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Combinatorial engineering approaches for improved lipid production in *Saccharomyces cerevisiae*

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Abstract

Triacylglycerols (TAGs) hold substantial medical, nutritional, and industrial value. However, environmental concerns demand more sustainable production alternatives. A promising approach is the fermentation of TAG-accumulating microbes. In this study, we engineered a previously developed high-lipid producing *Saccharomyces cerevisiae* strain to further increase lipid accumulation through a combinatorial approach. We obtained up to 327 mg·gCDW⁻¹ of lipid content by deleting *CKB1*, encoding a subunit of casein kinase 2 (CK2) related to the regulation of lipid metabolism, in combination with the deletion of *SEI1* encoding a seipin. The deletion of *CKB1* combined with reduced expression of *ERD1* and increased expression of *YFT2*, genes related to ER stability and lipid droplet production, respectively, led to 329 mg·gCDW⁻¹ of lipid content. Notably, in the highest lipid producer, only *ERD1* and *YFT2* were targeted. This strain reached up to 350 mg·gCDW⁻¹ of lipid content, a 41% increase in lipid accumulation. However, the strains with the highest lipid content also showed a reduction in growth rate and final biomass accumulation. The results in this study demonstrate that the outcomes of individual modifications depend on the genetic background, emphasizing the importance of identifying the appropriate combination of target genes to achieve the desired phenotype.

Keywords *Saccharomyces cerevisiae*, lipid accumulation, metabolic engineering, lipid droplets, cell factory, lipid metabolism

Introduction

The demand for fatty acids (FAs) for nutritional, medical and industrial purposes has been increasing over the past years (Rahmawati et al. 2024). This demand is currently mainly covered through production based on crops specialized in storing high amounts of lipids in the form of triacylglycerols (TAGs). Examples of this are the production of coconut oil or palm oil, where cultivation is only possible in tropical areas, which promotes deforestation and represents a threat to biodiversity (Fitzherbert et al. 2008, Meijaard et al. 2020). Other concerns that come along with these processes are the high CO₂ footprint and societal issues such as unsustainable working conditions and limited access to legal recourse in rural communities (Gesteiro et al. 2019, Afrizal et al. 2023). Therefore, finding alternatives to produce TAGs is of key importance. The use of microorganisms for this purpose is of interest, as microorganisms offer flexibility in FA composition and site of production, can be easily upscaled, and tackle the environmental concerns previously mentioned.

Cells can store lipids in lipid particles, also called lipid droplets (LDs). In eukaryotic cells, lipid droplets consist of a hydropho-

bic core of neutral lipids, mainly TAGs and steryl esters (SE), surrounded by a monolayer membrane of phospholipids (Zweytick et al. 2000).

Oleaginous yeasts are a group of yeast species defined by the phenotypic trait of accumulating more than 20% of their biomass as lipids. (Thorpe and Ratledge 1972). Oleaginous yeasts have been studied for the potential for industrial production of TAGs, although the limited knowledge about their metabolism and efficient genetic engineering tools still hampers the process. *Saccharomyces cerevisiae* has also been considered as a candidate to produce TAGs, although it is regarded as non-oleaginous. Nevertheless, some wild type strains of *S. cerevisiae* have been found to accumulate more than 20% of storage lipids (He et al. 2018). *Saccharomyces cerevisiae* is currently the preferred eukaryotic cell factory to produce biochemicals (Nielsen and Keasling 2016), including some FA-derived products (Runguphan and Keasling 2014). Its value as a cell factory is based on the vast knowledge about its metabolism, the range of efficient genetic engineering tools adapted for it, and other industrially appealing characteristics such as being generally regarded as safe (GRAS) and tolerant to inhibitors of fermentation and to low pH (Jullesson et al. 2015, Mans

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et al. 2015, Chen et al. 2016, Wu et al. 2022). Ferreira et al. (2018) developed a *S. cerevisiae* strain able to accumulate up to 25% of its dry weight as lipids, through the genetic modification of specific targets in the TAG synthesis and FA utilization pathways. They implemented a push-and-pull strategy to increase the activity of the first and last steps of the TAG synthesis. The developed strain expressed a deregulated version of acetyl-CoA carboxylase Acc1, which catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. It also overexpressed *PAH1* encoding phosphatidate phosphatase as well as *DGA1* encoding diacylglycerol acyltransferase. In addition, genes encoding the TAG lipases, responsible for most of the TAG lipase activity (Tgl3, Tgl4, and Tgl5) (Athenstaedt and Daum 2003), were deleted. Additionally deleted was the gene encoding sterol acyltransferase Are1, which competes with Dga1 for acyl-CoA. Beta-oxidation in the peroxisomes was inhibited by deletion of *POX1* encoding fatty-acyl coenzyme A oxidase. Finally, to avoid the utilization of glycerol-3-phosphate (G3P), glycerol-3-phosphate dehydrogenase Gut2, which catalyzes the conversion of G3P to dihydroxyacetone phosphate (DHAP) (Rønnow and Kielland-Brandt 1993), was eliminated to generate the strain RF11.

In this study we focused on regulatory mechanisms of the TAG synthesis pathway and genes related to LD generation whose modification have previously been shown to influence TAG accumulation in yeast.

Methods

Plasmid and strain construction

Strains, plasmids, gRNA sequences, and primers used in this study are listed and referenced in Table 1 and Supplementary Tables S1 and S2, respectively. Diagnostic primers, repair fragments, and plasmid assemblies were designed using Benchling (San Francisco, CA, USA).

Plasmid purifications from *Escherichia coli* were performed using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. Restriction enzymes used in this study were acquired from Thermo Fisher Scientific. All gRNA encoding sequences used in this study were cloned into and expressed from plasmid pMEL10.

Amplification of genes *DDL1* and *YFT2* was carried out by PCR using the genomic DNA of strain IMX581 (Mans et al. 2015) as template. *DDL1* was amplified in two fragments, introducing a silent mutation to eliminate a BsaI target sequence that otherwise would interfere with the Golden Gate reaction performed afterwards. Both fragments were then fused through fusion PCR and the resulting DNA fragment gel purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Both genes were assembled in pMC-URA-Cen-R1 (Otto et al. 2021), a Lvl-1 plasmid, through Golden Gate cloning using BsaI, together with the corresponding promoter and terminator.

From the Lvl-1 plasmids, *DDL1* and *YFT2* expression cassettes were assembled through Golden Gate assembly using BsmBI into the integrative vectors pMC-XI5 and pMC-X4, respectively. *DDL1* was assembled with the promoter *pTEF1* and terminator *tSSA1*, and *YFT2* with the promoter *pHHF1* and terminator *tENO1*. Before yeast transformation, the integrative vectors were linearized with NotI.

The repair fragments used for gene deletions were ordered through Eurofins Genomics (Ebersberg, Germany) as 120-bp oligos, denatured at 96°C for 10 min, and annealed by slow cooling to room temperature. Repair fragments consisted of 60 bp upstream and 60 bp downstream of the targeted region.

The low expression promoters *pREV1* and *pCYC1min* were PCR-amplified from the plasmids PYTK027 and FRP793_insul-(lexA-box)4-PminCYC1-Citrine-TCYC1, respectively, using primers that introduce 50 bp up- and downstream of the integration site, the 500 bp upstream of *ERD1*, for them to be used as repair fragments. The site was targeted by a gRNA guided CRISPR-Cas9 and substituted by the repair fragment used in each case.

Yeast transformation was performed using a PEG/LiOAc protocol (Gietz and Schiestl 2007). Genetic modifications were verified by PCR amplification, 1% agarose gel imaging and DNA sequencing through Eurofins Genomics (Ebersberg, Germany).

Growth media

E. coli was grown in LB medium consisting of 10 g·l⁻¹ peptone from casein (tryptone), 10 g·l⁻¹ NaCl, 5 g·l⁻¹ yeast extract. Using NaOH, the pH was adjusted to 7.0.

Yeast strains were grown in YPD medium consisting of 20 g·l⁻¹ glucose, 10 g·l⁻¹ yeast extract, 20 g·l⁻¹ peptone from casein; for growth on plates, 20 g·l⁻¹ agar were added. For growth assays and flask cultivations, strains were grown in Delft minimal medium (Verduyn et al. 1992) consisting of 20 g·l⁻¹ glucose, 5 g·l⁻¹ (NH₄)₂SO₄, 14.4 g·l⁻¹ KH₂PO₄, 0.5 g·l⁻¹ MgSO₄·7H₂O, 150 mg·l⁻¹ uracil and adjusted to pH 6.5. After sterilization, 2 ml·l⁻¹ trace element solution and 1 ml·l⁻¹ of vitamin solution were added. The trace metal solution consisted of FeSO₄·7H₂O 3.0 g·l⁻¹, ZnSO₄·7H₂O 4.5 g·l⁻¹, CaCl₂·2H₂O 4.5 g·l⁻¹, MnCl₂·4H₂O 1 g·l⁻¹, CoCl₂·6H₂O 300 mg·l⁻¹, CuSO₄·5H₂O 300 mg·l⁻¹, Na₂MoO₄·2H₂O 400 mg·l⁻¹, H₃BO₃ 1 g·l⁻¹, KI 100 mg·l⁻¹, Na₂EDTA·2H₂O 19 g·l⁻¹. The vitamin solution consisted of D-biotin 50 mg·l⁻¹, D-pantothenic acid hemicalcium salt 1 g·l⁻¹, thiamin-HCl 1 g·l⁻¹, pyridoxin-HCl 1 g·l⁻¹, nicotinic acid 1 g·l⁻¹, 4-aminobenzoic acid 0.2 g·l⁻¹, myo-inositol 25 g·l⁻¹. For selection of strains carrying the marker *URA3*, uracil was omitted. Plates for selection of *URA3*-carrying strains contained agar 20 g·l⁻¹, Complete Supplement Mixture (CSM) Drop-out -URA (Formedium, Hunstanton, United Kingdom) 0.77 g·l⁻¹, Yeast Nitrogen Base without amino acids 6.9 g·l⁻¹, D(+)-glucose 20 g·l⁻¹, and the pH was adjusted to 6.0.

Growth assays

Pre-cultures for the growth assays were performed in 96-well plates (EnzyScreen CR1496d) and grown in a Growth Profiler 960 (EnzyScreen, Heemstede, The Netherlands) at 30°C, 250 RPM, in Delft media supplemented with uracil. When all the strains reached stationary phase, growth assays were carried out in 96-well plates in the same Growth Profiler 960 at 30°C, 250 RPM, in Delft media supplemented with uracil, at a starting OD of 0.1 and culture volume of 250 µl. Three biological replicates per strain were used. The outside rows and columns of the 96-well plates were left unused. Data points were taken every 30 min for 40 to 50 h and analyzed using the growthrates package in R, version 4.5.1.

Table 1 Strains used in this study.

Strain name	Genotype	Parental strain	Reference
RF11	<i>MATaMAL2-8c SUC2 ura3-52 can1::pTEF1-cas9 pHXT7-ACC1** + pPGK1-PAH1 + pTEF1-DGA1 tgl3Δ tgl4Δ tgl5Δ are1Δ pox1Δ gut2Δ pxa1Δ</i>	IMX581	Ferreira et al. 2018
ACa	RF11 <i>gsy2Δ</i>	RF11	This study
ACb	RF11 <i>ckb1Δ</i>	RF11	This study
ACc	RF11 <i>sei1Δ</i>	RF11	This study
ACd	RF11 XI-5::pTEF1- DDL1-tSSA1	RF11	This study
ACe	RF11 X-4::pHHF1-YFT2-tENO1	RF11	This study
ACf	RF11 <i>pERD1Δ::pCYC1min</i>	RF11	This study
ACg	RF11 <i>pERD1Δ::pREV1</i>	RF11	This study
ACba	RF11 <i>gsy2Δ ckb1Δ</i>	ACa	This study
ACbac	RF11 <i>gsy2Δ ckb1Δ sei1Δ</i>	ACa	This study
ACbae	RF11 <i>gsy2Δ ckb1Δ X-4:: pHHF1-YFT2-tENO1</i>	ACba	This study
ACbc	RF11 <i>ckb1Δ sei1Δ</i>	ACb	This study
ACef	RF11 X-4::pHHF1-YFT2 <i>pERD1Δ::pCYC1min</i>	ACe	This study
ACeg	RF11 X-4::pHHF1-YFT2 <i>pERD1Δ::pREV1</i>	ACe	This study
ACefb	RF11 X-4::pHHF1-YFT2-tENO1 <i>pERD1Δ::pCYC1min ckb1Δ</i>	ACef	This study

Lipid droplet staining and visualization

Pre-cultures were performed in 4 ml of YPD liquid medium overnight in 10-ml culture tubes at 30°C, 200 RPM. Cultures were centrifuged in a VWR® Mega Star 1.6R refrigerated centrifuge (Model 521–2660; VWR International) at 1900 x g for 5 min, at room temperature, and stained with Nile red (Sigma-Aldrich, Cat. No. 72485, CAS 7385–67-3) following the protocol by Rostron and Lawrence (2017). Samples were then visualized under an inverted fluorescence microscope and processed with the LAS X software (DMI4000B, Leica Microsystems, Wetzlar, Germany) using the 1000x magnification. Fluorescence images were acquired using a DsRed channel with excitation at 558 nm (multiphoton excitation 1051 nm) and emission collected at 582 nm. The only change in the original staining protocol was that Slow Fade was not used.

Analysis of cell morphology

To evaluate potential differences in cell sizes and aggregation between yeast strains, flow cytometry analysis was performed for cell populations in stationary phase of the strains RF11, ACb, ACC, ACE, ACf, ACbc, ACef, and ACefb.

Pre-cultures of the strains RF11, ACbc, ACef and ACefb were performed in 4 ml of Delft liquid medium supplemented with uracil overnight in 10-ml culture tubes at 30°C, 200 RPM, in biological triplicates. Yeast strains were grown to stationary phase in 30 ml of Delft medium supplemented with uracil in 100-ml shake flasks at 30°C and 200 RPM. Their growth was monitored with a Cell Growth Quantifier and the DOTS software (Scientific Bioprocessing, Baesweiler, Germany). Cultures were then centrifuged in a VWR® Mega Star 1.6R refrigerated centrifuge (Model 521–2660; VWR International) at 1200 x g for 5 min and room temperature, the supernatant was discarded, and the pellet resuspended in PBS 1x,

pH 7.4. Cells were diluted to an OD of 0.35 in 5 ml of PBS 1x, pH 7.4 to further analyze them in the cell sorter.

Flow cytometry and cell sorting was performed using a Sony SH800 cell sorter (Sony Biotechnology, USA). Event detection was triggered on the forward scatter channel (FSC) with a threshold set to 5%. Detector gain values were set at 14 for FSC and 37.5% for the back scatter channel (BSC). Cell sorting was performed using a 130 μm sorting chip.

Populations were analyzed first by plotting FSC-Height (FSC-H) against FSC-Area (FSC-A) following the yeast display sorting gates presented by Pan et al. (2025), to visualize the distribution among singlets, doublets, aggregates of cells, and cell size changes among populations. Histograms were generated for FSC-A, FSC-H, and FSC-Width (FSC-W) for all events to study the cell size and aggregation level in the different strains. Histograms to study internal complexity of the cells were generated for BSC-A and BSC-H for all events. The histogram representing FSC-W was gated to isolate two populations, X for single cells and doublets, and Y for aggregates, and the percentage of the total population falling within each gate was collected (Supplementary Fig. S1). R software was used to process the data collected.

Total lipid quantification

Samples for lipid analysis were harvested after cultivation in shake flasks in 80 ml Delft medium supplemented with uracil. Their growth was monitored with the Cell Growth Quantifier and DOTS software until reaching stationary phase. The cultures were centrifuged at room temperature and 1200 x g for 5 min and the supernatant discarded. Pellets were washed with deionized water and centrifuged under the same conditions twice. Afterwards, the pellets were kept at -80°C until being freeze dried using a Christ

alpha 2–4 LSC (Christ Gefriertrocknungsanlagen, Osterode, Germany). Dried samples were weighed to obtain the total cell dry weight per 100 ml of culture, and about 100 mg of each culture were weighed and stored in glass vials in triplicates to perform lipid extraction.

Lipid extraction was based on the protocol of Bligh and Dyer (1959) with some modifications:

Biomass rupture was performed by adding 1.5 ml of 2 M HCl and shaking for 1 h at 90°C and 700 RPM in an Eppendorf® ThermoMixer® C (Eppendorf AG, Hamburg, Germany), then cooled down to RT.

Three millilitres of a chloroform:methanol solution (1:2) was added and the tubes were left shaking for 10 min at 1000 RPM, RT. Afterwards, 1 ml of chloroform and 0.8 ml of 0.88% KCl were added to remove non-lipid material compounds that may be trapped within micelles (Schneider and Daum 2006).

The tubes were vortexed again for 5 min at 1000 RPM and room temperature, and centrifuged for 5 min at 3000 RPM and room temperature. The non-polar chloroform phase was transferred to a new pre-weighed glass tube and dried overnight using a continuous flow of N₂. After evaporating the solvent, the tubes were weighed and total lipid weight per gram was calculated for each culture.

Fatty acid (FA) composition analysis

The FA composition was determined through gas chromatography (GC) coupled with flame ionization detection (FID) and mass spectrometry (MS). Lipids were extracted from freeze-dried biomass samples using the adapted Bligh and Dyer method described before in this study. Next, the samples were subjected to derivatization to fatty acid methyl esters (FAMES) as described by (Norlander et al. 2024). Lipids from each strain were extracted, derivatized and analyzed in biological triplicates.

Samples were analyzed using a Thermo Scientific TRACE GC 1300–1310™ gas chromatography (GC) system, complete with AI- and AS 1310 autoinjector and auto-sampler, equipped with a flame ionization detector (FID) and mass spectrometry (MS) detector.

The standard GLC-426 (Nu-Check-Prep, Elysian, MN, USA) was used for quantification and to assign FA identities based on retention times and to calculate response factors for individual FAs. The internal standards C17:0 (0.25 mg) and C15:0 (0.75 mg) were used to calculate the response coefficient of FA to account for lipids lost during the lipid extraction and methylation process.

GC controls using heptane were used to account for potential contaminations and background noise. If background FAs were detected in blanks, their average signal was subtracted from the corresponding sample peaks.

Based on the internal standards and FA corresponding response factors, the amount in grams for each FA before lipid extraction of each culture was calculated. The addition of those values gave the total amount of FA in the samples before extraction. The obtained values were used to calculate the g/g ratios of the individual FA species per biomass weight for each analysed sample.

FAs that were detected but were not included in the calibration standard could not be quantified. Nevertheless, their MS fragmentation patterns were compared to reference spectra to find tentative identifications (National Institute of Standards and Technology [NIST] 2020).

TAG quantification

Lipids were extracted from freeze-dried biomass samples using the adapted Bligh and Dyer method described above. This process was performed in biological triplicates for the strains RF11, ACefb, ACbc, and ACefb. Next, to isolate TAGs, lipid classes were separated by thin layer chromatography (TLC) as described by Dahlgren et al. 2026. Lipid classes were separated using a heptane:diethyl ether:acetic acid (65:35:1, v/v) mixture as the mobile phase, and the fractions corresponding to TAGs were marked with a pencil. The fractions were then wetted with mili-Q water, scraped off into a 10 ml glass tube with solvent-proof Teflon-lined screw-cap, and suspended in 500 µl methanol. The isolated TAGs were then sonicated and dried under a stream of N₂ at room temperature. Finally, the dried collected TAGs were subjected to derivatization to FAMES, analyzed by GC-FID and quantified as described in the previous section. For the separated and methylated TAG fraction a lower split ratio (10:1) was used during injection for GC-FID analysis.

Quantitative reverse transcription PCR (RT-qPCR)

Yeast strains were cultivated in triplicates in shake flasks in 100 ml Delft medium supplemented with uracil. Growth was monitored with the Cell Growth Quantifier and DOTS software until reaching mid exponential phase. Cultures were centrifuged at 4°C and 4100 × g for 10 min and the supernatant was discarded. Cells were immediately frozen in liquid nitrogen to prevent mRNA turnover (Piper et al. 2002). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germany), with cell disrupted using a FastPrep homogenizer (MP Biomedicals, USA). First-strand cDNA was synthesized from 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany). The synthesized cDNA was diluted 25-fold, and 2 µl of the diluted cDNA was used as the template for RT-qPCR. RT-qPCR was performed as previously described (Liu et al. 2016) using the DyNamo Flash SYBR Green qPCR kit (Thermo Fisher Scientific, USA) as template. *ACT1* was used as the reference gene for normalization of transcript levels. Primer sets are listed in [Supplementary Table S2](#).

Statistical analysis

Assumptions for parametric testing were evaluated by examining the normality of residuals with the Shapiro–Wilk test and assessing homogeneity of variances using Levene’s test. Due to the small sample size, normality was assumed in cases where the Shapiro–Wilk test had limited power.

Statistical analysis of the dry lipid weight was performed using the Kruskal–Wallis test, followed by Dunn’s post hoc test with Benjamini–Hochberg correction for multiple comparisons. Non-parametric tests were chosen because the homoscedasticity requirement to perform parametric tests was not met.

Statistical analysis of GC-FID data, FACS population data and growth data were performed using unpaired two-sample t-tests. *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method.

All statistical analyses were performed in R (version 2025.05.0 + 496). Homogeneity of variances was assessed

using `leveneTest()` from the `car` package, and normality was evaluated using `shapiro.test()` from base R. For direct pairwise comparisons of each strain versus the parental strain RF11, two sample unpaired t-tests were performed (`t.test()`, base R) and *P*-values were adjusted for multiple testing using the Benjamini-Hochberg procedure. Non-parametric tests were performed using the functions `kruskal.test()` from base R and `dunn.test()` from the `dunn.test` package.

Results and discussion

Characterization of regulatory mechanisms as targets for improved lipid formation in lipid-accumulating strain RF11

To increase total lipid accumulation, regulatory mechanisms of the lipid synthesis pathways are an attractive target, but their complexity and interconnectivity make this approach challenging. In this study, we implemented and combined in the strain RF11 regulation-related genetic modifications that were previously shown to influence lipid accumulation in yeast.

Cardiolipin is a phospholipid species mostly found in the inner mitochondrial membrane, and it plays a key role in mitochondrial function and stability (Jiang et al. 2000, Yadav and Rajasekharan 2017). It has also been previously shown that cardiolipin deficiency leads to upregulation of many genes related to TAG synthesis in *S. cerevisiae*, effectively increasing TAG accumulation (Yadav and Rajasekharan 2017).

The gene *DDL1* (Fig. 1) codes for DDHD domain-containing phospholipase A1, a mitochondrial matrix protein involved in remodelling mitochondrial phospholipids and hydrolysis of cardiolipin (CL) (Yadav and Rajasekharan 2016). *DDL1* overexpression in *S. cerevisiae* resulted in a 60% increase in TAG accumulation and a reduction in the CL content of the cells (Yadav and Rajasekharan 2016, Yadav and Rajasekharan 2017). Although it also led to a growth defect in solid media, growth in liquid media was mostly unaffected.

In this study, we implemented *DDL1* overexpression by introducing a second copy of the gene in RF11, under control of the promoter *TEF1*, producing the strain ACd. The strain increased lipid accumulation by 18% compared to RF11 (Fig. 2). However, also a significant reduction in the maximum growth rate ($\mu_{\max}$) was observed compared to RF11 (Supplementary Fig. S3B). This growth defect, not seen in a WT strain before (Yadav and Rajasekharan 2016), may result from the fact that RF11 already contains multiple modifications, and *DDL1* overexpression represented an additional metabolic burden. Alterations in the mitochondrial membrane may lead to the generation of reactive oxygen species, defective electron transporter chain and hindering of the ATP production.

In addition, the EtOH phase was elongated compared to RF11 (Supplementary Fig. S3). It has been reported that CL-defective *S. cerevisiae* strains, when grown in ethanol, show markers of ROS accumulation, and that CL remodelling may play a role in the oxidative stress response (Ji and Greenberg 2022). Arhar et al. (2021) reported an increased TAG accumulation in *S. cerevisiae* after deletion of *CKB1*, which encodes one of the two regulatory subunits of

casein kinase 2 (CK2). CK2 is a highly conserved kinase with many substrates, many of which may not have been identified yet (Meggio and Pina 2003). CK2 has also been proven to play a role in lipid synthesis by regulating the activity of key pathway enzymes. In *S. cerevisiae*, CK2 has been shown to phosphorylate the phosphatidate phosphatase Pah1 (Hsieh et al. (2016) and to promote the activity of diacylglycerol (DAG) kinase Dgk1 (Qiu et al. 2016) (Fig. 2). Sct1, an acyltransferase that catalyzes the conversion of acyl-CoA to lyso-PA has also been proven to be negatively regulated by CK2 (Arhar et al. 2021). CK2 was shown to play a role in the synthesis of key membrane-related components, such as sphingolipids (Fresques et al. 2015) or phosphatidic acid (Qiu et al. 2016). In this study, we implemented deletion of *CKB1* in the RF11 strain, generating strain ACb. The ACb strain showed a 27.2% increase in lipid accumulation (Fig. 2), with the lipid content reaching up to 31% of its dry cell weight (Fig. 2).

Investigation of membrane biogenesis and maturation targets to increase neutral lipid formation in lipid-accumulating yeast strains

Although targeting TAG biosynthesis and associated metabolic pathways has been proven to be a successful strategy to increase TAG accumulation in *S. cerevisiae*, the further development of oleaginous *S. cerevisiae* strains should include additional engineering targets. A promising approach is to focus on the budding and detachment of LDs from the ER, a process that involves a wide range of proteins and regulatory systems (Brasaemle and Wolins 2012).

SEI1 encodes a seipin involved in LD detachment and maturation from the ER. Teixeira et al. (2018) showed that the deletion of *SEI1* in a WT strain with the same genetic background as RF11, led to lipid accumulation of 13.9% of the dry cell weight, a 62% increase over the parental strain. They hypothesized that the reason for this could be a prolonged attachment of the LDs to the ER. On the other hand, Arhar et al. (2021) reported a reduction in TAG content when deleting *SEI1* in their engineered high TAG-accumulating *S. cerevisiae* strain. In this study, we introduced the *SEI1* deletion in RF11, producing strain ACc, and observed a reduction of the lipid accumulation to 16.8% of the dry cell weight. Interestingly, Arhar et al. (2021) and Teixeira et al. (2018), after deletion of *SEI1*, reported similar levels of neutral lipid accumulation, although these levels arose from opposite effects: Arhar et al. observed a decrease in neutral lipids, whereas Teixeira et al. reported an increase. In addition, a significant growth defect in liquid media was observed for strain ACc compared to RF11 (Supplementary Fig. S3B).

To further understand the effect of the *SEI1* deletion, we generated a *cbk1Δ sei1Δ* strain, ACbc. Interestingly, this strain slightly surpassed ACb in terms of lipid accumulation, reaching up to 32.9% of the dry cell weight (Fig. 2). The $\mu_{\max}$ observed in ACbc, although significantly lower than the $\mu_{\max}$ of RF11, did not differ much from ACc (Supplementary Fig. S3B).

In ACbc, stained LDs showed an almost amorphous structure and supersized phenotype under the fluorescence microscope (Fig. 3A). This phenotype was not observed in ACb or ACc carrying the individual deletions.

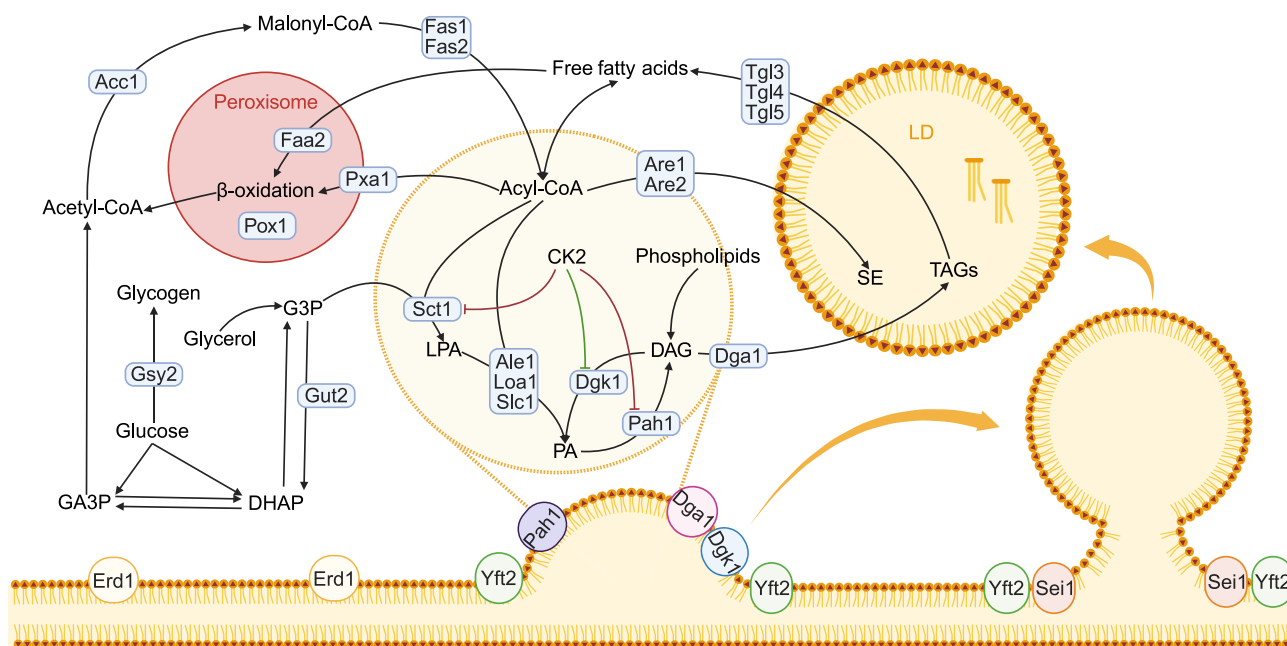


Figure 1 Schematic representation of the LD biogenesis and TAG biosynthetic pathways in *S. cerevisiae*. Coloured lines indicate the regulatory effects of CK2 on its targets, with green denoting positive regulation (targeting Dgk1) and red denoting negative regulation (targeting Sct1 and Pah1). For details, see text.

Grippa et al. (2015) previously showed that Sei1, together with the ER membrane protein Ldb16, stabilizes contact sites between LDs and ER, and that the absence of this complex leads to phospholipid packaging defects. This happens because of the increased diffusion of phospholipids between ER and LDs in the absence of the Sei1-Ldb16 complex. Furthermore, it has been previously shown that *SEI1* deletion leads to supersized and aggregated LD phenotypes (Szymanski et al. 2007), and that this effect is enhanced when seipin accessory factors are also altered (Wang et al. 2024). In fact, Wang et al. (2024) suggested that many seipin accessory factors could have mainly a regulatory role in controlling lipid packaging as well as protein sorting to the LD membrane. We speculate that *CKB1* deletion may lead to a deregulated flux of storage lipids towards forming LDs, which in combination with the altered LD formation machinery due to *SEI1* deletion, could collectively contribute to the increased lipid accumulation and alterations of the LD morphology as observed in ACbc.

Suppression of glycogen synthesis in lipid accumulating strains to increase the flux towards lipid synthesis

One potential target to further improve TAG accumulation is the glycogen synthesis pathway. In *S. cerevisiae*, glycogen synthesis is used to accumulate carbon and energy reserves during starvation conditions (Wilson et al. 2010). Glycogen is a homopolysaccharide, and its synthesis relies on two isoforms of the enzyme glycogen synthase: Gsy1 and Gsy2 (Farkas et al. 1991). Gsy activity is a potential competitor with the TAG biosynthesis for the available glucose, and reducing the flux towards glycogen synthesis could increase the glucose available to produce lipids. Arhar et al. (2021) tested this hypothesis and found that the deletion of *GSY2* led to an

increase in TAG accumulation by 35% compared to the wild type strain. In this study, we performed the same *GSY2* deletion in the strain RF11, generating the strain ACa. However, its lipid content did not significantly increase when compared to RF11.

We also tested the combination of *ckb1Δ* and *gsy2Δ* in the RF11 background, producing strain ACba, but combination of both deletions did not result in significant changes in the total lipid content of the strains (Fig. 2). These data suggests that glycogen synthesis is not a promising target to increase lipid accumulation in RF11 for our growth conditions. The reason could be that, due to prior modifications, the carbon flux in RF11 is already sufficiently diverted from glycogen synthesis towards lipid production.

We then tested the effect of the *SEI1* deletion in ACb and ACba, generating strains ACbc and ACbac respectively (Fig. 2). The comparison between ACbc and ACbac did not show differences in total lipid content, and ACbac did not show significant differences in lipid content compared to RF11. This supports the idea that the glycogen synthetic pathway is not a suitable metabolic target to improve lipid accumulation in RF11 under the used conditions. In addition, both ACba and ACbac, unlike ACb, showed a significantly reduced $\mu_{\max}$ compared to RF11.

Implementation of ER-biosynthesis and membrane-stability related modifications to increase lipid production in high-lipid accumulating *S. cerevisiae* strains

Yft2 belongs to the conserved Fat storage-Inducing Transmembrane (FIT) family ER proteins, which facilitate fat storage by partitioning energy-rich triglycerides into LDs (Moir et al. 2012). Yft2

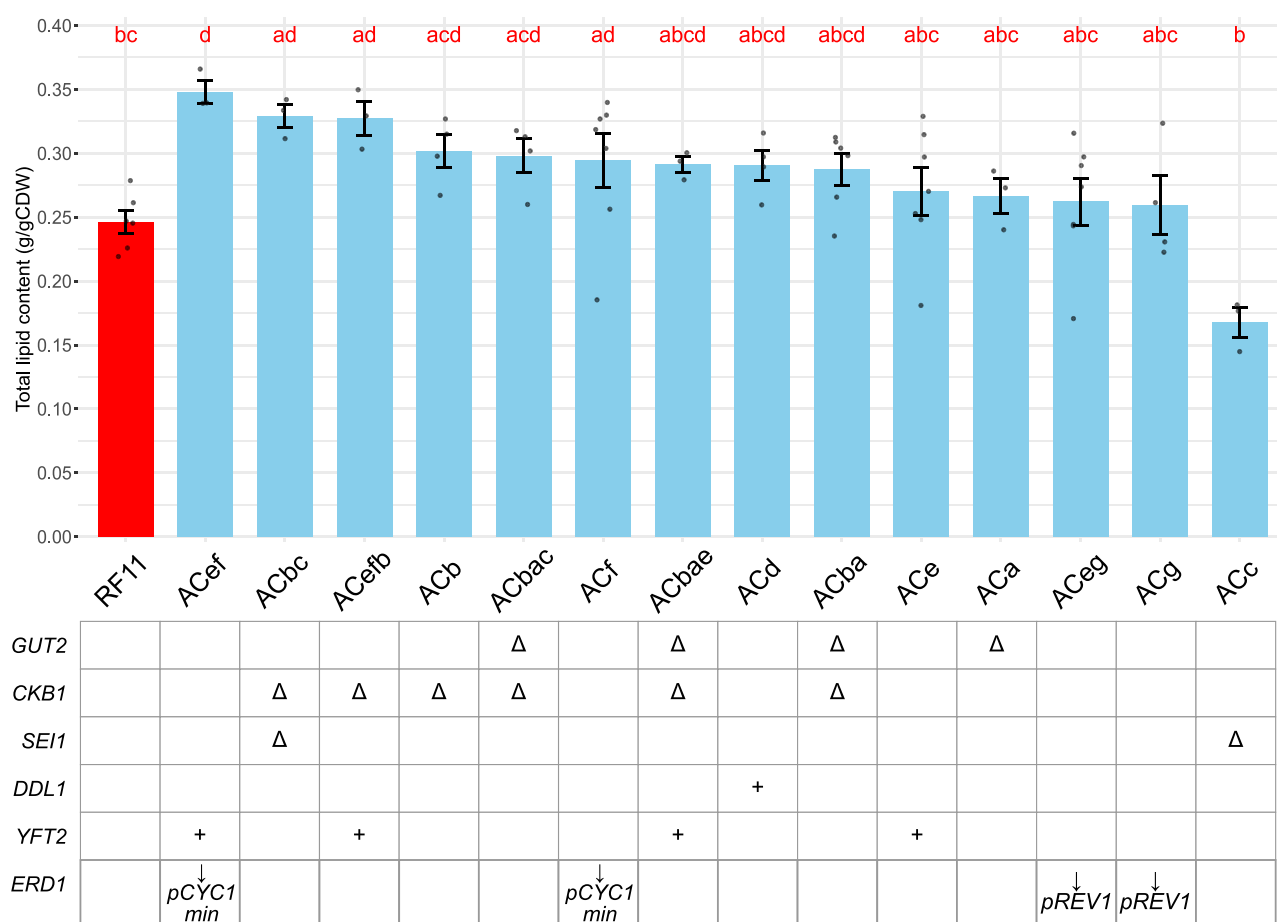


Figure 2 Total lipid weight and genotype matrix of strains developed in this study. Strains were grown in 80 ml of Delft medium supplemented with uracil, in 500-ml shake flasks until stationary phase was reached. Total lipids were extracted through the methanol-chloroform method, weighed, and ratios of dry lipid and biomass weight were calculated for each strain. Bars represent mean values, error bars represent standard error. Different red letters indicate statistically significant differences among strains, same letters indicate no significant difference. Individual data points are represented as jittered dots. Sample sizes were: RF11 ($n = 6$), ACa ($n = 3$), ACba ($n = 6$), ACC ($n = 3$), ACbac ($n = 4$), ACd ($n = 4$), ACbae ($n = 3$), ACef ($n = 3$), ACEg ($n = 7$), ACf ($n = 7$), ACE ($n = 7$), ACg ($n = 4$), ACb ($n = 4$), ACbc ($n = 3$), and ACefb ($n = 3$). Statistical significance was assessed using a Kruskal–Wallis test followed by Dunn’s multiple-comparison test with Benjamini–Hochberg correction.

has been shown to play an important role in lipid metabolism and transport of vesicles, as well as synthesis of the ER membrane in response to lipid metabolism perturbations and ER stress (Moir et al. 2012). *YFT2* was overexpressed in *S. cerevisiae* by Teixeira et al. (2018) successfully improving TAG accumulation. In this study, we integrated a second copy of the *YFT2* gene, expressed under control of the *HHF2* promoter, into the RF11 strain, resulting in strain ACe. ACe did not show significant differences in total lipid content compared to RF11. In addition to RF11, we also overexpressed *YFT2* in ACba generating the strain ACbae. Even in this background, lipid accumulation did not improve further.

It has been proven that genetic modifications that induce ER stress, such as protein-glycosylation related targets like *Erd1*, lead to increased LD formation (Fei et al. 2009, Teixeira et al. 2018). *Erd1* is a predicted membrane protein required for luminal ER protein retention that plays a key role in recycling of enzymes between Golgi compartments (Teixeira et al. 2018, Sardana et al. 2021). *Erd1* was shown to increase TAG content and LD formation in *S.*

cerevisiae when deleted, together with a slight reduction in final biomass.

Due to the potential ER stress provoked by *ERD1* deletion, which can be magnified in an extensively engineered strain such as RF11, we decided to substitute the native *ERD1* promoter with a low-activity promoter instead of completely deleting the gene. We tested the promoters *pREV1* and *pCYC1min*, generating, respectively, strains ACg and ACf. Both strains showed a higher variation in measured lipid accumulation than the rest of strains developed in this study, and a slight increase in lipid accumulation was observed for both strains compared to RF11 (Fig. 2). Only ACf showed a significant increase compared to RF11, reaching a total lipid content of 28.9% of the dry biomass weight. We suspected that the level of expression of *ERD1* under control of *pCYC1min* was more restricted than when *ERD1* was expressed using the *pREV1* promoter. This was supported by the significant reduction in the $\mu_{\max}$ for ACf compared to RF11, a growth defect that was not present in ACg. To support this hypothesis, we performed

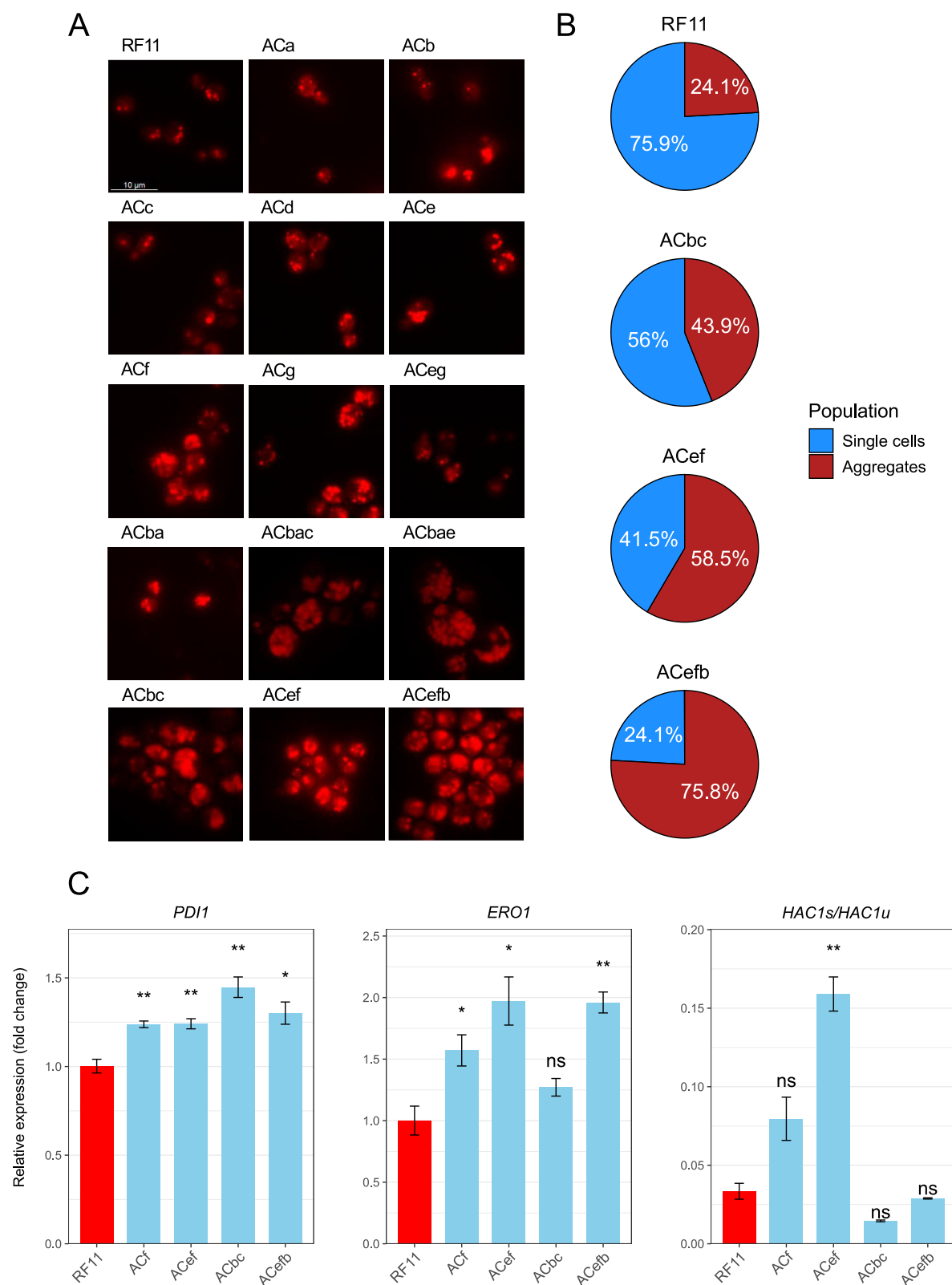


Figure 3 Analysis of phenotypical changes and ER stress response in engineered *S. cerevisiae* strains. (A) Fluorescence microscopy pictures of Nile Red-stained LDs of yeast strains. (B) Distribution of single cell and cell aggregates as percentage of total *S. cerevisiae* populations. (C) Relative expression of the ER stress response genes *PDI1*, *ERO1*, and the *HAC1s/HAC1u* ratio. Gene expression was analyzed by RT-qPCR. Expression levels were normalized to the reference gene *ACT1* and are presented relative to the reference strain RF11 using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean $\pm$ standard error of four biological replicates. Statistical significance was assessed using unpaired Welch's t-tests, with *P*-values adjusted for multiple comparisons using the Benjamini–Hochberg method. Asterisks indicate significant differences relative to RF11 (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001).

gene expression analysis of *ERD1* with RT-qPCR for strains RF11, ACg, ACf, ACef, and ACefb. Strains expressing *ERD1* under control of *pCYC1min* (ACf, ACef, and ACefb) showed significantly reduced *ERD1* expression, while *pREV1* did not significantly reduce *ERD1* expression in ACg (Supplementary Fig. S4).

It had previously been shown that lipid accumulation was enhanced when *ERD1* deletion was combined with *YFT2* overexpression (Teixeira et al. 2018). To further study the limitations of LD formation in RF11, we introduced the same promoter substitutions in ACe, generating the strains ACeg and ACef. In ACeg, lipid accumulation did not reach higher levels than in the strain with *YFT2* overexpression alone, supporting the hypothesis of an insufficient reduction in *ERD1* expression. In accordance, ACeg did not show any growth impairment, while ACef did (Supplementary Fig. S3).

Lipid accumulation in ACef increased by 41% compared to RF11, reaching 34.8% of the dry biomass weight (Fig. 2), the highest lipid weight per dry biomass obtained in this study. Thus, as observed for the *SEI1* deletion, the potential of *YFT2* overexpression to improve lipid accumulation depends on the strain background in which the modification is performed.

The fact that lipid accumulation was enhanced when *ERD1* downregulation was combined with *YFT2* overexpression may be due to a need for enhanced LD budding and maturation machinery in ACef. LD membranes can accumulate unfolded proteins to prevent aggregation (Fei et al. 2009, Welte and Gould. 2017). We speculate that LD biosynthesis could have been promoted to cope with reduced protein retention capability due to reduced *ERD1* expression. These results suggest that the protein abundance in the ER membrane requires fine tuning to improve the accumulation of lipids. Increased presence of Yft2 in a destabilized ER membrane may allow for a higher lipid accumulation by supporting the LD formation machinery.

We also tested the deletion of *CKB1* in ACef. The resulting strain, ACefb, showed a significant 32.9% increase of total lipid content compared to RF11, reaching up to 32.7% of the dry biomass weight, but no significant difference was observed compared to ACef (Fig. 2). Total TAG content was measured for the three best lipid producing strains developed in this study, ACef, ACefb, and ACbc, and although differences with the total TAG content of RF11 were not significant, a tendency towards higher TAG levels is suggested by the data (Supplementary Fig. S5). Given that total lipid content was previously shown to be significantly increased in these strains, these results suggest that the enhanced lipid accumulation is not exclusively driven by TAG but likely involves the accumulation of other lipid classes as well.

ACefb also showed the strongest supersize LD phenotype observed in this study (Fig. 3 A). Fei et al. (2011) hypothesized that changes in phospholipid composition such as increased level of phosphatidic acid in the LD membrane could promote spontaneous fusion of LDs in *S. cerevisiae*. The study also reported reduced levels of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and a tendency towards supersized LDs after deleting *CKB1*. We speculate that the effect of this previously proposed facilitation of LD fusion could be enhanced by the ER membrane destabilization and deregulation of lipid synthesis pathway, leading to the observed phenotype.

ER stress leads to the induction of the Unfolded Protein Response (UPR) through the splicing of the Hac1 transcription factor mRNA. The unspliced form of *HAC1* mRNA (*HAC1u*) under-

goes an Ire1-mediated splicing, generating the spliced form of *HAC1* mRNA (*HAC1s*), to activate the transcription of UPR target genes such as *PDI1* and *ERO1* (Patil and Walter 2001, Chen et al. 2017). This response can be induced by accumulation of misfolded proteins and alterations in the composition of lipid membranes, known as lipid bilayer stress (LBS) (Ishiwata-Kimata and Kimata 2023).

To test whether the different engineering strategies led to increased ER stress, we performed RT-qPCR analysis on selected strains (Fig. 3C). Indeed, *PDI1* expression was increased in ACf, ACef, ACefb, and ACbc, while *ERO1* expression was enhanced only in the first three strains, but not the latter. Interestingly, the *HAC1s/HAC1u* ratio was only significantly enhanced in ACef, but reduced again upon additional *CKB1* deletion (ACefb). It was previously shown in *Cryptococcus neoformans* and mammalian cells that CK2 itself can be involved in splicing of the mRNA of functional *HAC1* analogues (Choi et al. 2024, Hosoi et al. 2012).

Interestingly, ACb and all the strains developed in this study that carry the deletion of *CKB1* showed a reduced maximum specific growth rate in liquid media compared to RF11 (Supplementary Fig. S3). In addition, the final biomass of strains carrying the *CKB1* deletion was lower than the one of RF11 (Supplementary Table S3). We suggest that this is due to the highly regulatory role of CK2 in combination with the already existing modifications in RF11. The fact that no growth defect was reported when *CKB1* was deleted in other genetic backgrounds (Arhar et al. 2021) supports this theory. In addition, the increased diversion of carbon flux toward lipid biosynthesis can also play a role in the reduced final OD, especially in the highest lipid producers developed in this study such as ACefb and ACbc.

The stronger growth defect seen in ACf compared to ACg may be due to the increased ER due to the reduced expression of *ERD1*, especially in an extensively engineered strain such as RF11. Deletion of *Ckb1* in ACefb may have further increased this effect due to higher ER stress (Supplementary Fig. S3B).

Phenotypic alterations in engineered strains

When investigating changes in LD morphology with help of fluorescence microscopy, we also noticed a tendency for some of the strains to form cell aggregates (Fig. 3A). This was specifically the case for ACef, ACefb, and ACbc. In order to quantify this observation, we performed flow cytometry analysis using these three strains and RF11 as control. Measurements of the forward scatter width (FSC-W) were used to distinguish events representing single cells from event representing cell aggregates, as shown in Fig. 3B. The gating strategy is illustrated in Supplementary Fig. S1.

Flow cytometry results corroborated the microscopic observations. All three strains exhibited a significantly greater propensity for aggregate formation compared to RF11, with ACefb displaying the most pronounced effect (Fig. 3B). In ACefb, the proportions of singlet-like events and aggregates were 24.1% and 75.8%, respectively, representing an almost complete inversion of the distribution observed in RF11, which exhibited 75.9% singlets and 24.1% aggregates.

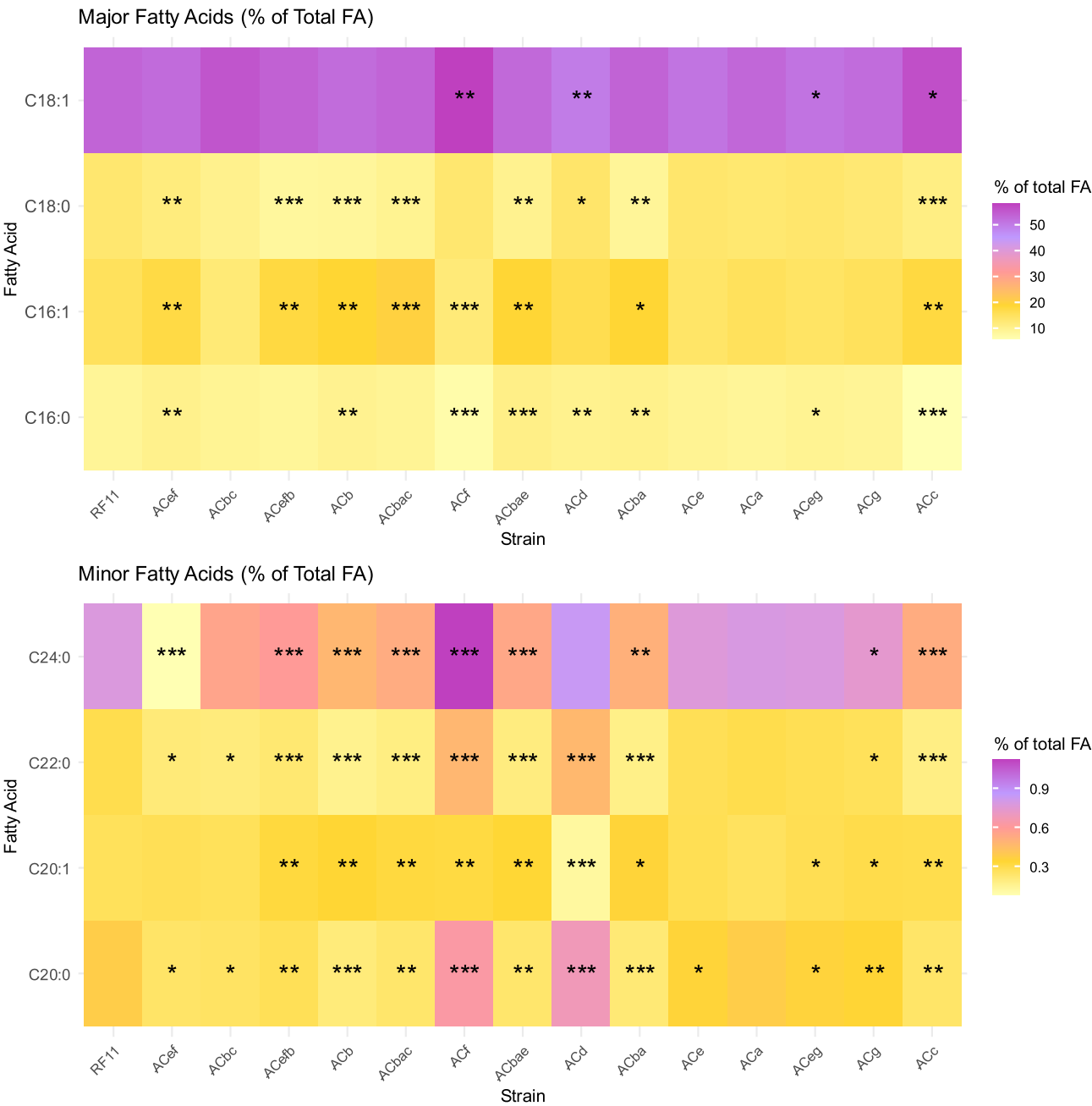


Figure 4 profile of developed strains. Relative abundance of FAs across strains presented as the percentage of each FA per total FA content of each strain. Significant differences in the content of each FA relative to RF11 are indicated by asterisks above each bar: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Bar plots with significant differences can be found in [Supplementary Fig. S1](#).

It has been previously been shown that ER membrane health is important for the correct delivery of membranes and proteins to the bud neck for cell division (Du et al. 2004). One possible explanation for the aggregation phenotype could be a defective cell division process, where membrane detachment might be incomplete. This could potentially result from a combination of factors, among which we could speculate changes in membrane detachment and maturation mechanisms associated with *SEI1* deletion, alterations in ER stability and protein retention related to changes in *YFT2* and *ERD1* expression levels, and possible effects on pathways related to phosphatidic acid

(PA) synthesis and other membrane components due to *CKB1* deletion. In line with this, CK2 has previously been implicated in eukaryotic cell division and proliferation (Guerra and Issinger 1999, Hermosilla et al. 2005), which could further support this interpretation. However, cell aggregation can also arise as a response to environmental and chemical stress in a process known as flocculation (Smukalla et al. 2008). Further research should be performed to understand the aggregation mechanisms observed in this study. The observed phenotypes may also be related to the growth defect observed in the three strains ([Supplementary Fig. S3B](#)). It is

important to consider that OD-based growth measurements may be influenced by cell aggregation due to altered light-scattering properties. In addition, the aggregation phenotype could potentially have affected lipid extraction efficiency, as cell clumping may hinder complete cell disruption, possibly leading to an underestimation of the total lipid content in these strains. Biomass estimation should not be influenced by the aggregation phenotype due to being performed gravimetrically on dry cell weight.

FA profiles of the strains developed in this study

To study potential changes in the FA profile of the developed strains, FAME analysis using GC-FID was carried out. C16:0, C16:1, C18:0, and C18:1 were the most abundant FAs in all strains, in line with previous literature (Ratray et al. 1975, Guo et al. 2018).

SEI1 deletion in ACc led to significant changes in all major and some minor FAs compared to RF11 towards an increase in monounsaturated FA content and reduction in saturated FA content in ACc compared to RF11 (Fig. 4 and Supplementary Fig. S2). Nevertheless, when *SEI1* and *CKB1* deletions were combined in ACbc, no significant changes in the major FA profile were detected, and only changes in C20:0 and C22:0 among the minor FAs were significant when compared to RF11.

Interestingly, with exception of ACbc, all strains carrying the *CKB1* deletion showed significant changes in the FA profile. Overall, strains carrying the *CKB1* deletion showed a decrease in the C18:0 content and an increase in the contents of C16:0 and C16:1. The content of C18:1 was only significantly reduced in ACbae (Fig. 4 and Supplementary Fig. S2). Regarding very-long-chain FAs, *CKB1* deletion led to a reduction in C24:0, C22:0, and C20:0, and an increase in C20:1 in all strains except ACbc (Fig. 4 and Supplementary Fig. S2). These results suggest that *CKB1* deletion modulates FA composition in RF11, likely reflecting the broad involvement of CK2 in lipid metabolic regulation and membrane homeostasis (Fresques et al. 2015, Qiu et al. 2016, Arhar et al. 2021).

ACd also showed a significantly altered content in most of the FAs, tending significantly towards a reduction of monounsaturated FAs and an increase of saturated FAs, except for the very-long-chain FA (VLCFA) C24:0 (Fig. 4 and Supplementary Fig. S2). As previously mentioned, *DDL1* influences the content of important mitochondrial and membrane phospholipids (Yadav and Rajasekharan 2016). Since phospholipid remodelling and mitochondrial metabolic activity are closely linked to FA desaturation and lipid homeostasis, *DDL1* overexpression may indirectly contribute to the altered FA profile observed in ACd.

Upon reduced expression of *ERD1* in ACf, a significant increase of C18:1 and reduction of C16:0 and C16:1 was observed. In addition, all the VLC FAs had a significantly increased content in ACf. Interestingly, *YFT2* overexpression in ACef led to a different FA profile, showing a reduction in C18:0 and most of the VLCFAs, and an increase in C16:0 and C16:1 content (Fig. 4 and Supplementary Fig. S2). This FA profile differs from the ones of ACf and ACe and supports the idea of considering the modifications of the background strain key to explain the effects of newly introduced modifications. These changes in FA profile could be expected due to the influence

of *YFT2* and *ERD1* on ER membrane stability and composition (Moir et al. 2012, Teixeira et al. 2018).

Conclusions

In this study, we developed high-lipid accumulating *S. cerevisiae* strains from the strain RF11, achieving total lipid contents of up to 34.8% of the dry biomass weight in the higher accumulating strain. Apart from the lipid synthesis pathway and its regulatory mechanisms, our results indicate that membrane synthesis and LD detachment and maturation from ER are promising targets to increase lipid production in yeast. Nevertheless, the results indicate that overly engineered yeast strains can lose viability upon further modification, highlighting the need to carefully fine-tune both the modifications and the chosen targets. An example of this is the tendency towards an aggregation phenotype of some strains (Fig. 3) and the reduction of $\mu_{\max}$ observed in some of the more heavily engineered strains developed in this study (Supplementary Fig. S3). The observed aggregation phenotype should be considered when discussing the industrial value of the developed strains, since this may cause mass transfer limitations and difficulties in process monitoring in batch cultivation settings. Nevertheless, aggregating phenotypes may be advantageous in other situations, such as facilitating biomass separation in certain cases.

Aggregation has also been proven to be advantageous in synthetic consortia of *S. cerevisiae* with sucrose-producing photosynthetic organisms, where after adaptive laboratory evolution, yeast cells tended towards aggregating phenotypes to utilize sucrose more efficiently (Koschwanetz et al. 2013).

In addition, our results demonstrate once again that the effect of genetic modifications strongly depends on the strain background. In this study, deletion of *SEI1* reduced significantly lipid accumulation and strongly altered FA profile except when combined with *CKB1* deletion, which led to a significant increase of lipid content and a less altered lipid profile compared to RF11. This supports the idea of the need for fine tuning of the engineering targets chosen, as well as the need for deeper understanding of regulatory mechanisms and pathway interactions to further increase lipid content in *S. cerevisiae*.

We have also shown in this study that CK2 may be required to sustain elevated *HAC1* splicing under the ER membrane stress conditions generated in ACef (Fig. 3C). The induction of *PDI1* and *ERO1* in *ckb1*Δ strains despite normalized *HAC1* splicing suggests that alternative UPR activation mechanisms are active, and further work is needed to clarify these mechanisms.

Finally, analysis of the FA profile of the produced strains shows that targets related to regulation of membrane composition and phospholipid synthesis, such as *CKB1* and *SEI1*, have a strong effect in the FA composition of the developed strains. These results suggest that, although increasing lipid accumulation in yeast is of interest, if the end goal is to tune the lipid profile for industrial production of specific products, the engineering targets must be considered for both purposes from the beginning to avoid interference between them.

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Supplementary material

Supplementary material is available at *FEMS* Journal online.

Conflicts of interest

Verena Siewers is shareholder of Melt&Marble.

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References

- Afrizal A, Hospes O, Berenschot W *et al.* Unequal access to justice: an evaluation of RSPO's capacity to resolve palm oil conflicts in Indonesia. *Agric Hum Values* 2023;**40**:291–304. <https://doi.org/10.1007/s10460-022-10360-z>.
- Arhar S, Gogg-Fassolter G, Ogrizović M *et al.* Engineering of *Saccharomyces cerevisiae* for the accumulation of high amounts of triacylglycerol. *Microb Cell Fact* 2021;**20**:147. <https://doi.org/10.1186/s12934-021-01640-0>.
- Athenstaedt K, Daum G. YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of *Saccharomyces cerevisiae*. *J Biol Chem* 2003;**278**:23317–23. <https://doi.org/10.1074/jbc.M302577200>.
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;**37**:911–17. <https://doi.org/10.1139/o59-099>.
- Brasaemle DL, Wolins NE. Packaging of fat: an evolving model of lipid droplet assembly and expansion. *J Biol Chem* 2012;**287**:2273–79. <https://doi.org/10.1074/jbc.R111.309088>.
- Chen X, Bisschops MMM, Agarwal NR *et al.* Interplay of energetics and ER stress exacerbates Alzheimer's amyloid- β (A β) toxicity in yeast. *Front Mol Neurosci* 2017;**10**:232. <https://doi.org/10.3389/fnmol.2017.00232>.
- Chen Y, Sheng J, Jiang T *et al.* Transcriptional profiling reveals molecular basis and novel genetic targets for improved resistance to multiple fermentation inhibitors in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2016;**9**:9. <https://doi.org/10.1186/s13068-015-0418-5>.
- Choi Y, Yu SR, Lee Y *et al.* Casein kinase 2 complex: A central regulator of multiple pathobiological signaling pathways in *Cryptococcus neoformans*. *mBio* 2024;**5**:e03275–23. <https://doi.org/10.1128/mbio.03275-23>.
- Dahlgren L, Jaison CA, Lindberg Yilmaz J. *et al.* Long-term storage of oat (*Avena sativa*) flour—an investigation of hydrolytic and oxidative rancidification. *Food Chem* 2026;**506**:148160. <https://doi.org/10.1016/j.foodchem.2026.148160>.
- Du Y, Ferro-Novick S, Novick P. Dynamics and inheritance of the endoplasmic reticulum. *J Cell Sci* 2004;**117**:2871–78. <https://doi.org/10.1242/jcs.01286>.
- Farkas I, Hardy TA, Goebel MG *et al.* Two glycogen synthase isoforms in *Saccharomyces cerevisiae* are coded by distinct genes that are differentially controlled. *J Biol Chem* 1991;**266**:15602–07. [https://doi.org/10.1016/S0021-9258\(18\)98448-6](https://doi.org/10.1016/S0021-9258(18)98448-6).
- Fei W, Shui G, Zhang Y *et al.* A role for phosphatidic acid in the formation of “supersized” lipid droplets. *PLoS Genet* 2011;**7**:e1002201. <https://doi.org/10.1371/journal.pgen.1002201>.
- Fei W, Wang H, Fu X *et al.* Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in *Saccharomyces cerevisiae*. *Biochem J* 2009;**424**:61–67. <https://doi.org/10.1042/BJ20090785>.
- Ferreira R, Teixeira PG, Gossing M *et al.* Metabolic engineering of *Saccharomyces cerevisiae* for overproduction of triacylglycerols. *Metab Eng Commun* 2018;**6**:22–27. <https://doi.org/10.1016/j.meten.2018.01.002>.
- Fitzherbert EB, Struebig MJ, Morel A *et al.* How will oil palm expansion affect biodiversity? *Trends Ecol Evol* 2008;**23**:538–45. <https://doi.org/10.1016/j.tree.2008.06.012>.
- Fresques T, Niles B, Aronova S *et al.* Regulation of ceramide synthase by casein kinase 2-dependent phosphorylation in *Saccharomyces cerevisiae*. *J Biol Chem* 2015;**290**:1395–403. <https://doi.org/10.1074/jbc.M114.621086>.
- Gesteiro E, Guisjarro L, Sánchez-Muniz FJ *et al.* Palm oil on the edge. *Nutrients* 2019;**11**:2008. <https://doi.org/10.3390/nu11092008>.
- Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2007;**2**:31–34. <https://doi.org/10.1038/nprot.2007.13>.
- Grippa A, Buxó L, Mora G *et al.* The seipin complex Fld1/Ldb16 stabilizes ER–lipid droplet contact sites. *J Cell Biol* 2015;**211**:829–44. <https://doi.org/10.1083/jcb.201502070>.
- Guerra B, Issinger OG. Protein kinase CK2 and its role in cellular proliferation, development and pathology. *Electrophoresis* 1999;**20**:391–408. [https://doi.org/10.1002/\(SICI\)1522-2683\(19990201\)20:2<391::AID-ELPS391>3.0.CO;2-N](https://doi.org/10.1002/(SICI)1522-2683(19990201)20:2<391::AID-ELPS391>3.0.CO;2-N).
- Guo Z, Khoomrung S, Nielsen J *et al.* Changes in lipid metabolism convey acid tolerance in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2018;**11**:297. <https://doi.org/10.1186/s13068-018-1295-5>.
- He Q, Yang Y, Yang S *et al.* Oleaginity of the yeast strain *Saccharomyces cerevisiae* D5A. *Biotechnol Biofuels* 2018;**11**:258. <https://doi.org/10.1186/s13068-018-1256-z>.
- Hermosilla GH, Tapia JC, Allende JE. Minimal CK2 activity required for yeast growth. *Mol Cell Biochem* 2005;**274**:39–46. <https://doi.org/10.1007/s11010-005-3112-2>.
- Hosoi T, Korematsu K, Horie N *et al.* Inhibition of casein kinase 2 modulates XBP1-GRP78 arm of unfolded protein responses in cultured glial cells. *PLoS ONE* 2012;**7**:e40144. <https://doi.org/10.1371/journal.pone.0040144>.
- Hsieh LS, Su WM, Han GS, Carman GM. Phosphorylation of yeast Pah1 phosphatidate phosphatase by casein kinase II regulates its function in lipid metabolism. *J Biol Chem* 2016;**291**:9974–90. <https://doi.org/10.1074/jbc.M116.726588>.
- Ishiwata-Kimata Y, Kimata Y. Fundamental and applicative aspects of the unfolded protein response in yeasts. *J Fungi* 2023;**9**:989. <https://doi.org/10.3390/jof9100989>.
- Ji J, Greenberg ML. Cardiolipin function in the yeast *S. cerevisiae* and the lessons learned for Barth syndrome. *J Inher Metab Dis* 2022;**45**:60–71. <https://doi.org/10.1002/jimd.12447>.
- Jiang F, Ryan MT, Schlame M *et al.* Absence of cardiolipin in the *crd1* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. *J Biol Chem* 2000;**275**:22387–94. <https://doi.org/10.1074/jbc.M909868199>.

- Jullesson D, David F, Pflieger B *et al.* Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. *Biotechnol Adv* 2015;**33**:1395–402. <https://doi.org/10.1016/j.biotechadv.2015.02.011>.
- Koschwanetz JH, Foster KR, Murray AW. Improved use of a public good selects for the evolution of undifferentiated multicellularity. *eLife* 2013;**2**:e00367. <https://doi.org/10.7554/eLife.00367>.
- Liu G, Bergenholm D, Nielsen J. Genome-wide mapping of binding sites reveals multiple biological functions of the transcription factor Cst6p in *Saccharomyces cerevisiae*. *mBio* 2016;**7**:e00559–16. <https://doi.org/10.1128/mBio.00559-16>.
- Mans R, van Rossum HM, Wijsman M *et al.* CRISPR/Cas9: a molecular swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2015;**15**:fov004. <https://doi.org/10.1093/femsyr/fov004>.
- Meggio F, Pinna LA. One-thousand-and-one substrates of protein kinase CK2? *FASEB J* 2003;**17**:349–68. <https://doi.org/10.1096/fj.02-0473rev>.
- Meijaard E, Abrams JF, Juffe-Bignoli D *et al.* Coconut oil, conservation and the conscientious consumer. *Curr Biol* 2020;**30**:R757–58. <https://doi.org/10.1016/j.cub.2020.05.059>.
- Moir RD, Gross DA, Silver DL *et al.* SCS3 and YFT2 link transcription of phospholipid biosynthetic genes to ER stress and the UPR. *PLoS Genet* 2012;**8**:e1002890. <https://doi.org/10.1371/journal.pgen.1002890>.
- National Institute of Standards and Technology (NIST). NIST/EPA/NIH Mass Spectral Library (NIST 20). Gaithersburg, MD. 2020.
- Nielsen J, Keasling JD. Engineering cellular metabolism. *Cell* 2016;**164**:1185–97. <https://doi.org/10.1016/j.cell.2016.02.004>.
- Norlander S, Dahlgren L, Sardari RRR *et al.* Effect of kilning on the macronutrient composition profile of three Swedish oat varieties. *Cereal Chem* 2024;**101**:382–96. <https://doi.org/10.1002/cche.10757>.
- Otto M, Skrekas C, Gossing M *et al.* Expansion of the yeast modular cloning toolkit for CRISPR-based applications, genomic integrations and combinatorial libraries. *ACS Synth Biol* 2021;**10**:3461–74. <https://doi.org/10.1021/acssynbio.1c00408>.
- Pan X, De Souza MO, Figueiras FM *et al.* Optimized single-cell gates for yeast display screening. *Protein Eng Des Sel* 2025;**38**:gzae018. <https://doi.org/10.1093/protein/gzae018>.
- Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol* 2001;**13**:349–55. [https://doi.org/10.1016/S0955-0674\(00\)00219-2](https://doi.org/10.1016/S0955-0674(00)00219-2).
- Piper MDW, Daran-Lapujade P, Bro C *et al.* Reproducibility of oligonucleotide microarray transcriptome analyses: an inter-laboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*. *J Biol Chem* 2002;**277**:37001–08. <https://doi.org/10.1074/jbc.M204490200>.
- Qiu Y, Hassaninasab A, Han GS *et al.* Phosphorylation of Dgk1 diacylglycerol kinase by casein kinase II regulates phosphatidic acid production in *Saccharomyces cerevisiae*. *J Biol Chem* 2016;**291**:26455–67. <https://doi.org/10.1074/jbc.M116.763839>.
- Rahmawati Z, Santoso L, Abdullah WNW *et al.* Biomass as an alternative feedstock to oleochemicals. *RSC Adv* 2024;**14**:28827–43. <https://doi.org/10.1039/D4RA04481A>.
- Ratray JB, Schibeci A, Kidby DK. Lipids of yeasts. *Bacteriol Rev* 1975;**39**:197–231. <https://doi.org/10.1128/br.39.3.197-231.1975>.
- Rønnow B, Kielland-Brandt MC. GUT2, a gene for mitochondrial glycerol 3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. *Yeast* 1993;**9**:1121–30. <https://doi.org/10.1002/yea.320091013>.
- Rostron KA, Lawrence CL. Nile red staining of neutral lipids in yeast. In: Pellicciari C, Biggiogera M (eds). *Histochemistry of Single Molecules: Methods in Molecular Biology*, Vol. **1560**, New York: Springer, 2017, 219–29. https://doi.org/10.1007/978-1-4939-6788-9_16.
- Runguphan W, Keasling JD. Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. *Metab Eng* 2014;**21**:103–13. <https://doi.org/10.1016/j.ymben.2013.07.003>.
- Sardana R, Highland CM, Straight BE *et al.* Golgi membrane protein Erd1 is essential for recycling a subset of Golgi glycosyltransferases. *eLife* 2021;**10**:e70774. <https://doi.org/10.7554/eLife.70774>.
- Schneider R, Daum G. Extraction of yeast lipids. In: Xiao W (ed.). *Yeast Protocol: Methods in Molecular Biology*, Vol. **313**, Totowa, NJ: Humana Press, 2006, 41–[x]. <https://doi.org/10.1385/1-59259-958-3:041>.
- Smukalla S, Caldara M, Pochet N *et al.* FLO1 is a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell* 2008;**135**:726–37. <https://doi.org/10.1016/j.cell.2008.09.037>.
- Szymanski KM, Binns D, Bartz R *et al.* The lipodystrophy protein seipin is found at endoplasmic reticulum lipid droplet junctions and is important for droplet morphology. *Proc Natl Acad Sci USA* 2007;**104**:20890–95. <https://doi.org/10.1073/pnas.0704154104>.
- Teixeira PG, David F, Siewers V *et al.* Engineering lipid droplet assembly mechanisms for improved triacylglycerol accumulation in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2018;**18**:foy060. <https://doi.org/10.1093/femsyr/foy060>.
- Thorpe RF, Ratledge C. Fatty acid distribution in triglycerides of yeasts grown on glucose or n-alkanes. *J Gen Microbiol* 1972;**72**:151–63. <https://doi.org/10.1099/00221287-72-1-151>.
- Verduyn C, Postma E, Scheffers WA *et al.* Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 1992;**8**:501–17. <https://doi.org/10.1002/yea.320080703>.
- Wang CW, Chen RH, Chen YK. The lipid droplet assembly complex consists of seipin and four accessory factors in budding yeast. *J Biol Chem* 2024;**300**:107534. <https://doi.org/10.1016/j.jbc.2024.107534>.
- Welte MA, Gould AP. Lipid droplet functions beyond energy storage. *Biochim Biophys Acta* 2017;**1862**:1260–72. <https://doi.org/10.1016/j.bbalip.2017.07.006>.
- Wilson WA, Roach PJ, Montero M *et al.* Regulation of glycolysis metabolism in yeast and bacteria. *FEMS Microbiol Rev* 2010;**34**:952–85. <https://doi.org/10.1111/j.1574-6976.2010.0220.x>.
- Wu Y, Li B, Miao B *et al.* *Saccharomyces cerevisiae* employs complex regulation strategies to tolerate low pH stress during ethanol production. *Microb Cell Fact* 2022;**21**:247. <https://doi.org/10.1186/s12934-022-01974-3>.

- Yadav PK, Rajasekharan R. Misregulation of a DDHD domain-containing lipase causes mitochondrial dysfunction in yeast. *J Biol Chem* 2016;**291**:18562–81. <https://doi.org/10.1074/jbc.M116.733378>.
- Yadav PK, Rajasekharan R. Cardiolipin deficiency causes triacylglycerol accumulation in *Saccharomyces cerevisiae*. *Mol Cell Biochem* 2017;**434**:89–103. <https://doi.org/10.1007/s11010-017-3039-4>.
- Zweytick D, Athenstaedt K, Daum G. Intracellular lipid particles of eukaryotic cells. *Biochim Biophys Acta* 2000;**1469**:101–20. [https://doi.org/10.1016/S0005-2736\(00\)00294-7](https://doi.org/10.1016/S0005-2736(00)00294-7).

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