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Engineering the lipid and fatty acid metabolism in *Yarrowia lipolytica* for sustainable production of high oleic plant oils

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Abstract:

Oleic acid is widely applied in the chemical, material, nutritional, and pharmaceutical industries. However the current production of oleic acid via high oleic plant oils is limited by the long growth cycle and climatic constraints. Moreover, global demand for high oleic plant oils, especially the palm oil, has emerged as the driver of tropical deforestation causing tropical rainforest destruction, climate change and biodiversity loss. In the present study, an alternative and sustainable strategy for high oleic oil production was established by reprogramming the metabolism of the oleaginous yeast *Yarrowia lipolytica* using a two-layer 'push-pull-block' strategy. Specifically, the fatty acid synthesis pathway was first engineered to increase oleic acid proportion by altering the fatty acid profiles. Then, the content of storage oils containing oleic acid was boosted by engineering the synthesis and degradation pathways of triacylglycerides. The strain resulting from this two-layer engineering strategy produced the highest titer of high oleic microbial oil reaching 56 g/L with 84% oleic acid in fed-batch fermentation, representing a remarkable improvement of 110-fold oil titer and 2.24-fold oleic acid proportion compared with the starting strain. This alternative and sustainable method for high oleic oils production shows the potential of substitute planting.

Keywords: Oleic acid; High oleic oils; Monounsaturated fatty acids; Triacylglycerides; *Yarrowia lipolytica*; Push-pull-block strategy; Metabolic engineering

1. Introduction

Oleic acid (trans-9-octadecenoic acid; C18:1 Δ9), is the most well-known omega-9 monounsaturated fatty acid, with wide applications in the chemical, material, nutritional, and pharmaceutical industries. As the most abundant fatty acid in human adipose tissue,[1,2] oleic acid causes reductions in cholesterol levels, blood pressure and several types of cancer.[3-5] Furthermore, oleic acid was also found to be beneficial to wound healing.[6] In the chemical industry, oleic acid, as a potential industrial renewable feedstock, has been widely applied to many oleochemicals such as oleyl alcohol, and oleate esters.[7,8] Besides, oleic acid is often used as a bio-based lubricant, because it has higher oxidative stability than polyunsaturated fatty acids and a wider usable temperature range than saturated fatty acids.[9,10] New generation of renewable surfactants based on oleic acid have been synthesized and investigated due to the manufacturing, economics, and technical and environmental performances.[11-13] Moreover, oleic acid is also widely used in materials, since it can be converted into ω-hydroxy nonanoic acids (the monomer for synthesizing polyethylene-like bio-based plastic) or dicarboxylic acids (the precursor of co-polyamide hot-melting adhesive), which is achieved via enzymatic cascade reactions involving hydratases, alcohol dehydrogenases, Baeyer-Villiger monooxygenases, and esterases.[14-16] Additionally, a catalyst-free method for continuous flow synthesis of azelaic acid and pelargonic acid from oleic acid has been developed using an ozone and oxygen mixture as the reagent for oxidative cleavage of double bond.[17]

Currently, high oleic plant oils such as soybean, palm, sunflower and peanut oil, are the main raw materials for oleic acid production for food and industrial applications.[5,18] However, the global supply of plant oils is limited by the long growth cycle, pests and diseases, and climatic constraints. In addition, global demand for oleic plant oils, especially palm oil has emerged as the driver of tropical deforestation, although parts of global palm oil production have been certified by the Roundtable on Sustainable Palm Oil (RSPO). Certification significantly reduced deforestation in participating plantations, but did not reduce fires or peatland removal.[19] In a word,

burning for clearing vegetation, a large amount use of chemical fertilizers and pesticides, and tropical rainforest destruction cause air, soil and water pollution, soil erosion, climate change and biodiversity loss in the oil palm planting process. This means that the production of oleic acid from palm oil is not sustainable in a certain sense. A simple and more "sustainable" method for the production of oil rich in oleic acid is therefore urgently needed, and microbial oils from oleaginous microorganisms have attracted increasing attention because oleaginous microorganisms can be engineered to accumulate lipids enriched in tailored fatty acids and their derivatives.[20-22] Compared to plant oils, microbial oils have the advantages of short life cycle, less labor required, less affection by venue, season and climate, easier to scale up, and can efficiently convert agricultural waste and renewable resources. Besides, several oleaginous microorganisms were proved to accumulate lipids on various inexpensive agro-industrial residues,[23] thus lowering the production cost. In microorganisms, oleic acid is a common precursor of all long-chain (C18-C22) and very-long-chain (>C22) value-added unsaturated fatty acids. Therefore, many groups have engineered suitable oleaginous microorganisms rich in oleic acid into cell factories for the production of various high-value-added fatty acids and their derivatives, such as α-linoleic acid (C18:2 Δ9Δ12, reduces carcinogenesis, atherosclerosis, inflammation and diabetes),[5,24] ricinoleic acid (used in food, textile, paper, plastics, perfumes, cosmetics, electronics, pharmaceuticals, paints, inks, and adhesives),[25] γ-decalactone (an important peach-flavored food additive),[26] long-chain polyunsaturated fatty acids (LCPUFAs, like arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid, beneficial for the treatment of cardiovascular and cerebrovascular diseases),[27-31] long-chain monounsaturated fatty acids (LCMUFAs, erucic acid, important renewable feedstock for plastics, cosmetics, nylon, and lubricant industries, or nervonic acid for the treatment of neurological diseases).[32] In general, the construction of an oil-producing microbial chassis enriched in oleic acid is necessary not only for high oleic acid production, but also for the biotechnological production of its derivatives. Furthermore, the production of high oleic microbial oils for the alternative of high oleic plant oils is more green and sustainable.

Previous studies have focused on engineering *Rhodotorula glutinis* and *Mortierella alpina* for oleic acid production.[5,33] For *R. glutinis*, a two-stage fermentation strategy was developed to increase the total lipid content, resulting in 8 g/L lipid production, accounting for 80% of dry cell weight (DCW). When 1% hexane with corn cob hydrolysate and in-situ oil capture was supplemented, the proportion of oleic acid in total fatty acids was increased to about 85%.[33] In addition, Sakamoto et al.[5] suppressed the expression of the endogenous *Δ6I* gene by introducing the W314Stop mutation in a Δ12 desaturase activity-defective mutant of *M. alpina*, which resulted in an oleic acid titer of 2.76 g/L, representing 68% of total fatty acids. Recently, the non-conventional oleaginous yeast *Yarrowia lipolytica* (generally regarded as safe) has been considered as a promising host due to its high intrinsic lipid-production capacity, known genetic background and efficient synthetic biology toolboxes.[34-37] Its large intrinsic pool of metabolic intermediates can be used to drive the biosynthesis of oleochemicals or plant natural products derived from fatty acids, acetyl-CoA and malonyl-CoA.[38-44] Tsakraklides et al.[45] engineered *Y. lipolytica* by replacing the native Δ9 desaturase, deleting the Δ12 fatty acid desaturase (FAD2) and expressing exogenous fatty acid elongases. Furthermore, a glycerol-3 phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT), which respectively catalyze the binding of oleic acid to the n-1 and n-3 positions of triacylglycerides (TAGs), were introduced into *Y. lipolytica* to boost oleic acid accumulation. Finally, these genetic engineering strategies led to the accumulation of 31.2 g/L of microbial oil rich in oleic acid.

In this study, the sustainable high-oleic oils production by *Y. lipolytica* cell factories was developed to reduce the dependence on palm plantation agricultural economy, curb deforestation of tropical rain forests and reduce greenhouse gas emissions, which will promote the development of sustainable cellular agricultural economy. Here, we attempted to engineer *Y. lipolytica* into an oleic acid hyperproducer using a two-layer 'push-pull-block' strategy (Fig. 1). In this strategy, the first layer aims at modulating the fatty acid profile through the up-regulation of precursor supply in the C18:0 module

(push-strategy), increase of the C18:1 Δ9 branch flux (pull-strategy), and the downregulation of the competing C18:2 Δ9Δ12 pathway module (block-strategy). The second layer aims at boosting lipid biosynthesis via the upregulation of precursor supply in the glycerol-3-phosphate (G3P) and malonyl-CoA modules (push-strategy), increasing the flux through the TAG accumulation pathway (pull-strategy), and blocking the degradation of TAGs and fatty acyl-CoAs (block-strategy). The final engineered strain produced 56.14 g/L high oleic microbial oil in fed-batch fermentation, representing a remarkable improvement of 110-fold oil titer and 2.24-fold oleic acid proportion compared to the starting strain in shake flasks, which showed the potential to substitute high oleic plant oils.

2. Materials and methods

2.1 Strains and culture conditions

All the strains included in this study are shown in Table 1. *Y. lipolytica* strain Po1f (ATCC MYA-2613) was used as the starting strain for engineering. The Ku70 gene was disrupted using the URA3-blaster method to improve the homologous recombination (HR) efficiency, resulting in strain Po1f-ΔKu70.[46] *Escherichia coli* DH5α, which was used for plasmid construction and propagation, was grown in Luria-Bertani medium supplemented with 100 mg/L ampicillin at 37 ℃ and 200 rpm. All yeast strains were cultured in YPD medium (20 g/L glucose, 10 g/L yeast extract, and 20 g/L tryptone) in addition to the medium for oil production in the shake flask at 28 ℃, 250rpm. The medium used for oil production contained 60 g/L glucose, 1.32 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base (YNB) without amino acids and ammonium sulfate (BBI, Sangon Biotech (Shanghai) Co. Ltd., China). The synthetic complete medium without uracil (SC-Ura) plates contained 20 g/L glucose, 6.7 g/L yeast nitrogen base (YNB) without amino acids (BBI, Sangon Biotech (Shanghai) Co. Ltd., China), 0.77 g/L CSM-Ura (Complete supplement mixture minus uracil, MP Biomedicals, USA), and 20 g/L agar. The synthetic complete medium without leucine (SC-Leu) plates were the same as the SC-Ura plates, except for replacing CSM-Ura

with CSM-Leu (Complete Supplement Mixture minus leucine, MP Biomedicals, USA). The URA3 maker was removed and selected against on YPD+FOA plates, which contained 20 g/L glucose, 10 g/L yeast extract, and 20 g/L tryptone, 0.8 g/L 5-fluoroorotic acid, and 20 g/L agar. Single yeast colonies were picked from the YPD plate and cultured in YPD liquid medium overnight. The resulting seed culture was used to inoculate 50 mL of fermentation medium in a 250 mL shake flask to an optical density (OD_{600}) of 0.1 and grown for 120 h at 250 rpm and 30 ℃. The fermentation medium for lipid production contained 1.7 g/L YNB (without amino acids and ammonium sulfate), 1.32 g/L ammonium sulfate, and 60 g/L glucose. After centrifuging the medium, the supernatant was used to measure the glucose content using a SENSEP SS-201 biosensor (Jiangsu Industrial Technology Research Institute, Nanjing, China). The biomass was quantified by lyophilizing the cells for 24 hours, and then measured the dry weight using an analytical balance.

2.2 Transformation of *Y. lipolytica*

Genomic DNA from *Y. lipolytica* was obtained using an E.Z.N.A.® Yeast DNA Kit (Omega Bio-Tek Inc., USA). The transformation of *Y. lipolytica* was performed using the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Orange, CA). URA3 was used as the genetic marker for counter selection on YPD+FOA plates. Gene deletion was performed by homologous recombination based on direct repeats. The recombinant strains were streaked on YPD+FOA plates and incubated for 2-3 days. Strains expressing URA3 did not grow in the presence of 5-FOA.

2.3 Gene deletion and overexpression

Disruption cassettes targeting Fad2 (YALIOB10153g), Scd (YALI0C05951g), Pex10 (YALI0C01023g), Tgl4 (YALI0F10010g) and Gut2 (YALI0B13970g) were constructed according to the URA3-blaster protocol.[46,47] For the construction of the Fad2 deletion cassette, homologous sequences spanning about 1.5 kb 5'- and 3'- of the Fad2 gene, named Fad2-up and Fad2-down, were respectively amplified from genomic DNA of *Y. lipolytica* using the primer pairs Fad2-up-F/R and Fad2-down-F/R. The resulting PCR fragments were digested with *Eco*RI and *Pac*I, followed by one-step cloning into the pUC-HUH vector with the HisG-URA3-HisG

(HUH) blaster cassette using the ClonExpress^{TM} MultiS one-step cloning kit (Vazyme, Nanjing, China), according to the manufacturer's instructions, to generate the vector pUC-HUH-ΔFad2. The pUC-HUH-ΔScd, pUC-HUH-ΔPex10, pUC-HUH-ΔTgl4, and pUC-HUH-ΔGut2 plasmids were constructed in the same way. The plasmid pUC-Leu-A08 was introduced into *Y. lipolytica* to supplement the auxotrophy marker (Leu). All the strains were ultimately prototrophic. The plasmids and primers used in this study are listed in ESI Tables S1 and S2, respectively.

The coding sequences of Dga1 (YALI0E32769g), Acc1 (YALI0C11407g), and Elo1 (YALI0F06754g), the promoters P_{TEFin}, P_{FBA}, P_{GPD}, P_{YAT1}, and P_{hp4d}, as wellas the terminators T_{Cyc1}, T_{pex10}, T_{pex20}, and T_{lip2} were all amplified by PCR from genomic DNA of *Y. lipolytica* or plasmids deposited in our laboratory. The heterologous genes, including BiScd from *Bos indicus* (GenBank Accession No. AWH90218.1), ChScd from *Capra hircus* (GenBank Accession No. AAL29305.1), BtScd from *Bos taurus* (GenBank Accession No. AAF22305.1), PgScd from *Puccinia graminis* (NCBI Reference Sequence: XP_003326562.1), MaElo2 from *Mortierella alpina* (GenBank Accession No. AB468587.1), and RnElo2 from *Rattus norvegicus* (NCBI Reference Sequence: NP_599210.1)[45,48] were codon-optimized for expression in *Y. lipolytica* (ESI Table S3) and ordered as fully synthetic DNA (GeneScript, Nanjing, China). The pUC-HUH-Scd-PgScd plasmid was constructed from the pUC-HUH-Scd vector for Scd deletion and PgScd overexpression. Other genes, promoters and terminators were cloned into different linearized plasmids with different genomic loci. All the genomic loci were selected based on previous reports that they can be used to integrate heterologous genes without impacting cell growth.[49,50] All the newly constructed plasmids were confirmed by DNA sequencing.

2.4 Lipid extraction and fatty acid quantification

A sample comprising approximately 2 mg of cell mass was weighed and suspended 500 μL 1M potassium hydroxide methanol solution with 100 μL of tridecanoic acid (C13:0) and vortexed 25 ℃ for 2 h. Then 40 μL of 98% sulfuric acid was added to the mixture to neutralize it. The fatty acid methyl esters (FAME) were extracted by adding 400 μL hexane and vortexing for 10 min. After centrifuging the

mixture, 500 μL of the hexane layer was transferred into a glass vial for GC analysis.

Gas chromatography-flame ionization (GC-FID) was conducted using an Agilent 8860 instrument equipped with a DB-23 column (60 m×0.25 μm×0.15 μm), using nitrogen as the carrier gas. The GC oven conditions were as follows: 100 ℃ (0.5 min), a 25 min ramp to 196 ℃, a 20 min ramp to 220 ℃, hold at 220 ℃ for 2 min. The split ratio was 50:1. Fatty acids were identified and quantified by comparison with commercial FAME standards normalized to methyl tridecanoate (C13:0). The total lipid content was calculated as the sum of total fatty acids of five FAMEs (Sigma-Aldrich, USA), including methyl palmitate (C16:0), methyl palmitoleate (C16:1 Δ9), methyl stearate (C18:0), methyl oleate (C18:1 Δ9), methyl linoleate (C18:2 Δ9Δ12), and cis-11-eicosenoic acid methyl ester (C20:1 Δ11).[51]

2.5 Bioreactor fermentations

Fed-batch fermentation was performed in a 5-L stirred bioreactor (T&J-Btype, J Bio-engineering Co., Ltd, Shanghai, China) with a working volume of 3 L at 30 ℃ and 300 rpm, medium described by Tsakraklides et al.[45] The overnight YPD culture (5 mL in a Falcon tube, 250 rpm, 30 ℃) was used to inoculate 100 mL of YPD medium in a 500 mL shake flask to an optical density (OD_{600}) of 0.05 and grown for 24 h (250 rpm, 30 ℃). Exponentially growing cells from the resulting preculture were used to aseptically inoculate the bioreactor to an initial OD_{600} of 0.2 and cultured for 132 h (0.3 vvm, pH 5.5, 30 ℃, and 300 rpm). The agitation speed was controlled between 300 and 1000 rpm to maintain minimum dissolved oxygen at 20% of the atmospheric value from 0-48 h and 0-5% after 48 h. The 800 g/L glucose substrate was supplemented at about 40 h and 96 h post-inoculation, respectively. The pH of the culture was constantly controlled at 5.50 via the automated addition of 10 N sodium hydroxide. A 2 mL aliquot of each sample was washed twice with water and dried by lyophilization, after which the cell mass was measured to obtain the DCW concentration and the total lipids were extracted to measure the lipid content.

3. Results and discussion

3.1 Altering the fatty acid profile of *Y. lipolytica*

The main lipids of *Y. lipolytica* are neutral triglycerides synthesized from fatty acids and glycerol-3-phosphate.[52] Acetyl-CoA from the cytoplasm is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC1). Subsequently, acetyl-CoA is used as the starting compound and malonyl-CoA as the elongation unit to continuously elongate fatty acids by C2 units until C16:0 or C18:0 acyl-CoA is synthesized via a type I fatty acid synthase (FAS). In the endoplasmic reticulum, C16:0 and C18:0 will be extended by two fatty acid elongases (ELO1 and ELO2) into long-chain (C18-C22) and very-long-chain (>C22) acyl-CoAs, or desaturated by the Δ9 desaturase (SCD) into monounsaturated fatty acyl-CoAs (C16:1 Δ9 and C18:1 Δ9).[53] A Δ12 desaturase (FAD2) catalyzes the conversion of C18:1 Δ9 into C18:2 Δ9Δ12. Finally, the fatty acyl-CoAs are converted by GPAT, lysophosphatidic acid acyltransferase (LPAT) and DGAT into TAGs, which are stored in the form of lipid droplets in vivo (Fig. 1).

In *Y. lipolytica*, the 6 major fatty acids C16:0, C16:1 Δ7, C16:1 Δ9, C18:0, C18:1 Δ9 and C18:2 Δ9Δ12 account for more than 99% of the total fatty acids (Fig. 3a). We explored the push-pull-block strategy of fatty acid synthesis to change the fatty acid profile of *Y. lipolytica* by increasing the proportion of C18:1 Δ9 in total fatty acids. First, the native Δ12 fatty acid desaturase was blocked to eliminate C18:2 Δ9Δ12 accumulation (block-strategy). Then, Δ9 desaturase was engineered to pull the conversion of C18:0 into C18:1 Δ9 (pull-strategy). Finally, the supply of C18:0 was increased by overexpressing different fatty acid elongases (push-strategy) (Fig. 2a).

3.1.1 Blocking the flow of oleic acid into the branching pathway by deleting the gene rncoding Δ12 fatty acid desaturase

Alpha-linoleic acid (C18:2 Δ9Δ12) is the only native polyunsaturated fatty acid produced by the initial strain *Y. lipolytica* Po1f-ΔKu70. It is produced from C18:1 Δ9 under the action of Δ12 fatty acid desaturase encoded by Fad2. Deletion of Fad2 eliminated C18:2 Δ9Δ12 and increased the proportion of C18:1 Δ9 from 37.23 to 49.26% in total fatty acids (Fig. 3a). It is worth noting that the two types of C16 monounsaturated fatty acids (i.e., C16:1 Δ7 and C16:1 Δ9. C16:1) are produced from C16:0 by Δ9 fatty acid desaturase, but C16:1 Δ7 can also be the result of β-oxidation, a single step two-carbon degradation of C18:1 Δ9.[54,55] Thus, the increase of C18:1 Δ9

also slightly increased the proportion of C16:1 Δ7 due to the oxidation of the former (Fig. 3a). At the same time, measurement of the DCW of the ΔFad2 strain showed that deleting Fad2 had a small positive effect on biomass accumulation (Fig. 3b), while the total lipid content of the ΔFad2 strain was similar to that of the original strain (Fig. 3b). In general, knocking out Fad2 is beneficial to yeast production of C18:1 Δ9, in terms of increasing both biomass and the proportion of C18:1 Δ9.

3.1.2 Pulling the conversion of stearic into oleic acid by overexpressing different Δ9 desaturases

SCD is the only Δ9 desaturase in *Y. lipolytica* that can convert C16:0 into C16:1 Δ9 and C18:0 into C18:1 Δ9.[45] To reduce the proportion of C16:1Δ9 and increase the proportion of C18:1 Δ9, we explored Δ9 desaturases from different sources with preference for C18:0, including BiSCD from *B. indicus*, ChSCD from *C. hircus*, BtSCD from *B. Taurus* and PgSCD from *P. graminis*. Subsequently, in order to reduce the by-product C16:1 Δ9 produced by the native SCD, we integrated these Δ9 desaturases into the Scd locus, and compared their specificity for C18:1 Δ9 while inactivating the native Scd. Accordingly, BiScd, ChScd, BtScd and PgScd were individually integrated at the Scd locus under the control of the strong P_{TEFin} promoter in the ΔFad2 strain to construct strains Po1f-ΔFad2-Scd::BiScd, Po1f-ΔFad2-Scd::ChScd, Po1f-ΔFad2-Scd::BtScd and Po1f-ΔFad2-Scd::PgScd, respectively. The four strains accumulated 52.82, 47.35, 51.34 and 56.07% C18:1 Δ9 among total fatty acids, respectively (Fig. 2b). Strain Po1f-ΔFad2-Scd::PgScd produced the highest C18:1 Δ9 proportion, which was 14% higher than that of Po1f-ΔFad2 (Fig. 3a). As excepted, the proportion of C16:1 Δ9 of Po1f-ΔFad2-Scd::PgScd was reduced from 2.24 to 0.26% of total fatty acids (Fig. 3a). This indicated that PgSCD had the highest specificity for C18:0 among the tested Δ9 desaturases, as well as effectively convert C18:0 to C18:1 Δ9 leading to pull C16:0 to C18:0 and reduce by-product C16:1 Δ9 obviously.

However, the Po1f-ΔFad2-Scd::PgScd strain still contained 14.21% C16:0, 0.26% C16:1 Δ9 and even 26.86% C18:0 among total fatty acids (Fig. 3a). These data show that PgSCD has also weak specificity for C16:0, and the expression level of a

single copy of PgScd is not sufficiently high to convert all C18:0 into C18:1 Δ9. The gene copy number strongly influences the expression level, and an appropriate gene copy number facilitates the accumulation of target products. For this reason, we expressed another copy of PgScd (*Y. lipolytica* P_{YAT1} promoter) to construct the 2Pg strain. With two copies of PgScd, the engineered strain (2Pg) produced 63.95% C18:1 Δ9 among total fatty acids from glucose, a 14% increase compared to the control strain (Po1f-ΔFad2-Scd::PgScd) and a 72% increase compared to the initial strain (Po1f-Δku70) (Figs. 2b and 3a). Subsequently, an additional copy of PgScd (*Y. lipolytica* P_{YAT1} promoter) was integrated to form the 3Pg strain, but it only produced 61.64% C18:1 Δ9 among total fatty acids (Fig. 2b). Thus, with the increasing PgScd copy number, the vast majority C18:0 was converted into C18:1 Δ9. However, a small amount of C18:0 was still not converted. In addition, the intensity of gene expression of the third copy of PgScd was not as strong as possible. The optimal PgScd expression level may be explored using different weak promoters to express the third copy of PgScd. In addition, the by-product proportion of C16:1 Δ9 in strain 2Pg was still measurable, reaching up to 0.38% (Fig. 3a).

As reported, Scd knockout prevents *Y. lipolytica* from automatically synthesizing C18:1 Δ9 and growing in a medium without C18:1 Δ9.[45] In addition, Scd overexpression can increase the total lipid content of *Y. lipolytica*, because saturated fatty acyl-CoAs with 16-20 carbons are converted into mono-unsaturated fatty acyl-CoAs, which reduces the potent allosteric inhibition of ACC1.[56] Consequently, to assess the growth and fermentation performance of the engineered strains expressing Δ9 desaturase, the biomass and lipid content was quantified. As can be seen in Figure 3b, the Po1f-ΔFad2-Scd::PgScd strain showed similar biomass to Po1f-ΔKu70 and lower biomass than Po1f-ΔFad2. This indicates that PgScd can compensate the lost function of the native SCD and provide the strain with sufficient essential fatty acids for growth. However, the expression of PgScd could not completely restore the growth of the Scd deleting strain, so that strain 2Pg had the lowest biomass (Fig. 3b). As expected, strain 2Pg accumulated 12.02% total lipids in DCW and 0.49 g/L C18:1 Δ9 (Fig. 3b), which represented 1.35- and 2.58-fold respective increases over the

initial strain Po1f-ΔKu70.

3.1.3 Pushing the supply of stearic acid by overexpressing different fatty acid elongases

Y. lipolytica contains two fatty-acid elongases, named ELO1 and ELO2.[57] ELO1 has been proved to play an important role in the elongation of exogenous fatty acids or de novo conversion of C14:0 into C16:0 and C18:0. However, ELO2 also converts C18:0 into longer-chain fatty acids, up to C24:0. Strain 2Pg demonstrated a sufficient capacity to convert C18:0 into C18:1 Δ9 with its 2 copies of PgScd. However, strain 2Pg still had 12.70% of C16:0 remaining, which indicates that it is necessary to continuously promote the conversion of C16:0 into C18:0 to provide enough C18:0 as precursor for the synthesis of C18:1 Δ9 (Fig. 3a). Consequently, expression of fatty acid elongases that specifically extend C16:0 into C18:0 was attempted to increase the precursor supply for the synthesis of C18:1 Δ9 and reduce the precursor supply of C16:1 Δ9.

It has been reported that fatty acid elongases such as MaELO2 from *M. alpina*[31] and RnELO2 from *R. norvegicus*[45,48] can convert C16:0 into C18:0. Thus, the two fatty acid elongases were expressed under the control of strong promoter P_{FBA} in the 2Pg strain to form the strains 2PgMaE and 2PgRnE, respectively. As shown in Figure 3c, strain 2PgMaE accumulated more C18:1 Δ9 (74.46% of total fatty acids) than 2PgRnE (69.27% of total fatty acids). This indicates that MaELO2 either has a stronger ability to convert C16:0 into C18:0 or simply has better expression and activity in *Y. lipolytica*. At the same time, strain 2PgMaE reduced the proportion of C16:0 from 12.70 to 7.75%, as hoped (Fig. 3a). However, the 2PgMaE strain produced 1.64% C20:1 Δ11, which was a 2.4-fold increase compared to 2Pg (Fig. 3a). This indicates that MaELO2 has weak specificity for C18:1 Δ9 in the formation of C20:1 Δ11. Consequently, to further increase the supply of C18:0, the native elongase ELO1 (*Y. lipolytica* P_{GPD} promoter) responsible for converting C14:0 into C16:0 and C18:0 was introduced into the 2PgMaE strain to construct strain 2Pg2E.[57] The optimal strain accumulated up to 82.31% C18:1 Δ9 among total fatty acids, which was 2.21 times higher than in the initial strain Po1f-Δku70 (Figs. 2c and 3a).

Although an additional copy of ELO1 was also introduced into strain 2Pg2E to construct 2Pg3E, it had a similar C18:1 Δ9 proportion. In addition, we measured the lipid content of all elongase-overexpressing strains, and the strain 2Pg2E exhibited a maximal lipid content of 14.55%. This may be caused by the fact that more saturated fatty acids were converted into unsaturated fatty acids, especially C18:1 Δ9. It was also found that the introduction of heterologous MaELO2 can decrease cell growth, while the endogenous ELO1 can restore the strain growth (Fig. 3b). Thus, we successfully constructed strain 2Pg2E with the highest C18:1 Δ9 proportion and lipid content while preserving a similar biomass yield to the initial strain.

Based on these data, it was clear that we have successfully increased the proportion of C18:1 Δ9 by modifying elongases and desaturases. Moreover, this means that other valuable fatty acids can be synthesized by expressing additional heterologous enzymes, such as those related to the elongation and desaturation pathways. Additionally, increasing the proportion of monounsaturated fatty acids without accumulating PUFAs produces an oil with higher oxidative stability while still providing TAGs with a wider useful temperature range for industrial applications (ESI Fig. S1).

3.2 Engineering *Y. lipolytica* to boost the lipid content

To further increase the lipid content, the 'push-pull-block' strategy of lipid synthesis was explored to boost the lipid content by further increasing the C18:1 Δ9 titer. We pushed the precursor supply of glycerol-3-phosphate and malonyl-CoA by overexpressing Acc1 and Gpd1, as well as deleting Gut2 (push-strategy), strengthened the TAG accumulation pathway by overexpressing Dga1 (pull-strategy), and blocked the degradation of TAGs and fatty acyl-CoAs by disrupting Pex10 and Tgl4 (block-strategy) (Fig. 4a).

3.2.1 Engineering the push and pull of lipid biosynthesis by overexpressing Acc1 and Dga1

DGA1 catalyzes the last step of TAG synthesis in *Y. lipolytica*. However, if only DGA1 is overexpressed, it will lead to an imbalance between the fatty acid and TAG synthesis pathways, which will adversely affect cell growth.[51] By contrast,

simultaneous overexpression of ACC1 and DGA1 was previously shown to push and pull the fatty acid and TAG synthesis pathways without accumulation of any intermediates, so that the lipid content is increased to 41.4%.[51] Consequently, we overexpressed ACC1 in the 2Pg2E strain to provide more malonyl-CoA precursor and thereby push the fatty acid biosynthesis, while also overexpressing DGA1 to pull the fatty acid accumulation in the form of TAGs. The lipid content of the resulting strains 2Pg2E-Acc1 and 2Pg2E-AD was increased to 30.23 and 45.21% of DCW (Fig. 4b), with C18:1 Δ9 titers of 1.37 and 2.6 g/L (Fig. 4b), respectively. The increased the proportion of C18:1 Δ9 of 2Pg2E-AD compared to 2Pg2E-Acc1 may be due to the fatty acid preference of the Dga1 enzyme (Table 2), as previous studies showed that different DGATs are specific for different fatty acids.[45] Moreover, coexpression of the two genes had almost no effect on biomass (Fig. 4b).

3.2.2 Deleting key genes to prevent the degradation of TAGs and fatty acyl-CoAs

The degradation of TAGs and free fatty acids results in a lower lipid accumulation capacity. In *Y. lipolytica*, TAGs can be hydrolyzed by lipases into free fatty acids and glycerol. The resulting free fatty acids are transformed into short-chain fatty acyl-CoAs and even acetyl-CoA through the β-oxidation pathway in the peroxisomes.[58,59] The most critical lipase in *Y. lipolytica* is TGL4, an active lipase localized at the interface of the lipid bodies. In addition, TGL3 was shown to be a positive regulator of TGL4.[60] Previous studies have demonstrated that the single or double deletion of the two genes can block the degradation of TAGs and increase the lipid accumulation capacity.[44,55,61] Moreover, deletion of the peroxisomal Pex10 gene disrupts the β-oxidation pathway and blocks the degradation of fatty acyl-CoAs. Thus, the genes Pex10 and Tgl4 were sequentially deleted to construct the strains 2Pg2E-AD-Δ and 2Pg2E-AD-2Δ., which accumulated 47.75 and 49.16% lipids in DCW, respectively (Fig. 4b). Compared with the 2Pg2E-AD strain, the lipid accumulation capacity of these two strains was initially not significantly improved. However, we speculated that deletion of both Pex10 and Tgl4 genes prevents lipid degradation in the later stages of fermentation. As expected, when the fermentation time was extended, the total lipid content of 2Pg2E-AD-Δ and 2Pg2E-AD-2Δ did not

decrease after 6 days of fermentation, while that of other strains decreased significantly in the later stage of fermentation (Fig. 5). This may be because the cells ran out of nutrients in the medium and had to upregulate the lipid degradation pathway. Notably, the Pex10 deletion strain could not degrade fatty acyl-CoAs, resulting in a large accumulation of free fatty acids degraded by Tgl4, which severely impaired the cell growth of strain 2Pg2E-AD-Δ (Fig. 4b). When Tgl4 was also deleted in 2Pg2E-AD-2Δ, the degradation of TAGs was prevented preventing the excessive accumulation of free fatty acids, so that the cell growth was restored (Fig. 4b).

3.2.3 Precursor engineering to provide more G3P for TAG biosynthesis

When strains use glucose as substrate, G3P is formed from dihydroxyacetone phosphate (DHAP) by GPD1. Additionally, the reverse reaction is catalyzed by FAD^+-dependent G3P dehydrogenase (GUT2). Previous research proved that the deficiency of Gut2 and overexpression of Gpd1 could boost the lipid accumulation.[54,55,62] Considering that G3P is also an indispensable precursor for TAG biosynthesis in addition to malonyl-CoA, GPD1 was overexpressed and GUT2 was deleted to enrich the supply of G3P. The resulting strain 2Pg2E-AD-3Δ accumulated up to 61.72% lipids in DCW, which was 26% more than strain 2Pg2E-AD-2Δ (Fig. 4b). Importantly, the proportion of C18:1 Δ9 in total fatty acids has increased up to 84.64% (Table 2). The titer of C18:1 Δ9 thus reached the highest level in shake flasks, up to 5.03 g/L (Fig. 4b). In the final strain, the ratio of unsaturated to saturated fatty acid rose to 8.13 (Table 2), which indicated that the strain produces a microbial oil suitable for bio-based lubricants and biodiesel production.[63]

3.3 Fed-batch fermentation

To evaluate the growth performance, fatty acid profile and total lipid content of the final strain 2Pg2E-AD-3Δ, it was cultured in a 5-L bioreactor for 6 days with glucose as the sole carbon source. The initial fermentation medium contained 150 g/L glucose, and 800 g/L glucose stock was supplemented twice during the fermentation. During the course of the 132 h fermentation, the lipid content continually increased to 76.98% of the DCW (Fig. 6a). Compared with the shake flask level, the lipid content was increased 5 times in the fed-batch fermentation. This can be ascribed to the

blockage of the degradation pathways of lipids and fatty acids, which led to the continuous accumulation of total lipids. The highest biomass reached 72.9 g/L at about 120 h, which was 9.81 times higher than in shake-flask fermentation (Fig. 6a). The intermediate decrease in biomass in the middle phase of fermentation may be caused by the addition of glucose, but the continuous increase in biomass in the later period was attributed to the accumulation of lipids. The composition of fatty acid methyl esters was also measured and it was found that the proportion of C18:1 Δ9 kept rising, eventually reaching 83.52% of the total fatty acids at about 132 h, which was similar to that of the shake flask experiments (Fig. 6b). The final strain produced 56.14 g/L total lipids, including 46.23 g/L C18:1 Δ9 in the fed-batch process at about 120 h, and the C18:1 Δ9 titer was 243 times that of the initial strain in shake flasks (Fig. 7). Overall, the lipid production performance of the strain was greatly improved in the fed-batch process without compromising the C18:1 Δ9 accumulation ability.

The oil content of DCW, the proportion of oleic acid in total fatty acids and the oleic acid titer in major oil plants and microorganisms were summarized (Table 3). To the best of our knowledge, the proportion of oleic acid in the final strain is higher than that of most high oleic plants. Besides, the titer of TAGs rich in oleic acid is the highest among microorganisms reported so far. Furthermore, the oleic acid ratio of the final strain is similar to that of high oleic plant oils, but the oil content far exceeds that of high oleic plant oils. The sustainable production of high oleic microbial oils by *Y. lipolytica* cell factories for the alternative of high oleic plant oils drives the development of sustainable cellular agricultural economy.

4. Conclusions

In this work, the oleic acid accumulation capacity of *Y. lipolytica* was greatly improved using a two-layer push-pull-block strategy. The modification of the fatty acid profile in the first layer was mainly focused on increasing the proportion of oleic acid in total fatty acids, and the second layer is mainly based on increasing the total lipid content to increase the oleic acid storage capacity of the strain. Finally, the oleic acid titer of the best strain produced 56.14 g/L high oleic microbial oil with 83.52%

oleic acid, over the initial strain in shake flasks. To the best of our knowledge, this is the highest titer of high oleic microbial oil reported so far. The final microbial oil rich in oleic acid have potential applications in bio-based lubricants, hydraulic fluids, electrical transformer oils and surfactants based on oleic acid, as well as being a potential industrial renewable feedstock for the production of many oleochemicals such as ω-hydroxy nonanoic acid, dicarboxylic acids, oleyl alcohol, and oleate esters.[7,11-14] Thus, the oleic acid hyperproducing *Y. lipolytica* strain developed in this study was demonstrated to be an industrially relevant chassis for the production of valuable oleic acid derivatives.

We gradually analyzed the subtle changes in fatty acid composition after each step of metabolic engineering to guide the subsequent stage of genetic modification. At the same time, by screening different fatty acid desaturases and elongases with specific substrate preferences, and then optimizing their copy number, optimal metabolic networks were engineered to obtain the highest oleic acid proportion. In terms of improving the total lipid accumulation, up- and downstream pathways were balanced, and genes were overexpressed to push and pull the flux towards desired pathways. Finally, the deletion of the fatty acid degradation pathway was effective for accumulating lipids in the late fermentation stage. Overall, these strategies drive the oleic acid hyperproducing *Y. lipolytica* as a promising platform for the production of renewable microbial oil with high oleic acid proportion, which allows a drop-in substitute for high oleic plant oils applications. The sustainable high oleic oils produced by *Y. lipolytica* cell factories reduce dependence on high oleic plants plantations, especially oil palm, contributing to develop sustainable cellular agricultural economy, and achieve global carbon peaking and carbon neutrality goals.

Conflicts of interest

The authors declare that they have no competing interests.

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Tables

Table 1 Strains used in this study.

Strain	Genotype or properties	Source
E. coli DH5α	supE44 ΔlacU169 (φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Takara
Y. lipolytica Po1f (ATCC MYA-2613)	MatA, Leu2-270, Ura3-302, Xpr2-322, Axp1-2	ATCC
Y. lipolytica Po1f-ΔKu70	Po1f ΔKu70::HisG-Ura-HisG	46
Y. lipolytica Po1f-ΔFad2	Po1f-ΔKu70 ΔFad2::HisG-Ura-HisG	This study
Y. lipolytica Po1f-ΔFad2-Scd::PgScd	Po1f-ΔFad2 ΔScd::HisG-Ura-HisG, P_{TEFin}-PgScd-cyc1t	This study
Y. lipolytica Po1f-ΔFad2-Scd::BiScd	Po1f-ΔFad2 ΔScd::HisG-Ura-HisG, P_{TEFin}-BiScd-cyc1t	This study
Y. lipolytica Po1f-ΔFad2-Scd::ChScd	Po1f-ΔFad2 ΔScd::HisG-Ura-HisG, P_{TEFin}-ChScd-cyc1t	This study
Y. lipolytica Po1f-ΔFad2-Scd::BtScd	Po1f-ΔFad2 ΔScd::HisG-Ura-HisG, P_{TEFin}-BtScd-cyc1t	This study
Y. lipolytica 2Pg	Po1f-ΔFad2-Scd::PgScd ΔLip1::HisG-Ura-HisG, P_{YAT1}-PgScd-cyc1t	This study
Y. lipolytica 3Pg	2Pg ΔIntE::HisG-Ura-HisG, P_{YAT1}-PgScd-cyc1t	This study
Y. lipolytica 2PgMaE	2Pg ΔScp2::HisG-Ura-HisG, P_{FBA}-MaElo2-pex10t	This study
Y. lipolytica 2PgRnE	2Pg ΔScp2::HisG-Ura-HisG, P_{FBA}-RnElo2-pex10t	This study
Y. lipolytica 2Pg2E	2Pg ΔScp2::HisG-Ura-HisG, P_{FBA}-MaElo2-pex10t, P_{GPD}-Elo1-pex20t	This study

Y. lipolytica 2Pg3E	2Pg2E ΔIntE::HisG-Ura-HisG, P_{GPD}-Elo1-pex20t	This study
Y. lipolytica 2Pg2E-Acc1	2Pg2E ΔIntC::HisG-Ura-HisG, P_{hp4d}-Acc1-mig1t,	This study
Y. lipolytica 2PgE-AD	2Pg2E ΔIntC::HisG-Ura-HisG, P_{hp4d}-Acc1-mig1t, P_{TEFin}-Dga1-lip2t	This study
Y. lipolytica 2Pg2E-AD-Δ	2Pg2E-AD ΔPex10::HisG-Ura-HisG	This study
Y. lipolytica 2Pg2E-AD-2Δ	2Pg2E-AD-Δ ΔTgl4::HisG-Ura-HisG	This study
Y. lipolytica 2Pg2E-AD-3Δ	2Pg2E-AD-2Δ ΔGut2::HisG-Ura-HisG, P_{TEFin}-Gpd1-xpr2t	This study

Table 2 Fatty acid composition of strains 2Pg2E-Acc1, 2Pg2E-AD, 2Pg2E-AD-Δ, 2Pg2E-AD-2Δ and 2Pg2E-AD-3Δ.

Strain	Fatty acid composition (%)									unsaturated /saturated fatty acid
	C16:0	C16:1 Δ7	C16:1 Δ9	C18:0	C18:1 Δ9	C18:2 Δ9Δ12	C20:0	C20:1 Δ11	Others	
2Pg2E-Acc1	7.13	0.28	0.29	9.09	80.22	0.00	0.50	2.19	0.30	4.96
2Pg2E-AD	5.07	0.32	0.35	8.37	82.37	0.00	0.60	2.62	0.29	6.10
2Pg2E-AD-Δ	6.03	0.00	0.67	8.15	80.92	0.00	0.86	2.50	0.88	5.65
2Pg2E-AD-2Δ	4.55	0.00	0.27	7.61	83.11	0.00	0.94	2.92	0.60	6.59
2Pg2E-AD-3Δ	4.18	0.00	1.09	6.20	84.64	0.00	0.54	3.14	0.21	8.13

Table 3 The oil content, the proportion of oleic acid in total fatty acids and the oleic acid titer in major oil plants and microorganisms.

Oil plant and microorganism	Initial oleic acid proportion (%)	Final oleic acid proportion (%)	Improvement (%)	Oil content (%)	High oleic oil (g/L)	References
Trachycarpus fortunei	39.0-45.0	-	-	41.0	-	19, 64
High oleic *Helianthus annuus*	87.0	-	-	50.0	-	64
Regular *Helianthus annuus*	17.0	-	-	50.0	-	
Glycine max	26.0	-	-	20.0	-	
Arachis hypogaea	52.0	-	-	45.0-52.0	-	
Rhodotorula glutinis	33.0	85.0	257.6	80.0	8.0	33
Mortierella alpine	37.3	71.6	192.0	49.2	4.1	5
Trichosporon capitatum	69.5	79.8	114.8	43.1	6.6	23
Yarrowia lipolytica	58.0	90.0	155.2	45.1	34.7	45
Yarrowia lipolytica	37.2	83.5	224.5	77.0	56.1	This study

Figures:

Fig. 1 The pathways for lipid and fatty acid biosynthesis in the engineered oleic acid hyperaccumulating *Y. lipolytica.* Dashed lines with arrows indicate multiple steps. The two-layer 'push-pull-block' strategy, including lipid and fatty acid metabolism, is shown outside and inside the dashed box. The pathways of lipid synthesis, accumulation, and degradation are shown outside the dashed box (layer I). The native fatty acid elongation and desaturation pathways are shown in the dashed box (layer II). Push, pull and block strategies are represented by green, blue and red arrows, respectively. Abbreviations: ACC1, acetyl-CoA carboxylase; Ac-CoA, acetyl-CoA; ACL1, ATP-citrate lyase 1; ACL2, ATP-citrate lyase 2; CIT, citrate; DAG, diacylglycerol; DGA1, acyl-CoA diacylglycerol acyltransferase 1; DGA2, acyl-CoA diacylglycerol acyltransferase 2; DHAP, dihydroxyacetone phosphate; ELO1, fatty acid elongase 1; ER, endoplasmic reticulum; FAA1, long chain fatty acyl-CoA synthetase; FAD2, Δ12 desaturase; FAS, fatty acid synthase; FBP, fructose 1,6-bisphosphate; FFA, free fatty acid; G3P, glycerol-3-phosphate; GAP, glyceraldehyde 3-phosphate dehydrogenase; GPD1, NAD+-dependent G3P dehydrogenase; GUT2, FAD+-dependent G3P dehydrogenase; ICT, isocitrate; LB, lipid body; LPA, lysophosphatidic acid; LRO1, phospholipid diacylglycerol acyltransferase; MaELO2, fatty acid elongase 2 from M. alpina; Mal-CoA, malonyl-CoA; MFE1, peroxisomal multifunctional enzyme type 1; MIT, mitochondria; OAA, oxaloacetate; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PER, peroxisome; PEX10, peroxisomal biogenesis factor 10; PgSCD, stearoyl-CoA desaturase or Δ9 desaturase from P. graminis; PL, phospholipid; POT1, 3-ketoacyl-CoA thiolase; POX1-6, fatty-acyl-CoA oxidase 1-6; Pyr, pyruvate; SCD, stearoyl-CoA desaturase or Δ9 desaturase; SCT1, glycerol-3-phosphate acyltransferase; SLC1, 1-acyl-sn-G3P acyltransferase; TAG, triacylglyceride; TCA, tricarboxylic acid;

TGL3, triacylglycerol lipase 3; TGL4, triacylglycerol lipase 4; VLFA, very-long chain fatty acid; α-KG, α-ketoglutarate.

Fig. 2 A simplified pathway schematic illustrating the overexpression or deletion of different genes in the fatty acid biosynthesis pathway to improve the C18:1 Δ9 proportion among total fatty acids using the 'push-pull-block' strategy (a). Heterologous expression of Δ9 fatty acid desaturases and elongases in *Y. lipolytica*. The optimal copy number of the optimal gene was explored. All transformants were grown in nitrogen-limited media for 120 h and the proportion of C18:1 Δ9 in total fatty acids was quantified (b and c). Values shown are the averages of triplicate cultures and error bars represent standard deviations. Abbreviations: ACC1, acetyl-CoA carboxylase; ELO1, fatty acid elongase 1; FAD2, Δ12 desaturase; FAS, fatty acid synthase; MaELO2, fatty acid elongase 2 from *M. alpina*; PgSCD, stearoyl-CoA desaturase or Δ9 desaturase from *P. graminis*; SCD, stearoyl-CoA desaturase or Δ9 desaturase.

Fig. 3 Engineering *Y. lipolytica* to alter its fatty acid profile. All transformants were grown in nitrogen-limited media for 120 h and subjected to methanolysis prior to gas chromatography to analyze the lipid profiles (a). The C18:1 Δ9 titer and lipid content are shown (b). To evaluate the effect of genetic modification on the growth, all strains were cultured in YPD medium for 24 hours with the same inoculum amount, and the biomass was recorded as the OD_{600} (b). The values shown are the averages of triplicate cultures and error bars represent standard deviations.

Fig. 4 Engineering *Y. lipolytica* to boost the lipid content. A simplified pathway schematic illustrating the deletion or overexpression of different genes in the lipid biosynthesis pathway to improve the total lipid content via the 'push-pull-block' strategy (a). All transformants were grown in nitrogen-limited media for 120 h and analyzed by gas chromatography to obtain the C18:1 Δ9 titer (b) and lipid content (b). To evaluate the effect of genetic modifications on the growth, all strains were cultured

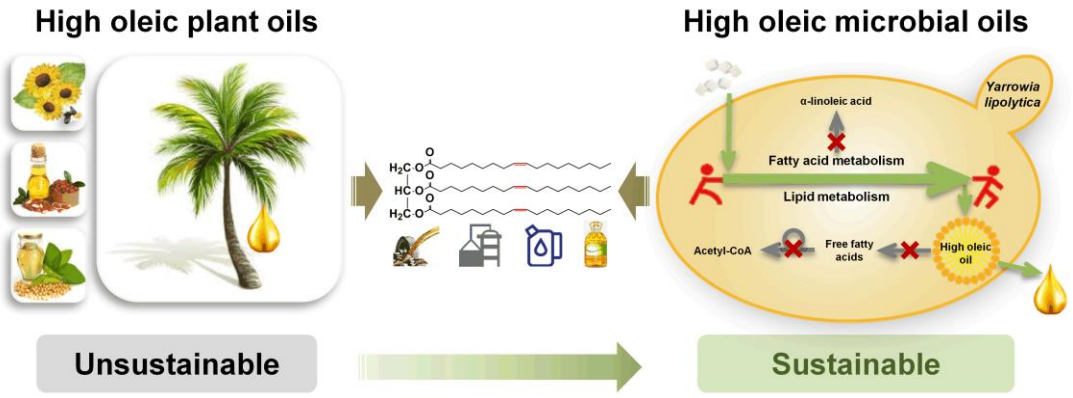
in YPD medium for 24 hours with the same inoculum amount, and the biomass was reported as the OD_{600}. Values shown are the average of triplicate cultures and error bars represent the standard deviations. Abbreviations: ACC1, acetyl-CoA carboxylase; DGA1, Acyl-CoA diacylglycerol acyltransferase 1; DHAP, dihydroxyacetone phosphate; FAS, fatty acid synthase; FAs, fatty acids; G3P, glycerol-3-phosphate; GPD1, NAD+-dependent G3P dehydrogenase; GUT2, FAD+-dependent G3P dehydrogenase; PEX10, peroxisomal biogenesis factor 10; TAG, triacylglyceride; TGL4, triacylglycerol lipase 4; β-O, β-oxidation.

Fig. 5 Time courses of lipid production in strains 2Pg2E, 2Pg2E-Acc1, 2Pg2E-AD, 2Pg2E-AD-Δ, 2Pg2E-AD-2Δ and 2Pg2E-AD-3Δ.

Fig. 6 Evaluation of the final strain in a 5-L bioreactor and the composition of the resulting microbial oil. The glucose consumption, DCW and lipid content (measured as fatty acid methyl esters) were measured twice daily (a). All samples were analyzed with all fatty acid fractions characterized by gas chromatography. The fraction of the final time point with the highest C18:1 Δ9 proportion is shown (b).

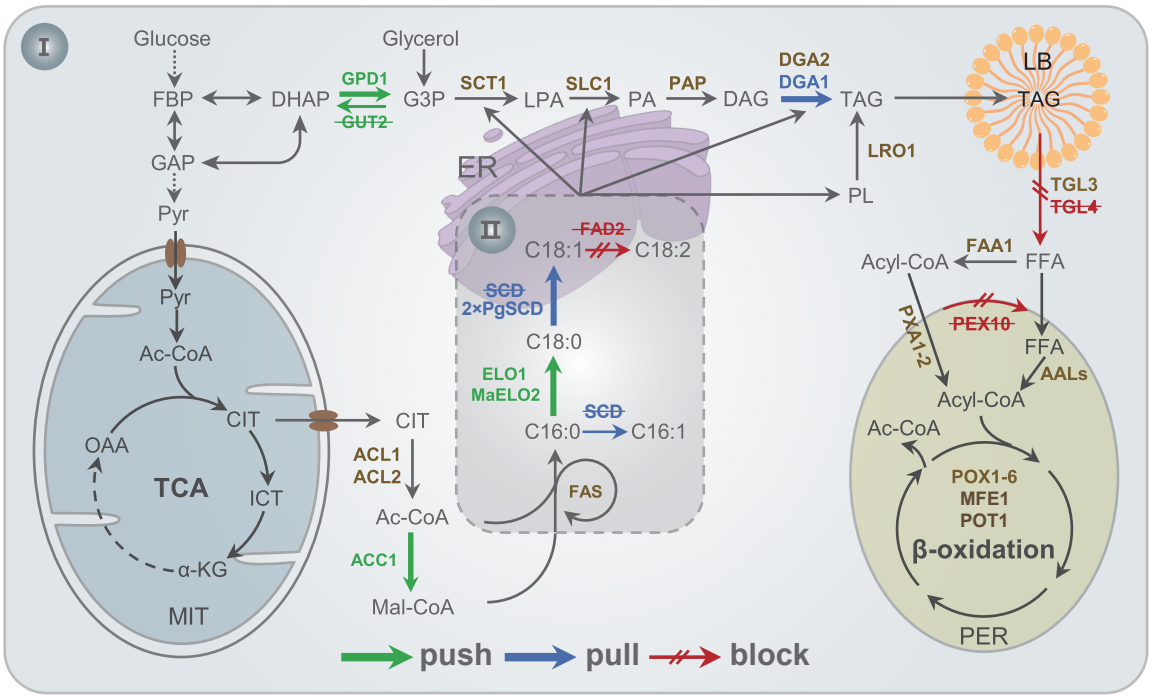
Fig. 7 Improvement of oleic acid production in different *Yarrowia lipolytica* cell factories by engineering the lipid and fatty acid pathways using the 'push-pull-block' strategy and fed-batch fermentation.

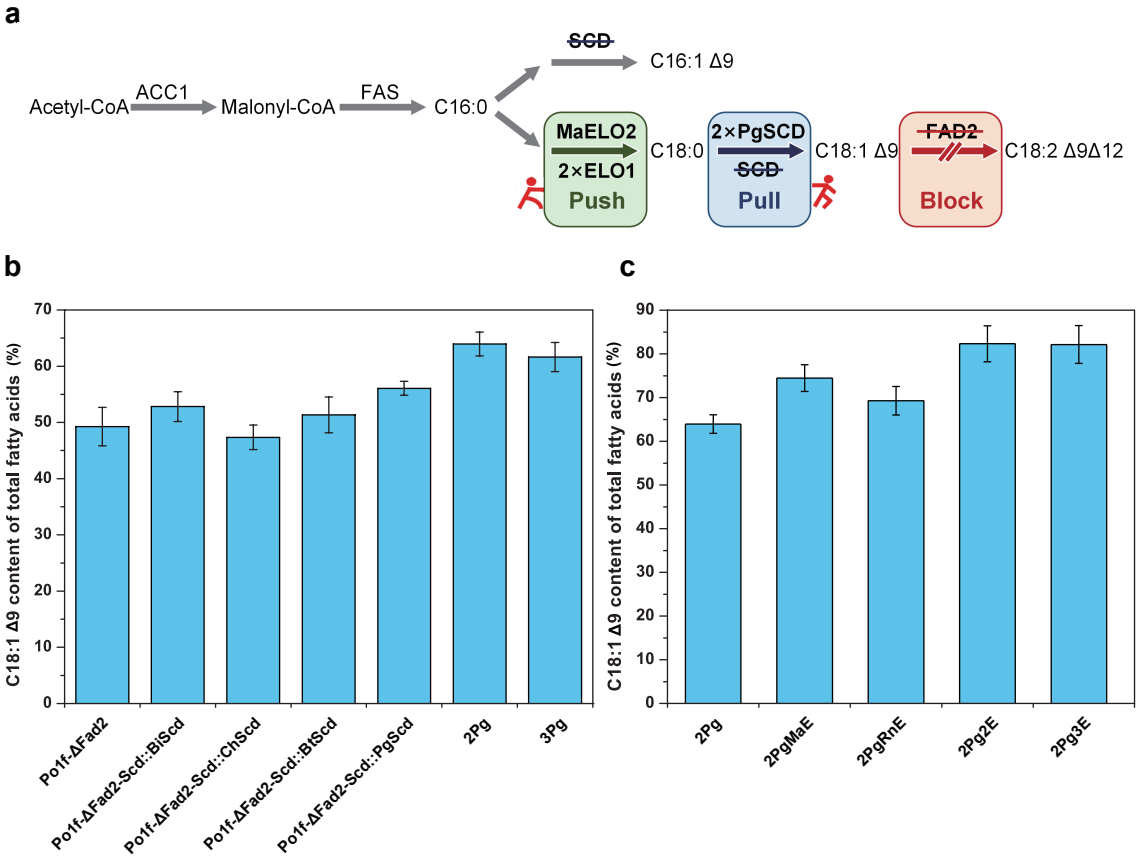
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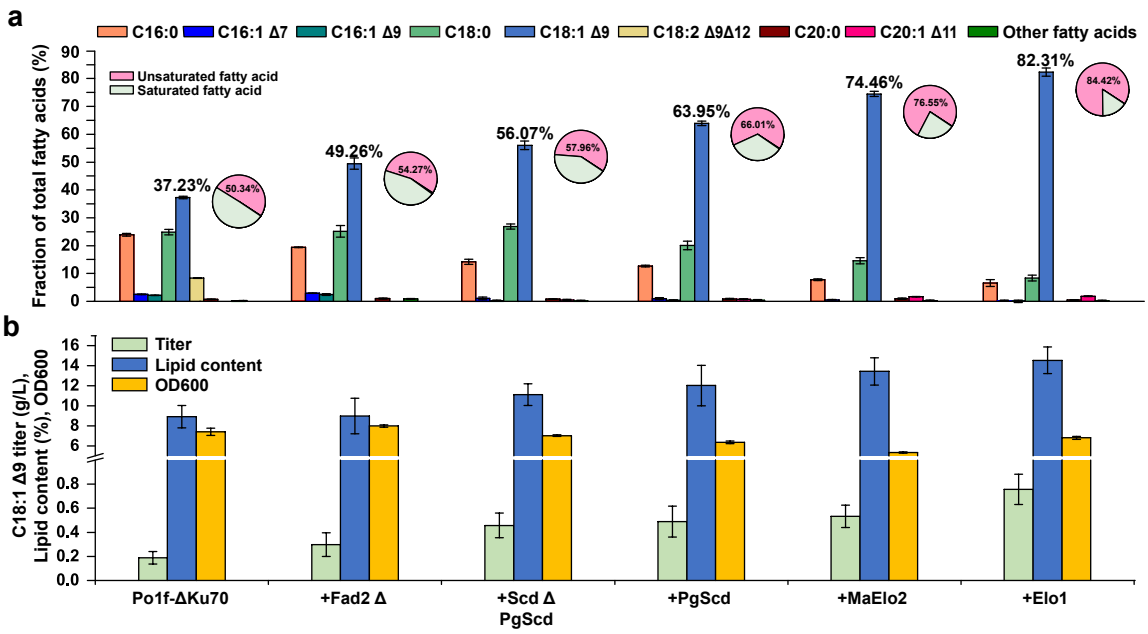


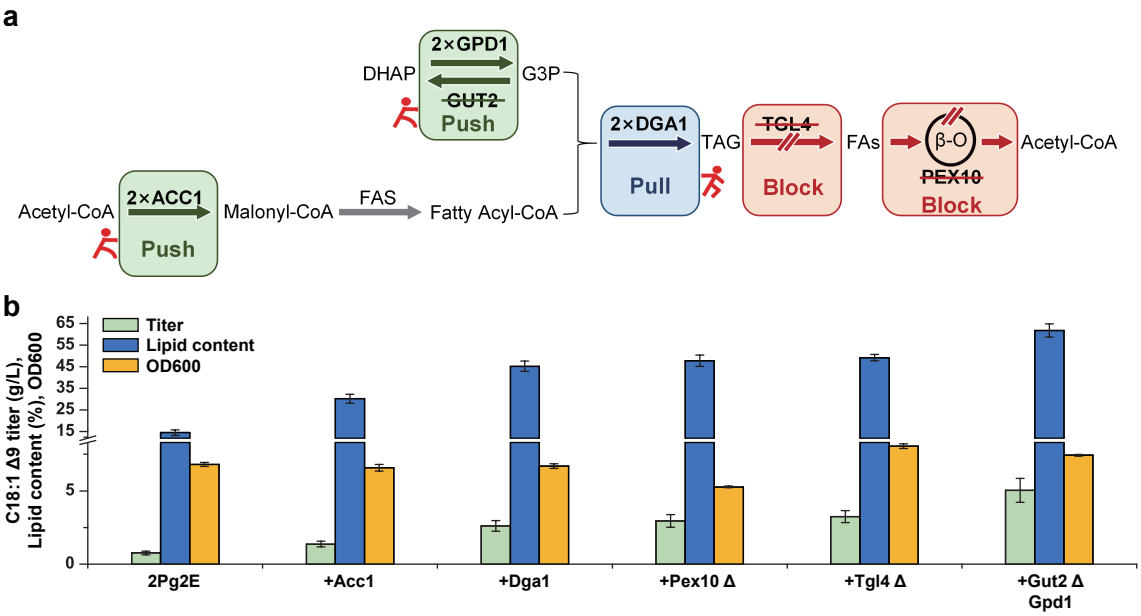
Synopsis

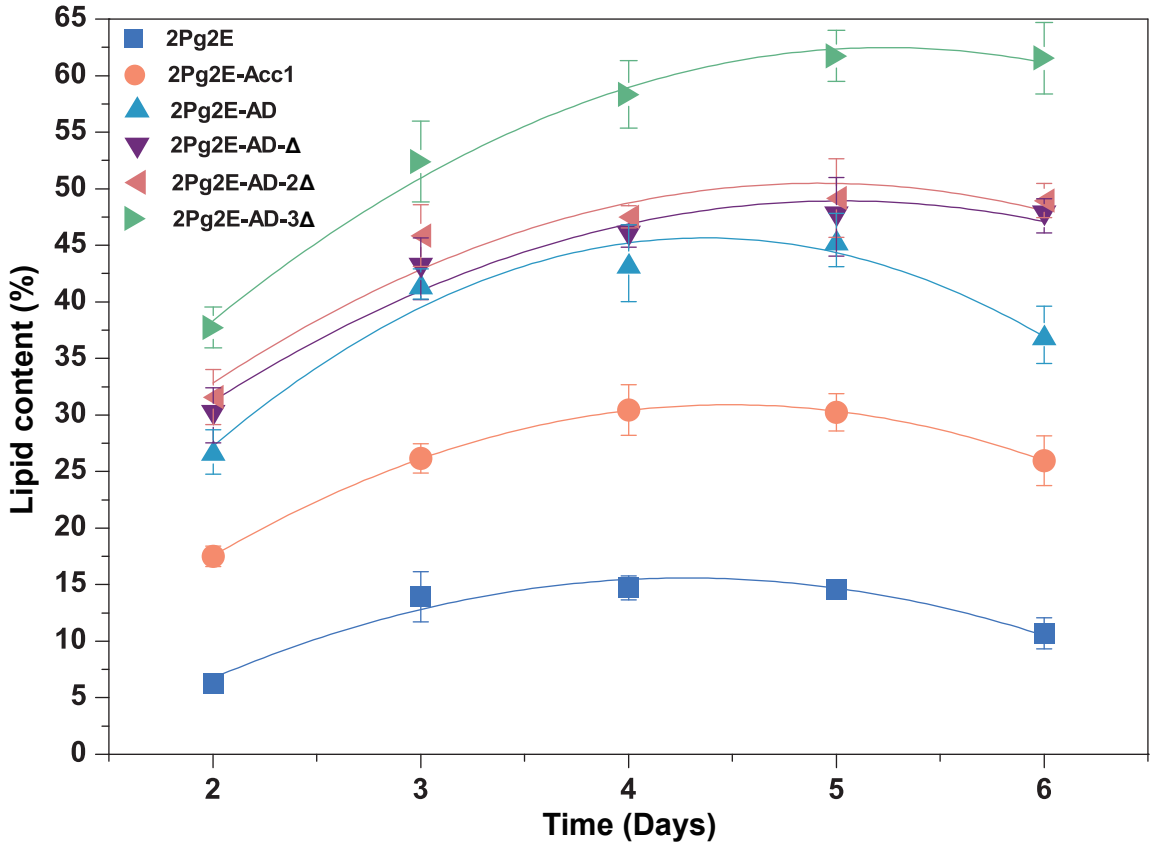
Yarrowia lipolytica cell factories were engineered for sustainable production of high oleic plant oils with two-layer 'push-pull-block' strategy.

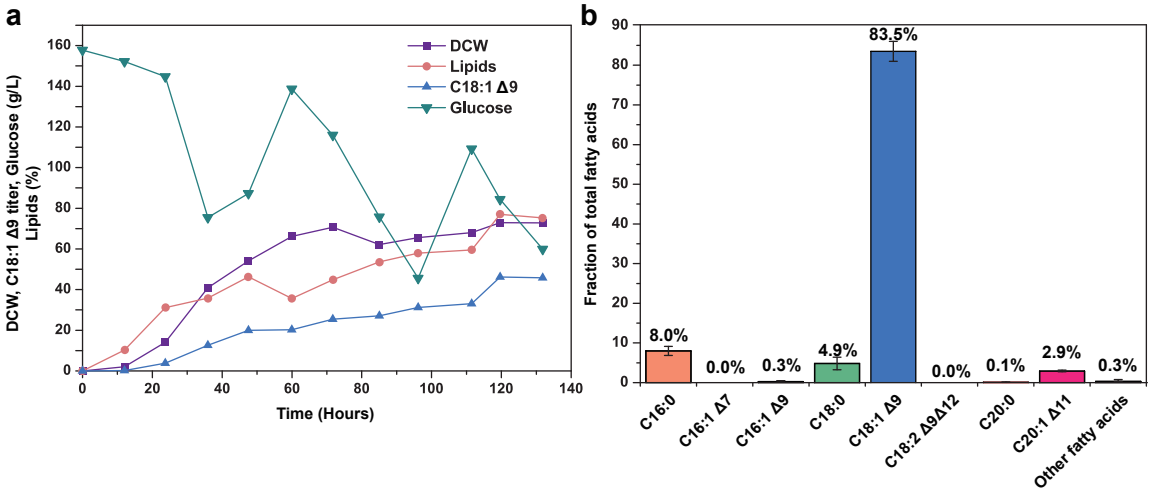

I
Glucose
Glycerol
FBP
DHAP
GPD1
GUT2
G3P
SCT1
LPA
SLC1
PA
PAP
DAG
DGA2
DGA1
TAG
LB
TAG
GAP
Pyr
ER
LRO1
PL
TGL3
TGL4
II
FAD2
C18:1
C18:2
FAA1
Acyl-CoA
FFA
SCD
2×PgSCD
C18:0
PEX10
PXA1-2
FFA
AALs
ELO1
MaELO2
SCD
C16:0
C16:1
Acyl-CoA
Pyr
Ac-CoA
CIT
CIT
OAA
TCA
ICT
ACL1
ACL2
Ac-CoA
Ac-CoA
POX1-6
MFE1
POT1
FAS
ACC1
α-KG
β-oxidation
Mal-CoA
MIT
PER
push
pull
block


a
Acetyl-CoA
ACC1
Malonyl-CoA
FAS
C16:0
SCD
C16:1 Δ9
MaELO2
2×ELO1
Push
C18:0
2×PgSCD
SCD
Pull
C18:1 Δ9
FAD2
Block
C18:2 Δ9Δ12
b
C18:1 Δ9 content of total fatty acids (%)
Po1f-ΔFad2
Po1f-ΔFad2-Scd::BiScd
Po1f-ΔFad2-Scd::ChScd
Po1f-ΔFad2-Scd::BtScd
Po1f-ΔFad2-Scd::PgScd
2Pg
3Pg
c
C18:1 Δ9 content of total fatty acids (%)
2Pg
2PgMaE
2PgRnE
2Pg2E
2Pg3E


a
C16:0
C16:1 Δ7
C16:1 Δ9
C18:0
C18:1 Δ9
C18:2 Δ9Δ12
C20:0
C20:1 Δ11
Other fatty acids
Unsaturated fatty acid
Saturated fatty acid
Fraction of total fatty acids (%)
37.23%
50.34%
49.26%
54.27%
56.07%
57.96%
63.95%
66.01%
74.46%
76.55%
82.31%
84.42%
b
Titer
Lipid content
OD600
C18:1 Δ9 titer (g/L), Lipid content (%), OD600
Po1f-ΔKu70
+Fad2 Δ
+Scd Δ PgScd
+PgScd
+MaElo2
+Elo1


a
2×GPD1
DHAP
G3P
GUT2
Push
2×DGA1
TAG
TGL4
FAs
β-O
Acetyl-CoA
Pull
Block
PEX10
Block
2×ACC1
Acetyl-CoA
Malonyl-CoA
FAS
Fatty Acyl-CoA
Push
b
Titer
Lipid content
OD600
C18:1 Δ9 titer (g/L), Lipid content (%), OD600
65
55
45
35
25
15
5
0
2Pg2E
+Acc1
+Dga1
+Pex10 Δ
+Tgl4 Δ
+Gut2 Δ Gpd1


2Pg2E
2Pg2E-Acc1
2Pg2E-AD
2Pg2E-AD-Δ
2Pg2E-AD-2Δ
2Pg2E-AD-3Δ
Lipid content (%)
Time (Days)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
2
3
4
5
6


a
DCW, C18:1 Δ9 titer, Glucose (g/L)
Lipids (%)
Time (Hours)
DCW
Lipids
C18:1 Δ9
Glucose
b
Fraction of total fatty acids
8.0%
0.0%
0.3%
4.9%
83.5%
0.0%
0.1%
2.9%
0.3%
C16:0
C16:1 Δ7
C16:1 Δ9
C18:0
C18:1 Δ9
C18:2 Δ9Δ12
C20:0
C20:1 Δ11
Other fatty acids