/S1157A (ACC1**), as well as the last two steps of TAG formation: phosphatidic phosphatase (PAH1) and diacylglycerol acyltransferase (DGA1), ultimately leading to 129 mg gCDW⁻¹ of TAGs. Disruption of TAG lipase genes TGL3, TGL4, TGL5 and sterol acyltransferase gene ARE1 increased the TAG content to 218 mg gCDW⁻¹. Further disruption of the beta-oxidation by deletion of POX1, as well as glycerol-3-phosphate utilization through deletion of GUT2, did not affect TAG levels. Finally, disruption of the peroxisomal fatty acyl-CoA transporter PXA1 led to accumulation of 254 mg gCDW⁻¹. The TAG levels achieved here are the highest titer reported in *S. cerevisiae*, reaching 27.4% of the maximum theoretical yield in minimal medium with 2% glucose. This work shows the potential of using an industrially established and robust yeast species for high level lipid production.

**1. Introduction**

In recent years, there has been an increasing demand for oils with specialized properties. Most oils found in nature are mainly composed of triacylglycerols (TAGs). Natural producers with specific TAG compositions, such as exotic plants, are usually poorly understood at the metabolic level, and lack available tools for introducing genetic modification, which ultimately makes them difficult to engineer. Consequently, there is an important need for alternative organisms that can accumulate high lipid amounts and be easily engineered for tailored oil production.

Although certain microorganisms are known to accumulate high levels of lipids, *Saccharomyces cerevisiae* remains the preferred cell factory for production of biochemicals (Nielsen and Keasling, 2016; Krivoruchko and Nielsen, 2015). It is a Generally Regarded As Safe (GRAS) organism with high tolerance to low pH and fermentation inhibitors. One of the major advantages of *S. cerevisiae* is the vast knowledge available about its physiology and metabolism, providing a solid background for engineering approaches (Jülleson et al., 2015). It is the most tractable organism, with several available tools for genome engineering, such as the CRISPR-Cas system, which has proven to be highly efficient (Dicarlo et al., 2013; Jakočiūnas et al., 2015; Zalatan et al., 2015).

TAGs are composed of three fatty acid chains esterified with a glycerol backbone. As such, the first key step towards increasing the TAG production potential in *S. cerevisiae* is the upregulation of the fatty acid biosynthesis. Fatty acids are synthesized as acyl-CoAs by the fatty acid synthetase complex, encoded by the genes *FAS1* and *FAS2* (Schweizer et al., 1984). Malonyl-CoA is used in this process as a substrate for iterative elongation of acyl chains, and is synthesized from acetyl-CoA by the enzyme acetyl-CoA carboxylase (Acc1). This enzyme is tightly regulated at the transcriptional and protein-level, rendering it a major regulation step in fatty acid biosynthesis (Hasslacher et al., 1993; Blank et al., 2017; Bozaqueñ-Morais et al., 2017). It is inhibited by ...
Snf1 phosphorylation, and the expression of a double mutant Acc1<sup>S659A/S1157A</sup> with two removed phosphorylation sites, here shortened Acc1<sup>++</sup>, has been shown to increase production of malonyl-CoA and fatty acid-derived products up to 3-fold compared to the wild-type (wt) strain (Shi et al., 2014).

The second point of focus is increasing the flux of acyl chains towards TAG formation. TAG assembly is initiated with glycerol-3-phosphate dehydrogenase (Gut2) improved the cylglycerol acyltransferase Dga1 or the phospholipid: diacylglycerol diacylglycerol (DAG), which is then used as a substrate by the dia-dephosphorylated by the phosphatidic acid phosphohydrolase Pah1 to phosphatidic acid (PA). PA is then derived products up to 3-fold compared to the wild-type (wt) strain (Shi et al., 2014).

2.1. Plasmid and strain construction

Strains, plasmids, links to plasmid maps, gRNA sequences and primers used in this study are listed in Table 1 and Additional File 1. Strain IMX581, and plasmids pROS10, pROS10, pME13 and pME10 were obtained from EUROSCARF (Frankfurt, Germany). Diagnostic primers and repair fragments were designed using the Yeaststriction webtool (http://yeastrstriction.tiw.tudelft.nl/). Gene deletions were performed and verified as described in (Mans et al., 2015). DNA fragments for DGA1 and PAH1 were amplified from genomic DNA of CEN.PK 113-7D (provided by P. Kötter, University of Frankfurt, Germany). Sequences for PGK1p and TEF1p were amplified from pSP-G1 (Partow et al., 2010). Integrative plasmids were based on pCfB389 (XI-2-locx-P-MntMX4) and pCfB2055 (X-2-locx-P-KanMX4), a derivative of pCBS353 (Jensen et al., 2014). The KanMX4 cassette from pCfB2055 was subcloned with XhoI–SalI into pCfB389 to generate pMG95 (XI-2-locx-P-KanMX4). The sequences for PGK1p, TEF1p, PAH1 and DGA1 were assembled into an integrative vector using pMG95 as backbone, generating pMG97. Sequences were assembled into integrative plasmids using QPEC (Quan and Tian, 2011). Construction of HXT7p-ACC1<sup>++</sup> has been described elsewhere (Bergenholm et al., submitted). For integration, generated integrative vectors were linearized with NotI and transformed directly using a PEG/LiOAc technique (Gietz et al., 2007). Correct integration was confirmed using primers located up- and downstream of the integration sites. For construction of HXT7p-ACC1<sup>++</sup> PAH1 + DGA1, the KanMX4 marker was consecutively removed by expressing the cre recombinase gene from pSH65 as described (Jensen et al., 2014).

2.2. Growth medium

<i>S. cerevisiae</i> strains with auxotrophs were grown on YPD plates containing 20 g L<sup>-1</sup> glucose, 10 g L<sup>-1</sup> yeast extract, 20 g L<sup>-1</sup> peptone from casein and 20 g L<sup>-1</sup> agar. URA3 plasmid carrying strains were grown on selective growth medium containing 6.9 g L<sup>-1</sup> yeast nitrogen base without amino acids (Formedium, Hungstanton, UK), 0.77 g L<sup>-1</sup> complete supplement mixture without uracil (Formedium), 20 g L<sup>-1</sup> glucose and 20 g L<sup>-1</sup> agar. KanMX4 plasmid carrying strains were grown on YPD plates supplemented with 200 mg L<sup>-1</sup> Genistic<sup>®</sup>. Shake flask cultivations were performed in minimal medium containing 20 g L<sup>-1</sup> glucose, 5 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 14.4 g L<sup>-1</sup> KH<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O adjusted to pH 6. After sterilization, 2 mL L<sup>-1</sup> trace element solution and 1 mL L<sup>-1</sup> of vitamin solution were added. The composition of the trace element and vitamin solution has been reported earlier (Verduyn et al., 1992).

2.3. Shake flask cultivations

Biological triplicates were pre-cultivated in 5 mL minimal medium at 200 rpm and 30 °C for 18 h. Subsequently, the pre-culture was diluted into 15 mL minimal medium in a 100 mL shake flask to an OD<sub>600</sub> of 0.1. Shake flasks were incubated at 200 rpm and 30 °C for 72 h. A spectrophotometer (Genesis 20, Thermo Fisher Scientific, Waltham, MA, USA) was used to measure cell density at the end of the shake flask cultivations.

2.4. Lipid quantification

Samples for lipid analysis were taken at the end of the shake flask cultivations. The samples were centrifuged at 3000g for 5 min and the supernatant was discarded. Cell pellets were then washed with 50 mL deionized water, centrifuged at 3000g for 5 min and the supernatant was discarded. The pellets were kept at −20 °C for 10 min and then freeze-dried using a Christ alpha 2–4 LSC (Christ Gefriertrocknungsanlagen, Osterode, Germany). Lipids were extracted using microwave extraction and analyzed by HPLC–CAD using 10 mg of dried cell biomass as previously described (Khoormung et al., 2013).

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Parental strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMX581</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td>CEN.PK113-5D</td>
<td>Mans et al. (2015)</td>
</tr>
<tr>
<td>ADP</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td>IMX581</td>
<td>This study</td>
</tr>
<tr>
<td>RF08</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td></td>
<td>ADP</td>
</tr>
<tr>
<td>RF09</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td></td>
<td>RF08</td>
</tr>
<tr>
<td>RF10</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td></td>
<td>RF09</td>
</tr>
<tr>
<td>RF11</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td></td>
<td>RF10</td>
</tr>
<tr>
<td>RF12</td>
<td>MATa ura3–52 can1::car9-natNT2</td>
<td></td>
<td>RF11</td>
</tr>
</tbody>
</table>

References:

Hung et al., 2016
Jensen et al., 2014
Kohlwein et al., 2013
Runguphan and Keasling, 2014
Shi et al., 2014
Verduyn et al., 1992
Waltham, MA, USA
Bergenholm et al., submitted
Jensen et al., 2014
Jensen et al., 2014
Jensen et al., 2014
Jensen et al., 2014
Hung et al., 2016
2.5. Lipid droplet staining

After 72 h of shake flask cultivation, 100 μL of cell culture were transferred into a 1.5 mL Eppendorf tube, centrifuged and the pellet washed with 1 mL of deionized water. Cells were then centrifuged at 3000g for 5 min and resuspended with 100 μL phosphate-buffered saline (PBS). Resuspended cells were treated with 1 μL of BODIPY 493/503 (Thermo Fisher Scientific, Waltham, MA, USA) solution (1 mg·mL⁻¹ in ethanol) and kept at 4°C in the dark for 10 min. Fluorescent microscope pictures were analyzed using a Leica AF 6000 inverted microscope using GFP filter settings (Leica Microsystems, Wetzlar, Germany) and processed with the Leica Application Suite (LAS) software.

3. Results

3.1. Push-and-pull strategy: Overexpression of ACC1**, PAH1 and DGA1 leads to high accumulation of TAGs

We first co-overexpressed genes responsible for the first and last steps of TAG biogenesis, termed push-and-pull strategy. In that regard, we genomically integrated in chromosome X one copy of the double mutant version of ACC1, ACC1<sup>DM9A:S157T</sup> (ACC1**), under control of the strong promoter HXT7p induced at low glucose concentrations (Jensen et al., 2014; Reifenberger et al., 1997), as the majority of fatty acids are produced during the ethanol phase (Teixeira et al., 2017, Bergenholm et al., submitted) (Fig. 1). We further engineered the strain by genomically integrating in chromosome XI one additional copy of PAH1 and DGA1 each, expressed under control of the strong constitutive promoters PGK1p and TEF1p, respectively, resulting in the ADP strain (Table 1) (Jensen et al., 2014). This combination of overexpressed genes led to 129 mg·gCDW⁻¹ of TAGs after 72 h shake flask cultures in 2% glucose minimal medium, a higher than 10-fold increase in comparison to the reference strain (Fig. 2). The accumulation of TAGs also led to an increase of the number of lipid droplets (Fig. S1). Notably, the integration of these genes did not alter the final OD of the resulting strain compared to the reference strain (Fig. S2).

3.2. Removal of the major TAG lipases and sterol acyltransferase increases TAG levels

In <i>S. cerevisiae</i>, three TAG lipases, namely Tgl3, Tgl4, and Tgl5, are responsible for most of the TAG lipase activity, and their deletion has been shown to lead to an increased level of TAGs (Athenstaedt and Daum, 2003).

![Fig. 1. Simplified schematic representation of the key metabolic fluxes targeted in this study. Highlighted in green are genes overexpressed, ACC1** (acetyl-CoA carboxylase), DGA1 (diacylglycerol acyltransferase) and PAH1 (phosphatidic acid phosphohydrolase). Highlighted in red are deleted genes, GUT2 (glycerol-3-phosphate dehydrogenase), ARE1 (acyl-CoA: sterol acyltransferase), TGL3/4/5 deletion), the glycerol-3-phosphate dehydrogenase subunit of peroxisomal ABC-transporter), POX1 (fatty acyl-CoA oxidase), and FAA2 (fatty acyl-CoA synthetase). Marked in black are unmodified genes that are also involved in lipid metabolism: FAS1/2 (fatty acid synthetase); GPT2 and SCT1 (GPAT genes); LOA1, SLC1 and ALE1 (GPAT genes); and ARE2 (acyl-CoA: sterol acyltransferase). For the metabolic compounds: G3P: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate, Gro-3-P: glycerol-3-phosphate; PA: phosphatidic acid; PAH1: phosphatidic acid phosphohydrolase; FFA: free fatty acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](Image 262x66 to 398x201)

![Fig. 2. TAG quantification of the different engineered strains. TAGs were quantified for strains with the implemented push-and-pull approach (ACC1**, DGA1 and PAH1 overexpression) and lacking TAG lipases (TGL3/4/5 deletion), sterol acyltransferases (ARE1 deletion), β-oxidation (POX1 deletion), glycerol utilization (GUT2 deletion), the acyl-CoA peroxisomal transporter (PXA1 deletion) and fatty acyl-CoA synthetase (FAA2 deletion). Strains were grown for 72 h in minimal medium containing 2% glucose and TAGs were quantified from biological quadruplicates. *p value < .05 (Student’s T-Test: two-tailed, two-sample equal variance).](Image 311x366 to 555x723)
The neutral lipids stored in lipid droplets are normally composed of 50% TAGs and 50% sterol esters (SEs) (Kohlwein et al., 2013). An SE results from the esterification of an acyl-CoA with a hydroxyl group of a sterol, catalyzed by acyl-CoA: sterol acyltransferases encoded by the S. cerevisiae genes ARE1 and ARE2 (Yang et al., 1996). We proposed to remove sterol ester formation through deletion of ARE1 and ARE2 in order to obtain a larger fraction of the neutral lipids in form of triacylglycerols. Removal of the major lipase genes TGL3, TGL4 and TGL5 and deletion of ARE1 resulted in the new strain RF08. Although ARE2 has been reported to be the major SE synthase (Sandager et al., 2002), compared to strain ADP, with a lowing strain construction. RF08 showed a 68% increase in TAGs levels we faced major technical di

by RF11 of 1.76 g

order to obtain a larger fraction of the neutral lipids in form of tria-

levels, often making it a di

4. Discussion

Lipid metabolism in eukaryotes is complex due to its crosstalk with essential cellular processes, such as synthesis of signaling molecules and cellular membranes. For this reason, it is tightly regulated at several levels, often making it a difficult target for metabolic engineering.

Here, we started by implementing a typical push-and-pull strategy towards TAG accumulation. For that, engineering of Acc1 at the transcriptional and phosphorylation level was a fundamental step due to the importance of this enzyme in controlling fatty acid metabolism. The initial approach of overexpressing genes encoding an Acc1 with two removed phosphorylation sites, together with Pah1 and Dga1 resulted in TAG accumulation levels up to 12% of dry cell biomass, more than 10-fold above the levels in the reference strain. A similar strategy of overexpressing ACC1 and DAG1 in Y. lipolytica reached to a lipid content of 41.4% (Tai and Stephanopoulos, 2013). Previous studies based on individual expression of these three genes have reported similar titer improvements, with more pronounced effect for Acc1 (Hofbauer et al., 2014, Hung et al., 2016, Runguphan and Keasing, 2014). Acetyl- and malonyl-CoA constitute the building blocks of fatty-acid biosynthesis, and these results show the importance of the Acc1 level and activity being a central metabolic knot of the lipid metabolism in S. cerevisiae.

Next we removed most lipase activity and part of the sterol ester synthesis activity and observed an TAG increase of up to 80%, reaching close to 22% of the dry cell weight. These results strongly indicate that in the ADP strain, lipid homeostasis mechanisms are counterbalancing TAG accumulation with TAG hydrolysis, therefore limiting further production. Supporting this, previous reports have shown that lipase activity at transcriptional and protein level is affected by neutral lipid levels (Klein et al., 2016). Based on similar experiments realized in S. cerevisiae, we speculate that the removal of the lipases has had the major role in increasing TAG levels (Sandager et al., 2002; Athenstaedt and Daum, 2003).

In contrast with previous results obtained in Y. lipolytica, the disruption of the glycerol consumption gene, GUT2, did not lead to a significant increase in TAGs (Dulermo and Nicaud, 2011). Here, we hypothesize that the flux towards TAG biosynthesis is not constrained by the availability of Gro-3-P. This diverges from studies made in Y. lipolytica, where TAGs can accumulate to 80% of the cells’ dry weight. This high lipid content in Y. lipolytica might indicate that fatty acid synthesis exceeds Gro-3-P availability in this organism, making Gro-3-P a limiting factor in TAG synthesis, which does not seem to be the case in S. cerevisiae. While not tackled in this study, recent studies have reported an improvement of TAG levels when overexpressing heterologous GPAT and LPAT genes, which suggests a potential additional engineering strategy reach higher TAG titters (Wei et al., 2017). Over-expression of the endogenous GPAT and LPAT genes could be considered as well (Fig. 1).

Additionally, neither the disruption of the first reaction of the β-oxydation, catalyzed by Pox1, nor removal of the import activation of FFAs into the peroxisomes catalyzed by Faa2, affected the TAG levels. In S. cerevisiae, in normal condition, a wild-type strain only produces very low levels of FFAs, as they are directly and quickly re-activated by fatty acyl-CoA synthetases to fatty acyl-CoAs (Zhou et al., 2016). Although previous studies have shown that POX1 disruption increased TAGs in S. cerevisiae and Y. lipolytica (Valle-Rodríguez et al., 2014, Dulermo and Nicaud, 2011), the removal of the major TAG lipases further decreased the supply of FFAs, which could explain the null eff-

fect of POX1 disruption. Additionally, the null effect from removing

3.3. Removal of the fatty acyl-CoA peroxisomal transporter further increases TAG levels

In S. cerevisiae, acyl-CoA is degraded back to acetyl-CoA by the peroxisomal β-oxidation pathway, and inactivation of this pathway leads to improved production of acyl-CoA derived chemicals (Valle-Rodríguez et al., 2014). Removal of the first step of the β-oxidation encoded by POX1, resulted in the strain RF09 which accumulated 204 mg gCDW⁻¹ of TAGs, a 9% decrease (p > .05) in comparison to RF08 (Fig. 2).

Next, we targeted the Gro-3-P utilization pathway by deleting the glycerol-3-phosphate dehydrogenase encoding gene GUT2. Gut2 catalyzes the conversion of Gro-3-P to dihydroxyacetone phosphate (DHAP) (Rønnow et al., 1993). Removal of GUT2 resulted in the strain RF10, which accumulates TAGs at a level of 222 mg gCDW⁻¹, similar to the level of RF08 (Fig. 2).

We deleted PAX1 encoding a subunit of the peroxisomal transporter involved in the import of acyl-CoA (Shani et al., 1995). The resulting strain RF11 reached a TAG level of 254 mg gCDW⁻¹, a 96% increase in comparison to the ADP strain (p value < .05), and a 14% increase in comparison to RF10 (p value < .05) (Fig. 2). The TAG titers produced by RF11 of 1.76 gL⁻¹ from 20 gL⁻¹ glucose translates to 27.4% of the maximum theoretical yield when considering the theoretical yield to be 0.32 gTAG gGlucose⁻¹ (Table 2) (Caspea and Nielsen, 2013). Notably, the titer achieved in RF11 is the highest reported in S. cerevisiae (Table S1). Finally, we deleted a gene encoding a peroxisomal fatty acid activating enzyme, FAA2, leading to strain RF12. The final strain accumulated a similar level of TAG (248 mg gCDW⁻¹) compared to RF11.

4. Discussion

Lipid metabolism in eukaryotes is complex due to its crosstalk with essential cellular processes, such as synthesis of signaling molecules and cellular membranes. For this reason, it is tightly regulated at several levels, often making it a difficult target for metabolic engineering.

<table>
<thead>
<tr>
<th>Strain</th>
<th>mgTAG/gDCW</th>
<th>Biomass (gDCW/L)</th>
<th>Titer (gTAG/L)</th>
<th>Yield (gTAG/gGlac)</th>
<th>%Theoretical Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMX581</td>
<td>7.45 ± 1.51</td>
<td>7.83 ± 0.07</td>
<td>0.05 ± 0.01</td>
<td>0.003 ± 0.001</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>ADP</td>
<td>129.30 ± 10.05</td>
<td>7.90 ± 0.26</td>
<td>1.02 ± 0.10</td>
<td>0.051 ± 0.005</td>
<td>16.0 ± 1.6</td>
</tr>
<tr>
<td>RF08</td>
<td>217.78 ± 12.43</td>
<td>7.95 ± 0.18</td>
<td>1.73 ± 0.10</td>
<td>0.087 ± 0.005</td>
<td>27.0 ± 1.6</td>
</tr>
<tr>
<td>RF09</td>
<td>201.23 ± 10.18</td>
<td>8.00 ± 0.22</td>
<td>1.61 ± 0.05</td>
<td>0.080 ± 0.003</td>
<td>25.1 ± 0.8</td>
</tr>
<tr>
<td>RF10</td>
<td>221.86 ± 20.77</td>
<td>6.74 ± 0.17</td>
<td>1.50 ± 0.17</td>
<td>0.075 ± 0.009</td>
<td>23.4 ± 2.7</td>
</tr>
<tr>
<td>RF11</td>
<td>254.02 ± 19.87</td>
<td>6.91 ± 0.04</td>
<td>1.76 ± 0.15</td>
<td>0.088 ± 0.007</td>
<td>27.4 ± 2.3</td>
</tr>
<tr>
<td>RF12</td>
<td>247.87 ± 10.13</td>
<td>6.73 ± 0.27</td>
<td>1.67 ± 0.12</td>
<td>0.083 ± 0.006</td>
<td>26.1 ± 1.8</td>
</tr>
</tbody>
</table>

* Assuming 0.32 g/g as theoretical maximum yield.
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