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Model-Driven Engineering of *Yarrowia lipolytica* for Improved Microbial Oil Production

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ABSTRACT

Extensive usage of plant-based oils, especially palm oil, has led to environmental and social issues, such as deforestation and loss of biodiversity, thus sustainable alternatives are required. Microbial oils, especially from *Yarrowia lipolytica*, offer a promising solution because of their similar composition to palm oil, low carbon footprint and ability to utilise low-cost substrates. In this study, we employed the Design-Build-Test-Learn (DBTL) approach to enhance lipid production in *Y. lipolytica*. We systematically evaluated predictions from the genome-scale metabolic model to identify and overcome bottlenecks in lipid biosynthesis. We tested the effect of predicted medium supplements (glutamate, leucine, methionine and threonine) and genetic intervention targets, including the overexpression of ATP-citrate lyase (*ACL*), acetyl-CoA carboxylase (*ACC*), threonine synthase (*TS*), diacylglycerol acyltransferase(*DGA1*), the deletion of citrate exporter gene (*CEX1*) and disruption of β-oxidation pathway (*MFE1*). This work revealed the critical roles of *ACC*, *ACL*, *TS* and *DGA1* and the interaction of these genes with elevated intracellular citrate availability in lipid biosynthesis. Combining *TS* and *DGA1* overexpression in the *Δmfe_Δcex* background achieved a remarkable 200% increase in lipid content (56% w/w) and a 230% increase in lipid yield on glycerol. These findings underscore the potential of *Y. lipolytica* as an efficient microbial cell factory for fatty acid production. Our study advances the understanding of lipid metabolism in *Y. lipolytica* and demonstrates a viable approach for developing sustainable and economically feasible alternatives to palm oil.

1 | Introduction

Plant-based oils are extensively used in food, feed, chemical, personal care and cosmetic products to enhance texture, flavour and shelf-life (Holley and Patel 2005; Desbois 2012). Palm oil, in particular, is favoured as an inexpensive source of these functional components (Rustan and Drevon 2005). However, the rising demand for palm oil has led to the destruction of native tropical forests in many countries across Asia, South America and Africa, has severe consequences for local communities and contributes to climate change (Vijay et al. 2016; Abubakar, Ishak, and Makmom 2021; Murphy, Goggin, and Paterson 2021). Therefore, there is an urgent and critical need to develop sustainable alternatives to palm-based fatty acids and oils.

Microbial oils present a valuable alternative to traditional plant-based oils because of their sustainability and versatility. They can be produced using renewable resources and waste

substrates, minimising ecological impact and promoting circular economy practices. Among the oil-producing microorganisms, oleaginous yeasts contain more than 20% of their total biomass in lipids (Salvador López, Vandeputte, and Van Bogaert 2022). Oleaginous yeasts are often considered superior for commercial applications because of their fast growth, high lipid content and high volumetric productivity (Sitepu et al. 2014). The most extensively studied oleaginous yeast have been *Cutaneotrichosporon oleaginosus*, *Rhodotorula toruloides* and *Yarrowia lipolytica* (Abeln and Chuck 2021).

Yarrowia lipolytica commonly accumulates lipids up to 20%–30% of its biomass when cultured on glucose or similar carbon sources (Beopoulos et al. 2009; Abeln and Chuck 2021). Moreover, *Y. lipolytica* is non-pathogenic and regarded as food-grade yeast, thus its oil can be used for food-related applications (Zinjarde 2014; Amalia et al. 2020). Under nitrogen-limiting conditions, *Y. lipolytica* produces fatty acids comparable to that of palm, composed of 15% palmitic acid (C16:0), 13% stearic acid (C18:0), 51% oleic acid (C18:1), and 21% linoleic acid (C18:2) (Carsanba, Papanikolaou, and Erten 2018). It is able to grow and produce lipids on a wide range of substrates including agro-industrial residues and crude glycerol (Papanikolaou and Aggelis 2003; Rywińska et al. 2013; Sara, Brar, and Blais 2016; Caporusso, Capece, and De Bari 2021). Because of these advantages, this oleaginous yeast is flagged as an attractive microbial-cell factory to sustain a bio-based circular economy for industrial implementation.

Lipid synthesis in *Y. lipolytica* occurs via *de novo* synthesis by metabolising hydrophilic substrates (Fabiszewska et al. 2019). When nitrogen is limited in the medium, the excess carbon is directed to fatty acid synthesis via channelling mitochondrial citrate to the cytoplasm. ATP-citrate lyase (*ACL*) converts citrate into acetyl-CoA and oxaloacetate then acetyl-CoA carboxylase (*ACC*) converts acetyl-CoA to malonyl-CoA that is the primary precursor for fatty acid elongation (Fabiszewska et al. 2019; Poontawee et al. 2023). Followed by fatty acid elongation, synthesised acyl-CoA chains are finally incorporated into triacylglycerol (TAG) by diacylglycerol acyltransferase (*DGA1*).

Several research groups worked on strategies for increasing lipid accumulation in *Y. lipolytica* and developed metabolic engineering tools and strategies mainly focused on maximising the flux toward lipid biosynthesis (Larroude et al. 2019; Wang et al. 2020). This was achieved by increasing the availability of precursors such as acetyl-CoA. Tai and Stephanopoulos 2013 explored the push and pull strategy for lipid accumulation by overexpression of *ACC1* and *DGA1*. Overexpression of *ACL* from *Mus musculus* in *Y. lipolytica* enhanced the citrate conversion to acetyl-CoA resulting in higher lipid contents (Zhang et al. 2014). Additionally, the secretion of citric acid to the extracellular environment is identified as one of the main limitations on the utilisation of intracellular citrate for lipid biosynthesis especially when *Y. lipolytica* grows on glycerol (Moeller et al. 2011; Sagnak et al. 2018; Wang et al. 2020). Recently, (Erian et al. 2020) identified the first citrate exporter of *Y. lipolytica*. On the other hand, the interplay between citrate secretion, intracellular citrate availability and lipid accumulation in *Y. lipolytica* has not been assessed systematically. Furthermore minimising the flux toward one of the competing metabolic pathways was achieved

by deleting genes related to the β-oxidation pathway, which degrades the intracellular fatty acids, (Dulermo and Nicaud 2011; Blazeck et al. 2013). Blazeck et al. (2014), combined the overexpression of *DGA1* and deletion of *PEX10* and *MFE1*, genes responsible for the β-oxidation of fatty acids, coupled with leucine biosynthetic capacity which improved the lipid accumulation (Blazeck et al. 2014). In addition to these strategies, (Kim et al. 2019) analysed the genome-scale metabolic model (GEM) of *Y. lipolytica* and successfully predicted some of the established genetic engineering strategies for higher lipid accumulation including overexpression of diglyceride acyltransferase and *ACC*, and knockout of reactions involved in one-carbon/methionine metabolism to boost lipid production. Although the main limitations leading to lower lipid accumulation levels in *Y. lipolytica* were highlighted over the last two decades, these bottlenecks have not been addressed systematically.

In this study, we followed the Design-Build-Test-Learn (DBTL) approach, a streamlined method for iterating the steps of strain development. This approach integrates systems biology and metabolic engineering to develop *Y. lipolytica* into a sustainable and more productive fatty acid production platform. We intertwined the predictions from the GEM of *Y. lipolytica* with previously addressed bottlenecks for improved lipid accumulation and ultimately defined an efficient strain design strategy. The identified genetic intervention strategy was experimentally validated with iterations in the built and test step.

2 | Experimental Procedures

2.1 | Comparative Flux Sampling Analysis Using the GEM

We used Comparative Flux Sampling Analysis (CFSA) to identify suitable strategies by using the iYali4, v4.1.2 model (Kerkhoven et al. 2016; van Rosmalen et al. 2024). In brief, the model was used to simulate scenarios of maximum growth, maximum lipid production and slow growth. In each scenario flux sampling was used to characterise the metabolic space and reactions with the highest changes between scenarios were selected as targets for further inspection as described by (van Rosmalen et al. 2024). The N-limiting medium was adjusted by using glycerol exchange (y001808) and urea exchange (y002091) reactions. CFSA was performed (number of samples $= 30{,}000$, optimality $= 0.90$, flux fraction $= 1.25$, $KS1 = KS2 \geq 0.75$, mean absolute change ≥ 0.01 and standard deviation in production ≤ 50) using the lipid exchange reaction (xlipid_export) as a target for the production scenario and xBIOMASS for growth scenario. CFSA is available at GitLab.

2.2 | Strains, Media and Growth Conditions in Shake-Flask

Yarrowia lipolytica strains used in this study were derived from wild-type *Y. lipolytica* CBS8108 strain obtained from Westerdijk Fungalbio Diversity Institute (Utrecht, The Netherlands) and maintained on Yeast extract Peptone Dextrose (YPD) agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar. The maintained cultures were stored at 4°C

for up to a week. *Escherichia coli* Zymo 10B (Zymo Research, Orange, CA) was used for all cloning purposes throughout this study and maintained on Luria-Bertani (LB) agar (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 15 g/L agar) with ampicillin (100 μg/mL) at 37°C.

The inoculum was prepared by transferring a single colony of *Y. lipolytica* into 10 mL YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L) in 50 mL tubes and incubated at 30°C, 250 rpm for 18 h in a shaking incubator. Wild-type and other built *Y. lipolytica* transformants were cultivated into minimal media consisting of glycerol as carbon source and urea as nitrogen source with set ratios of C/N 140 (g/g) (C/N 163 (mol/mol)) (Duman-Özdamar et al. 2022). Methionine (2 mM), threonine (2 mM), leucine (2 mM) and glutamate (2 mM) were added into C/N 140 (g/g) cultivation medium, and only wild-type was tested in these experiments. Cultures were incubated at 30°C, 250 rpm for 120 h in a shaking incubator. Cells were harvested at the end of incubation and centrifuged at 1780 g, 4°C for 20 min. All experiments were performed in triplicates.

2.3 | Plasmid Construction and Preparation for Transformation

Restriction enzymes and Q5 High-Fidelity DNA polymerase used in cloning were obtained from New England Biolabs (Ipswich, MA). Genomic DNA (gDNA) of *Y. lipolytica* was prepared using YeaStar Genomic DNA Kit (Zymo Research, Irvine, CA). PCR products and DNA fragments were purified with GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA). The primers and plasmids used are described in Tables S1 and S2, respectively. Assembly of the plasmids was performed by using NEBridge Golden Gate Assembly Kit (BsaI-HF v2) (New England Biolabs, Ipswich, MA). All constructed plasmids were verified by whole plasmid sequencing (Eurofins, Germany).

ACL1, ACC containing introns and threonine synthase (*TS*) genes from *C. oleaginosus* were amplified from pUC57-ACL, pUC57-ACC and pUC57-TS plasmid (Duman-Özdamar et al. 2024) by using primers ACL_BsaI_D_Fw and ACL_BsaI_E_Rv, ACC_BsaI_D_Fw and ACC_BsaI_E_Rv, TS_BsaI_D_Fw and TS_BsaI_E_Rv, respectively (Table S1). Diacylglycerol acyltransferase 1 gene (*DGA1*, Accession Number: XM_504700) and homologous upstream and downstream parts of the citrate exporter gene of *Y. lipolytica* (*CEX1*, Accession Number: XM_503062.1) were amplified from the gDNA by using primers DGA_D_BsaI_Fw and DGA_E_BsaI_Rv, Cex1Up_BsaI_A_Fw and Cex1Up_BsaI_B_Rv, Cex1Down_BsaI_C_Fw and Cex1Down_BsaI_M_Rv, Cex1Down_BsaI_L_Fw and Cex1Down_BsaI_M_Rv. Amplified parts were cloned into the pCR-Blunt vector using Zero Blunt PCR Cloning Kit by following the instructions from the supplier (Invitrogen, Waltham, MA). These amplified parts were assembled with the parts from the *Yarrowia lipolytica* Golden Gate tool kit (Addgene kit #1000000167) by facilitating BsaI restriction sites; promoter (pCR4Blunt-TOPO- P1 TEF-8UAS), terminator (pCR4Blunt-TOPO-TLip2 (E-L)), two markers for antibiotic resistance (hygromycin, pCR4Blunt-TOPO-M-hph, and nourseothricin, pCR4Blunt-TOPO-M-Nat), MFE homologous sites with NotI restriction sites (pCR4Blunt-TOPO-MFE-NotI_Up,

pCR4Blunt-TOPO-MFE-NotI_Down) and backbone plasmid (pSB1A3) (Larroude et al. 2019).

Assembled plasmids were transformed into *E. coli* Zymo 10B cells (Cat #T3020; Zymo Research, Irvine, CA, The US) by following the supplier's instructions, and the transformed strains were stored at −80°C. *E. coli* cells were grown overnight in 10 mL LB broth with 100 μg/mL ampicillin or 50 μg/mL kanamycin in 50 mL falcon tubes shaking at 250 rpm at 37°C. Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Cat #K0503; Thermo Fisher Scientific, MA, The US). Isolated plasmids were prepared for transformation by releasing the assembled cassette cut with NotI restriction enzyme according to the supplier's instructions (NEB, Ipswich, MA, The US).

2.4 | Transformation and Selection for Transformants

Electrocompetent *Y. lipolytica* cells were prepared by following the protocol established by (Duman-Özdamar et al. 2024). Approximately 1 μg linearized vector was mixed with 50 μL electrocompetent *Y. lipolytica* cells and incubated on ice for 5 min. Electroporation was performed using a pulse of 0.8 kVolt, 1000 Ohm, 25 μFarad (Bio-Rad, CA, The US) in 2 mm electroporation cuvettes. 1 mL of YPD broth was added immediately after pulsing. The cells were transferred to a 2 mL Eppendorf tube, and incubated for 2.5 h at 30°C, gently mixing the cells every 30 min by inversion. 100 μL cells were spread onto YPD agar plate containing 300 μg/mL nourseothricin and/or 200 μg/mL hygromycin for primary selection. The negative control was plated on YPD agar. Incubation of the plates was done at 30°C for 48 h. Grown colonies were randomly selected and streaked into a YPD agar plate including 400 μg/mL nourseothricin and/or 300 μg/mL hygromycin for secondary selection. Transformants were confirmed via colony PCR. The primers in Table S1 and DreamTaq Green PCR Master Mix (2×) were used by following the instructions from the supplier (Thermo Fisher Scientific, MA, The US, #K1081).

2.5 | Analytical Methods

The growth of *Y. lipolytica* strains was monitored by measuring the OD_{600}. Measured absorbance was converted into dry cell weight for *Y. lipolytica* as explained by Duman-Özdamar et al. (2022).

The total fatty acids were determined quantitatively with a gas chromatograph (GC), 7830B GC systems (Aligent, Santa Clara, CA, The US) equipped with a Supelco Nukol 25,357 column (30 m × 530 × 1.0 μm; Sigma-Aldrich, St. Louis, MO, The US), hydrogen as a carrier gas. Samples were prepared as described by Duman-Özdamar et al. (2022). Chloroform was evaporated under nitrogen gas and the remaining lipid in the tubes was dissolved in hexane before GC analysis.

Glycerol, citric acid, erythritol, mannitol and arabitol were determined via HPLC analysis (Waters Alliance e2695, Milford, MA) with an RSpak KC811 column (ID = 8 mm, length = 300 mm, Shodex, NY) with a guard column RSpak KC-G (ID = 6 mm and

the length = 50 mm, Shodex, NY). The column was operated at 60°C with 3 mM H_2SO_4 as the mobile phase and a flow rate of 0.1 mL/min for 20 min. Peaks for components were detected and quantified with a refraction index detector (2414 RI Detector, Waters, Milford, MA). Peak integration and other chromatographic calculations were performed using Empower 3 software (Waters, Milford, MA). Identification and quantification of the corresponding compounds were achieved via comparisons to the standard curves (Figure S1).

2.6 | Regression Model

All computational analysis was performed with R version 4.0.2 (R Core Team 2020). The relationship between the responses (Y), factors (x), and selected two-factor interactions were expressed by fitting a linear regression: $Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j$. β_o represents the interception coefficient, β_i is the linear coefficient

and β_{ij} is the interaction coefficient. The quality of the regression equations was assessed according to the coefficient of determination (R^2). Statistical analysis of the model was performed using analysis of variance (ANOVA) and $p < 0.05$ was considered significant.

3 | Results

3.1 | Design

In the Design step of the DBTL approach (Figure 1), we evaluated the metabolic capabilities, and selected genes affecting lipid accumulation in *Y. lipolytica* for subsequent modulation, and designed experiments for the characterisation of newly built strains.
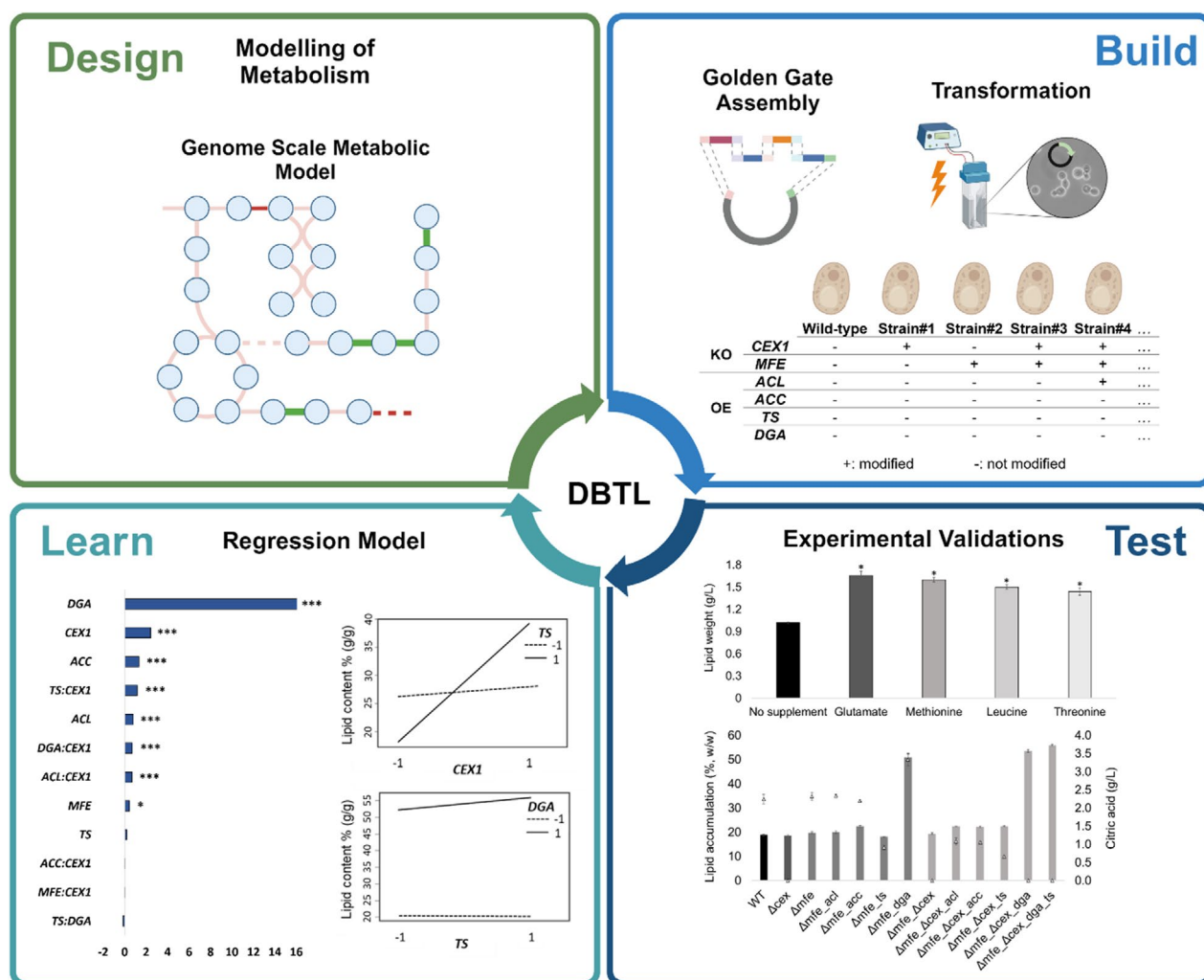

FIGURE 1 | Overview of the DBTL approach Design: We analysed the GEM of *Y. lipolytica* (*i*Yali4, v4.1.2) to identify a genetic intervention strategy for enhancing the lipid content. Build: Plasmids were constructed via the golden gate assembly approach and confirmed via whole plasmid sequencing. Predicted target genes were overexpressed or knocked out via homologous recombination. Test: Predicted genetic interventions from the amino acid synthesis pathway were tested with wild-type (WT) by supplementing the corresponding amino acids to validate predictions and establish an efficient medium for increased lipid accumulation. Finally, the performance of the built transformants were characterised at C/N 140 (g/g) minimal medium. Learn: Regression models were fitted to evaluate the main effects and two-factor interactions of genetic interventions on lipid content.

3.1.1 | Comparative Flux Sampling Analysis on GEM

CFSA on the GEM model was performed to investigate genetic engineering strategies for improved lipid synthesis with *Y. lipolytica*. Flux distributions for each reaction were evaluated by simulating maximum production, and maximum growth (van Rosmalen et al. 2024). As a result, CFSA highlighted 70 overexpression targets (that could be clustered in 35 groups), 19 knock-out candidates (belonging to 17 groups), and 21 knock-down targets (belonging to 10 groups) (a complete list of targets and distribution plots are available at GitLab).

Reactions from the fatty acid synthesis and fatty acid elongation pathway, (fatty-acyl-CoA synthase [*ACS*] and stearoyl-CoA desaturase [*SCD*]) were suggested as overexpression targets (Table 1). Additionally, targets from pyruvate metabolism providing acetyl-CoA, (pyruvate kinase [*PK*], pyruvate decarboxylase [*PD*], acetaldehyde dehydrogenase [*ALDH*] and acetyl-CoA synthase [*ACS*]) and *ACC*, which converts acetyl-CoA to malonyl-CoA, were predicted as overexpression targets. Furthermore, several reactions from the phosphate pathway (PPP) (i.e., glucose 6-phosphate dehydrogenase (*G6PD*) and phosphogluconate dehydrogenase [*PGD*]

TABLE 1 | Selected genetic interventions (overexpression, knock down, knock-out) predicted to increase the lipid production of *Y. lipolytica* using CFSA on the genome-scale metabolic model. The complete list of predicted targets is available at GitLab.

Intervention	Pathway	Target name
Overexpression	Fatty acid biosynthesis	Acetyl-CoA C-acetyltransferase
		Acetyl-CoA carboxylase
	Fatty acid elongation	Oleoyl-CoA desaturase
		Fatty-acyl-CoA synthase
		Stearoyl-CoA desaturase
	Pyruvate metabolism	Acetaldehyde dehydrogenase
		Acetyl-CoA synthetase
		Pyruvate decarboxylase
	Pentose phosphate pathway (oxidative)	6-phosphogluconolactonase
		Glucose 6-phosphate dehydrogenase
		Phosphogluconate dehydrogenase
	Pentose phosphate pathway (non-oxidative)	Ribulose 5-phosphate 3-epimerase
		Transketolase 1, transketolase 2
	Glycolysis	Glucose-6-phosphate isomerase
		Glyceraldehyde-3-phosphate dehydrogenase
		Phosphoglycerate kinase
		Triose-phosphate isomerase
		Enolase
		Phosphoglycerate mutase
		Pyruvate kinase
	Amino acid metabolism	Glutamate synthase
		Methionine synthase
		Methionine adenosyltransferase
Knock-down		2-isopropylmalate synthase
		L-threonine deaminase
Knock-out		Acetolactate synthase
		2-isopropylmalate hydratase
		Glutamate dehydrogenase (NADP)
	One carbon pool by folate	Formate-tetrahydrofolate ligase
		Methenyltetrahydrofolate cyclohydrolase

providing NADPH, ribulose 5-phosphate 3-epimerase [*RPE1*] and transketolase [*TKL1*, *TKL2*]) were listed as overexpression targets. (Wasylenko, Ahn, and Stephanopoulos 2015) reported the oxidative PPP as a primary NADPH source for lipid synthesis and (Dobrowolski and Mirończuk 2020) tested the increased NADPH availability via overexpression of *TKL1* and *DGA1* and reported around 1.5-fold increase in lipid content of *Y. lipolytica*.

CFSA predicted genetic intervention targets from amino acid metabolism including glutamate synthase (*GltS*) and methionine synthase (*MS*) for overexpression (Table 1). In addition, knock-out (acetolactate synthase [*ALS*], 2-isopropylmalate hydratase [*IPMS*]) and knock-down targets (2-isopropylmalate synthase [*LeuA*], L-threonine deaminase [*TD*]) related to threonine and leucine metabolism were highlighted. Lastly, reactions involved in one-carbon/methionine metabolism, methenyltetrahydrofolate cyclohydrolase (*MTHFC*) and formate-tetrahydrofolate ligase (*FTHFL*) were predicted as knock-out targets.

Subsequently, the predictions from the metabolic model intertwined with previous knowledge obtained for *Y. lipolytica* and *C. oleaginosus* (Duman-Özdamar et al. 2024). Combining all the outcomes led to the identification of amino acid supplements (glutamate, methionine, threonine and leucine), and four overexpression targets (*ACL1*, *ACC*, *TS* of *C. oleaginous* and *DGA1* of *Y. lipolytica*) (Figure 2). CFSA or analysis of GEMs in general will not predict targets from competing pathways as their activity is not considered under the optimality conditions the model is set to operate. Therefore we additionally investigated the metabolism to investigate competing mechanisms such as the β-oxidation pathway and citrate secretion to the extracellular environment (Blazeck et al. 2013; Madzak 2021). Selected overexpression targets were combined with the knock-out of citrate exporter protein (*CEX1*) (Odoni et al. 2019; Erian et al. 2020), and knock-out of a multifunctional enzyme (*MFE*) catalysing the second step of the β-oxidation pathway (Liu et al. 2021).

3.2 | Build

In the Build step, selected, genetic interventions were implemented (Figure 1). Plasmids were assembled using Golden Gate Assembly and confirmed via whole plasmid sequencing. Overexpression of *ACL*, *ACC*, *TS* (from *C. oleaginosus*) and *DGA1* (from *Y. lipolytica*) was achieved by knocking out *CEX1*, and/or *MFE1* via homologous recombination. In total, we constructed 12 strains in which we knocked out *CEX1*, and *MFE1* individually and in combination (Figure 3A). After characterising the background strains, we overexpressed the selected targets on *Δmfe* and *Δmfe_Δcex* background strains (Figure 3B,C). Integration of the genes was confirmed via colony PCR (Figure S2).

3.3 | Test

In the test step, we validated predictions from the amino acid synthesis pathway obtained on the design step via supplementation and observed the effect on the performance of *Y. lipolytica*.
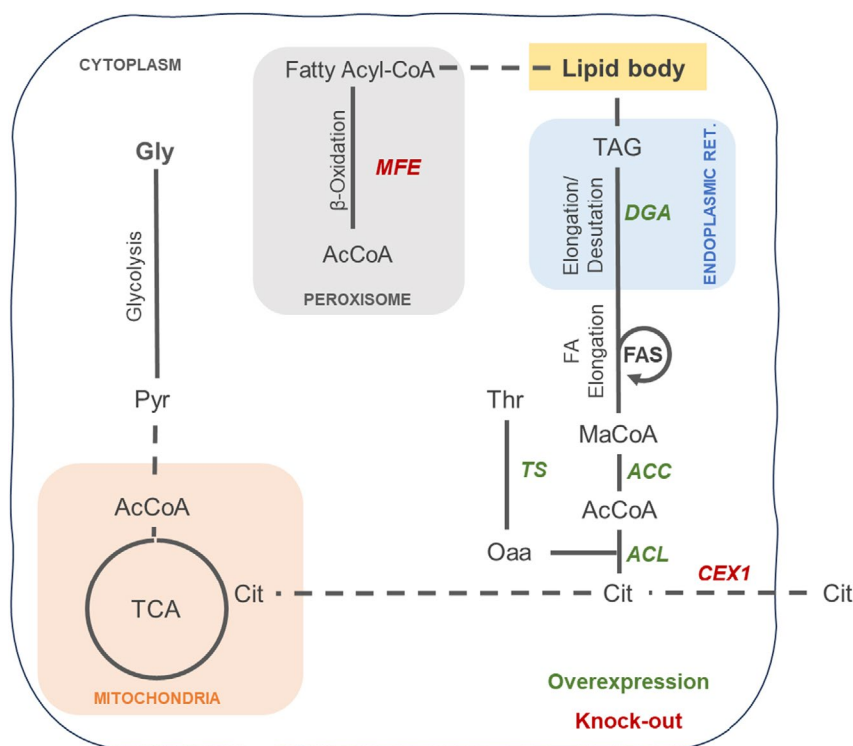

FIGURE 2 | Summary of selected genetic interventions for improved lipid production in *Y. lipolytica*. FA, fatty acids; TCA, tricarboxylic acid cycle; Metabolite abbreviations: AcCoA, Acetyl-coenzyme A; Cit, citrate; Gly, Glycerol; MaCoA, malonyl-coenzyme A; Oaa, oxaloacetate; Pyr, Pyruvate; TAG, triacylglycerols; Thr, threonine.
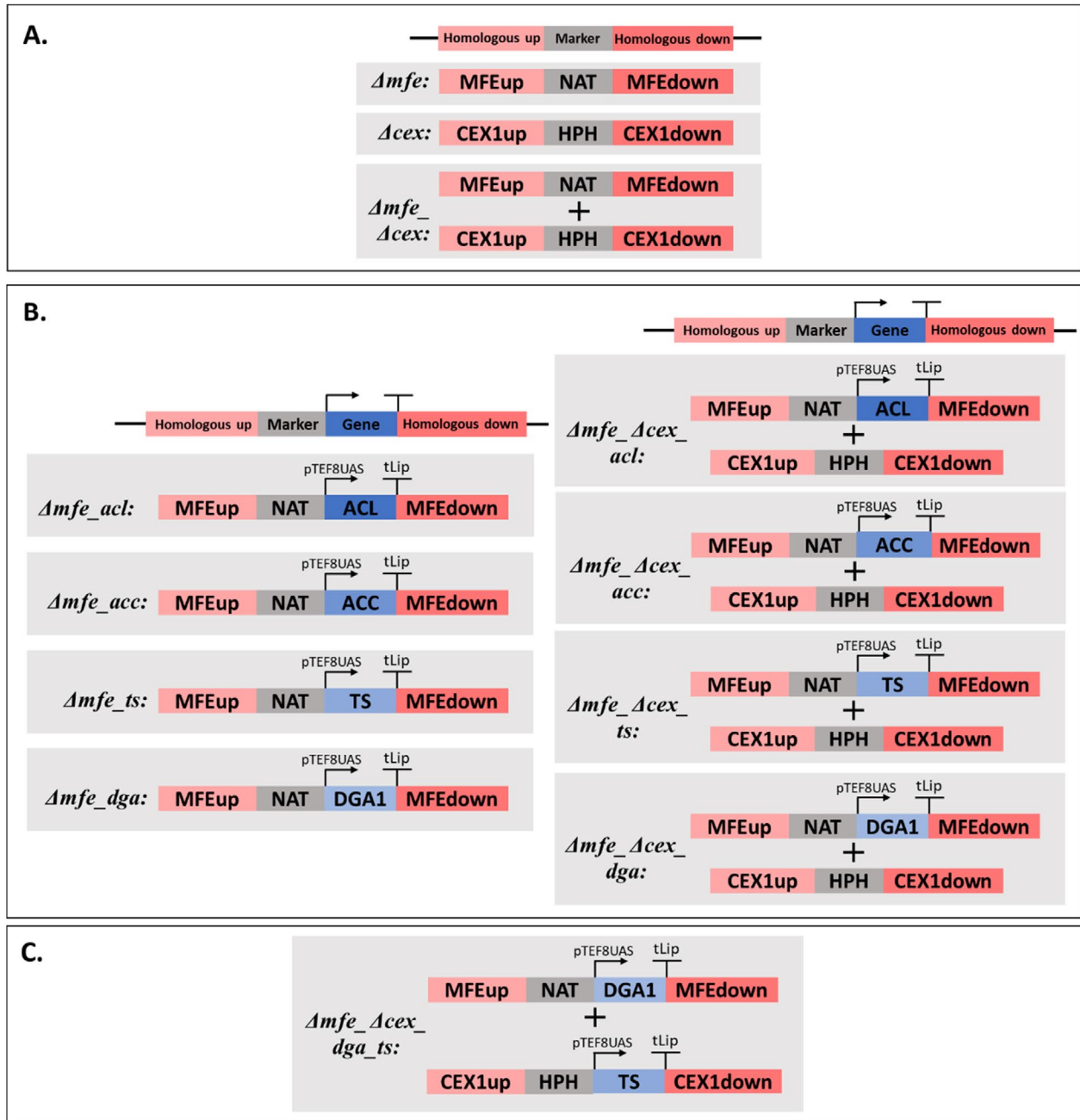
FIGURE 3 | Overview of built step. (A) Build 1: Background strains, *Δcex*, *Δmfe* and *Δmfe_Δcex*, were built by transforming the assembled plasmids. (B) Build 2: Single overexpression strains were built by overexpressing the selected targets (*ACL*, *ACC*, *TS* and *DGA1*) in *Δmfe* and *Δmfe_Δcex* background strains. (C) Build 3: Double overexpression strain was built by overexpression of *DGA1* and *TS* in *Δmfe_Δcex*.

Furthermore constructed transformants in the build step were characterised at C/N 140 (g/g) (C/N 163 mol/mol) cultivation medium containing glycerol as a carbon source and urea as a nitrogen source (Figure 1).

3.3.1 | Testing Amino Acid Supplements

The predictions from the metabolic model related to the amino acid metabolism were experimentally validated by supplementing methionine, threonine, leucine and glutamate into a nitrogen-limited cultivation medium (C/N140 g/g)

(Duman-Özdamar et al. 2022). Although these additions did not alter the lipid accumulation, total lipid production was 1.5-fold increased on average due to higher biomass concentrations (Table 2). Furthermore, biomass yield and lipid yield on glycerol were improved by around 1.6-fold with amino acid supplements. The addition of glutamate and threonine reduced extracellular citric acid by around 1.5-fold whereas it decreased by 1.6-fold with methionine and 2-fold with the addition of leucine. Supplementing amino acids did not affect the total content of saturated and unsaturated fatty acids, however, we observed around 7.5% shift of polyunsaturated fatty acids (PUFAs) to monounsaturated fatty acids (MUFAs) (Table S3).

TABLE 2 | Lipid content, dry cell weight, biomass and lipid yield on consumed glycerol of *Y. lipolytica*, and extracellular citrate concentrations upon supplementation of glutamate, methionine, leucine, or threonine into C/N140 (g/g) minimal medium (at 120h).

Supplement	Dry cell weight (g/L)	Lipid content (%, w/w)	$Y_{X/S}$ (g dcw/ g consumed glycerol)	$Y_{P/S}$ (g lipid/ g consumed glycerol)	Citric acid (g/L)
No supplement	5.38 ± 0.14	18.94 ± 0.34	0.14 ± 0.004	0.026 ± 0.001	2.41 ± 0.13
Glutamate	$9.34 \pm 0.55*$	17.85 ± 0.66	$0.23 \pm 0.004*$	$0.042 \pm 0.001*$	$0.92 \pm 0.02*$
Methionine	$8.80 \pm 0.21*$	18.20 ± 0.50	$0.22 \pm 0.011*$	$0.041 \pm 0.001*$	$1.49 \pm 0.02*$
Leucine	$8.24 \pm 0.29*$	18.18 ± 0.32	$0.21 \pm 0.006*$	$0.038 \pm 0.001*$	$1.13 \pm 0.04*$
Threonine	$7.74 \pm 0.23*$	18.63 ± 0.68	$0.21 \pm 0.004*$	$0.039 \pm 0.002*$	$0.94 \pm 0.03*$

Note: Differences with control (no supplement) were evaluated using a *t*-test.
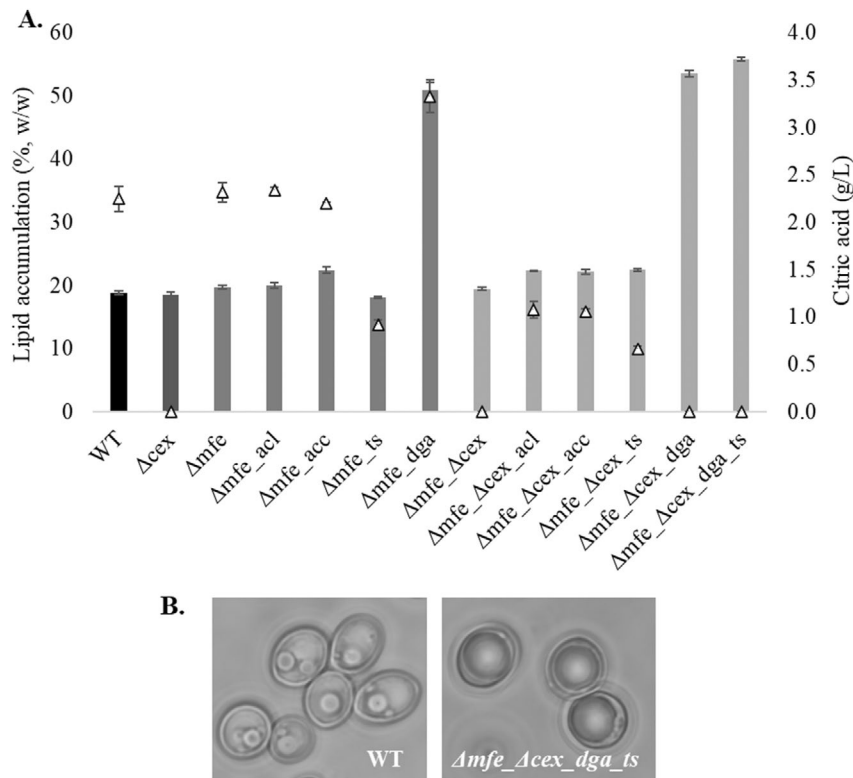*$p \leq 0.05$.

FIGURE 4 | (A) Lipid accumulation % (w/w) (bars), and extracellular citric acid (g/L) (triangles) of WT and *Y. lipolytica* transformants at C/N140 (g/g) minimal medium at 120h. (B) Microscope images of WT and *Δmfe_Δcex_dga_ts* at 120h showing that *Δmfe_Δcex_dga_ts* visibly more saturated with lipids.

3.3.2 | Characterisation of Constructed *Y. lipolytica* Transformants

Background strains (*Δcex*, *Δmfe* and *Δmfe_Δcex*) and the overexpression of *ACL1*, *ACC*, *TS* and *DGA* with *Δmfe* and *Δmfe_Δcex* backgrounds were characterised at C/N 140 (g/g) medium, which was identified as optimum C/N ratio for WT, in order to evaluate the effect of genetic interventions on lipid content, growth and extracellular citric acid concentration (Figure 4A) (Duman-Özdamar et al. 2022).

Secretion of citrate was blocked successfully with the knockout of *CEX1*, whereas there was no significant effect on lipid

content, lipid weight and dry cell weight (Table S4). We obtained a slight increase in lipid accumulation in *Δmfe* and *Δmfe_Δcex* compared to WT.

Overexpression of *ACL* resulted in a lipid content of 22.4% (w/w) when with simultaneous knock-out of *MFE1* and *CEX1*, which represents a 15% increase compared to *Δmfe_Δcex*. Although *Δmfe_acc* and *Δmfe_Δcex_acc* accumulated around 22% (w/w) lipids and provided around 14% increase with respect to WT, a reduction in the lipid content was observed with *Δmfe_ts* (18.1%, w/w). On the other hand, *Δmfe_Δcex_ts* enhanced the lipid accumulation by 15% (22.45%, w/w). Although there was no extracellular citrate measured with

Δmfe_Δcex, we detected a leakage of citrate in *Δmfe_Δcex_acl*, *Δmfe_Δcex_acc* and *Δmfe_Δcex_ts* experiments which was declined approximately 2-fold compared to WT, *Δmfe_acl*, *Δmfe_acc* and *Δmfe_ts*. In addition to citrate, mannitol (for all strains), erythritol (for all strains excluding *Δmfe_acl*) and arabitol (only *DGA1* overexpressed strains) were detected at 120 h (Table S5).

The overexpression of *DGA* provided a sharp increase in lipid production, *Δmfe_dga* produced 51% (w/w) with around 3-fold increase in total lipid (g/L), *Δmfe_Δcex_dga* accumulated 53.5% (w/w) lipids with 3.3-fold improvement in total lipid (g/L). There was no extracellular citrate detected with *Δmfe_Δcex_dga*, while *Δmfe_dga* secreted 1.4-fold higher citrate compared to *Δmfe*. Lastly, we overexpressed the *TS* and *DGA1* in the *Δmfe_Δcex* strain by considering improved lipid content and declined extracellular citrate concentrations in *Δmfe_dga*, *Δmfe_Δcex_dga* and *Δmfe_Δcex_ts* strains. The ultimate increase was obtained with *Δmfe_Δcex_dga_ts* accumulated 56% (w/w) lipids (Figure 4B), which is a 2.8-fold increase in lipid content and a 3-fold improvement in $Y_{P/S}$ compared to WT. As a result of these improvements, the total lipid (g/L) was enhanced by 3.35-fold compared to WT.

Regarding the fatty acid composition of accumulated lipids, the knock-out of *MFE1* resulted in a lower content of very long-chain fatty acids (VLCFAs) in combination with all overexpressed genes and knock-out of *CEX1* compared to WT (Table S6). When *ACC* and *TS* overexpressed in *Δmfe* and *Δmfe_Δcex*, the content of saturated fatty acids (C16:0, C18:0) decreased by around 8%, and the content of MUFAs (C16:1, C18:1) increased by around 10%. Furthermore, *Δmfe_Δcex_acl* overexpression produced 7% lower saturated fatty acids and 8% higher MUFAs. On the other hand, we obtained the highest content of saturated fatty acids (on average 33.5%) with *Δmfe_dga*, *Δmfe_Δcex_dga* and *Δmfe_Δcex_dga_ts*, while the content of MUFAs was around 12% higher compared to WT.

3.4 | Learn

In the Learn step, we evaluated the impact of the tested genetic interventions both as main effects and selected 2-factor interactions (2Fi, *MFE:CEX1*, *ACL:CEX1*, *ACC:CEX1*, *TS:CEX1*, *DGA:CEX1* and *TS:DGA*) on the lipid content by fitting a second-order polynomial equation (Figure 1). ANOVA was conducted to evaluate the statistical significance and suitability of the model and the quality of the model fit was assessed using the coefficient of determination ($R^2 = 99.89\%$), and the significance was confirmed via $p < 2.2e\text{-}16$ (Table S7).

The results highlighted the significant and positive effect of *DGA1*, *ACL*, *ACC* overexpression, *CEX1*, and *MFE1* knock-out on lipid content (Figure 5A). Among the indicated main effects, the greatest impact was observed by the modification of *DGA1* followed by *CEX1*. The outputs of the regression analysis showed positive significant interactions of *TS:CEX1* nevertheless, the main effect of *TS* and 2Fi of *TS:DGA* is insignificant on lipid content (Figure 5B). Furthermore, the model demonstrated a positive significant effect of *ACL:CEX1* and *DGA:CEX1*.

4 | Discussion

An effort has been made to elucidate the lipid accumulation mechanism of *Y. lipolytica*. Developed genetic engineering tools, available GEM models and it is analysis for providing genetic engineering strategies enable a systematic approach to
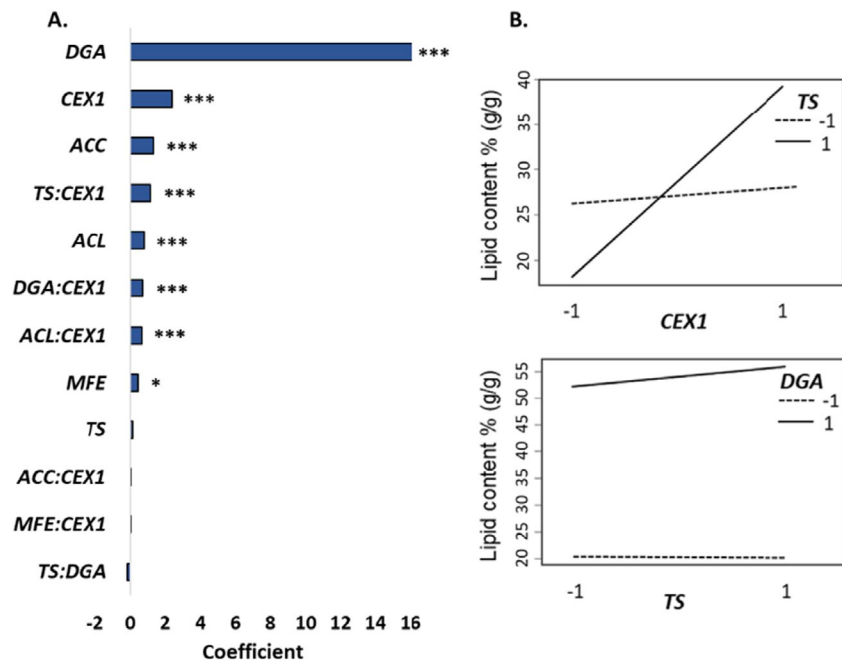

FIGURE 5 | Regression model to evaluate the effects of tested genetic interventions and selected two-factor interactions on lipid content. (A) Coefficients of the model. ***indicates corrected $p \leq 0.001$, ** ≤ 0.01 and * ≤ 0.05 compared to control group (WT). (B) Interaction plot of *CEX1:TS* representing the positive significant interaction and interaction plot of *TS:DGA1* representing insignificant case.

establish this oleaginous yeast as a sustainable fatty acid production platform (Beopoulos et al. 2009; Beopoulos, Nicaud, and Gaillardin 2011; Wang et al. 2020). In this study, we deployed the DBTL approach and focused on the lipid production potential of *Y. lipolytica* by intertwining the predictions from the GEM model, previous findings and known bottlenecks for lipid accumulation of oleaginous yeasts with rounds of genetic interventions. We tested the effect of some amino acid supplements and characterised the built strains with shake flask experiments in N-limiting conditions. Statistical analysis was used to evaluate the gathered experimental data and evaluate the possible interactions between selected interventions on the lipid content of *Y. lipolytica*. The effect of the selected genetic interventions on the lipid content and the results from previous works were summarised in Table 3.

When designing an efficient metabolic engineering strategy, it is important to understand not only the key reactions that are contributing to production but also the competing metabolic pathways that can affect productivity (Ratledge and Wynn 2002; Beopoulos et al. 2009; Wen and Al Makishah 2022). Blazeck et al. (2013) showed that the deletion of *MFE1* and *PEX10* related to the β-oxidation pathway increased the lipid accumulation by 19% (Table 3). Furthermore, especially when *Y. lipolytica* grows on glycerol, the secretion of citric acid into the extracellular environment is identified as one of the main limitations for the utilisation of intracellular citrate for lipid biosynthesis. (Moeller et al. 2011; Sagnak et al. 2018; Wang et al. 2020). Recently, the first citrate exporter of *Y. lipolytica*, was identified by (Erian et al. 2020). In our metabolic engineering strategy, we implemented the knock-out of *MFE1* and *CEX1* to increase the intracellular citrate availability and prevent cleavage of fatty acid via the β-oxidation pathway. In the background strains *Δmfe and Δmfe_ Δcex*, a slight increase in lipid content was observed, and we were able to block citrate secretion in the *Δcex* and *Δmfe_ Δcex*.

Analysis of the metabolic model predicted *ACC* as a suitable target, which initiates lipid synthesis by providing malonyl-CoA. Tai and Stephanopoulos (2013) reported that overexpression of *ACC* improved lipid content by around 2-fold. In this work, we observed that lipid accumulation of *Δmfe_acc* improved by 19% and *Δmfe_Δcex_acc* by 18% compared to WT. Despite these improvements, the lipid content of *Y. lipolytica* did not exceed 23% (w/w). On the other hand, in our previous work, *ACC* overexpression in *C. oleaginosus* increased lipid content by 30%, up to 50% (w/w) (Table 3) (Duman-Özdamar et al. 2024). This indicates that pushing acetyl-CoA to lipid synthesis is a more beneficial approach for *C. oleaginosus*. Furthermore, the fitted polynomial model represented a positive significant effect of *ACC* on lipid content but no significant interaction between ACC:CEX1. This suggests that preventing the secretion of citrate is not beneficial to increasing accumulated lipids when overexpressing ACC.

In addition to the ACC, ACL was identified as a critical reaction. Dulermo et al. (2015) reported that the knock-out of *ACL* in *Y. lipolytica* led to repression of the lipid synthesis. On the other hand, overexpressing the *ACL* of *Y. lipolytica* did not alter the lipid synthesis that was encountered by overexpressing the *ACL* of *Mus musculus* (Blazeck et al. 2014; Zhang et al. 2014). This was explained by the lower citrate affinity of

Y. lipolytica ACL compared to the *Mus musculus ACL*. Also, the authors reported that this overexpression redirected a significant amount of the cytosolic citrate to the lipid synthesis pathway. As the *ACL* of *Y. lipolytica* showed lower affinity to citrate, we overexpressed *ACL* of *C. oleaginous* providing a 37% increase in the lipid content of *C. oleaginosus* (Duman-Özdamar et al. 2024). We obtained a significant increase in lipid accumulation with only *Δmfe_Δcex_acl* (by 19%), while there was around 2-fold declined extracellular citric acid compared to WT. In support of these results, statistical analysis revealed a positive significant interaction of CEX:ACL on lipid content showing that the simultaneous modification of their expression levels is a successful approach to increase lipid synthesis. On the other, hand the lipid accumulation obtained with this transformant was still limited, as also observed with *ACC* overexpression indicating that increasing only the precursors (acetyl-CoA and malonyl-CoA) is not sufficient to achieve high lipid contents with *Y. lipolytica*.

In addition to predictions from the fatty acid synthesis pathway, CFSA predicted genetic intervention targets from the amino acid synthesis pathway related to glutamate, methionine, threonine and leucine metabolism. Wei et al. (2017) simulated the supplement of L-serine, L-threonine and L-aspartate and reported the supplements enhanced TAG production. We observed that supplementing glutamate, methionine, leucine and threonine did not alter the specific lipid accumulation however, it affected the fatty acid composition. Thus these amino acid supplements could be used as a strategy to alter fatty acid composition. In addition, due to an increase in biomass concentration, these supplements enhanced the total lipid (g/L) and lipid yield on glycerol (g/g) by around 1.6-fold. Furthermore, Kim et al. (2019) performed simulations on the GEM model of *Y. lipolytica* and reported that the threonine synthesis pathway was predicted as an overexpression target for improving lipid content. On the other hand, Park, Ledesma-Amaro, and Nicaud (2020) indicated that the lipid content of *Y. lipolytica* declined due to the overexpression of homoserine kinase and *TS* (Table 3). In our previous work, we overexpressed *TS* in *C. oleaginosus* and obtained a 36% increase in lipid content (Duman-Özdamar et al. 2024). Therefore the effect of *TS* overexpression was tested for *Y. lipolytica* by constructing *Δmfe_ts* and *Δmfe_Δcex_ts* transformants. Although the lipid content of *Δmfe_ts* declined by around 5%, we obtained around 19% increase in the lipid content of *Δmfe_Δcex_ts*. In both cases measured extracellular citrate concentrations declined by 2.4-fold with *Δmfe_ts* and 3.4-fold with *Δmfe_Δcex_ts*. Besides these results, our model represented that the main effect of *TS* on lipid content is not significant however interaction of TS:CEX1 has a positive effect on lipid content. In all, these results indicate when the intracellular citrate concentration is higher, *TS* overexpression supports lipid accumulation possibly by balancing the over-production of oxaloacetate (Kim et al. 2019; Duman-Özdamar et al. 2024).

The citric acid secretion was prevented in the *Δcex* strain successfully. On the other hand, when *ACL*, *ACC* and *TS* were overexpressed we detected secreted citric acid (up to 1 g/L, 2-fold lower than WT). Colony PCR was performed at the end of cultivation for all *Δcex* strains and we again confirmed the *Δcex* genotype. Altogether this indicates other exporters are able to transport citrate in the genome of this yeast (Lazar et al. 2017; Erian et al. 2020).

TABLE 3 | Lipid content of engineered *Y. lipolytica*.

Gene	Intervention	Gene source	Lipid content change	Carbon source	References
CEX1	Knock-out	—	No significant difference	Glycerol	This work.
MFE1	Knock-out	—	Lipid content was increased from 16% to 19% (w/w)	Glucose	(Blazeck et al. 2013)
	Knock-out	—	Lipid content was increased from 18.86% to 19.74% (w/w)	Glycerol	This work.
ACL	Knock-out	—	*Δacl1* mutant presents lower FA content and a higher citrate and mannitol production	Glucose	(Dulermo et al. 2015)
	Overexpression	*Mus musculus*	Lipid content increased from 7.3% to 23.1% (w/w)	Glycerol	(Zhang et al. 2014)
		Y. lipolytica	No significant difference	Glucose	(Blazeck et al. 2014)
		C. oleaginosus	When combined with *Δmfe_Δcex*, lipid content was increased from 18.86% to 22.38% (w/w)	Glycerol	This work
			When tested with *C. oleaginosus*, lipid content was increased from 37.8% to 51.8% (w/w)	Glycerol	(Duman-Özdamar et al. 2024)
ACC	Overexpression	*Y. lipolytica*	Lipid content was increased from 8.77% to 17.9% (w/w)	Glucose	(Tai and Stephanopoulos 2013)
			ACC1 and DGA1 overexpressed. Lipid content was increased from 8.77% to 41.4% (w/w)		
		C. oleaginosus	When combined with *Δmfe*, lipid content was increased from 18.86% to 22.39% (w/w)	Glycerol	This work
		C. oleaginosus	When tested with *C. oleaginosus*, lipid content was increased from 37.8% to 50% (w/w)	Glycerol	(Duman-Özdamar et al. 2024)
TS	Supplement	—	Simulations on *i*YL_2.0 resulted in a 2-fold increase in lipid productivity	Glucose	(Wei et al. 2017)
	Overexpression	*C. oleaginosus*	When combined with *Δmfe_Δcex*, lipid content was increased from 18.86% to 22.45% (w/w)	Glycerol	This work
			When tested with *C. oleaginosus*, lipid content was increased from 37.8% to 51.4% (w/w)	Glycerol	(Duman-Özdamar et al. 2024)
Threonine synthesis pathway	Overexpression	*Y. lipolytica*	Overexpression of the threonine synthesis pathway decreased lipid content from 19% to 14.4% (w/w)	Glucose	(Park, Ledesma-Amaro, and Nicaud 2020)

(Continues)

TABLE 3 | (Continued)

Gene	Intervention	Gene source	Lipid content change	Carbon source	References
DGA1	Overexpression	*Y. lipolytica*	Around a 2-fold increase in lipid content (50% w/w)	Glucose	(Friedlander et al. 2016)
			Lipid content was increased from 8.77% to 33.8% (w/w)	Glucose	(Tai and Stephanopoulos 2013)
			When combined with *Δmfe_Δcex*, lipid content was increased from 18.86% to 53.53% (w/w)	Glycerol	This work
DGA1 + TS	Overexpression	*Y. lipolytica*	When combined with *Δmfe_Δcex*, lipid content was increased from 18.86% to 55.87% (w/w)	Glycerol	This work

Another selected gene was *DGA1*, catalysing the last step of TAG biosynthesis of *Y. lipolytica* (Beopoulos et al. 2012; Tai and Stephanopoulos 2013). *DGA1* overexpression showed a push effect of fatty acid synthesis and provided around a 2-fold increase in lipid accumulation (Tai and Stephanopoulos 2013; Friedlander et al. 2016). In our work, we overexpressed *DGA1* in *Δmfe* and *Δmfe_Δcex* which resulted in a 2.7-fold and 2.8-fold increase in lipid content respectively. *DGA1* has the highest positive effect on lipid content among tested genetic interventions and DGA:CEX1 represented a positive interaction. However, the identification of a significant interaction does not necessarily reflect a physical interaction between the enzyme and the metabolite. For instance, *ACL*, *TS* and *DGA1* represented a significant interaction with increased citrate availability because when intracellular citrate availability is high, a high level of lipid accumulation is achieved. Ultimately, we combined the overexpression of *TS*, as it was leading to the lowest extracellular citrate concentrations compared to other targets, and *DGA1* in the *Δmfe_Δcex* background that resulted in around 200% increase in lipid content (56% w/w) and a 230% increase in $Y_{P/S}$ (0.085 g/g).

Overall, these findings show that probably reactions catalysing the late stage of TAG formation in *Y. lipolytica*, pulling free fatty acids into lipid bodies, are the rate-limiting steps of lipid accumulation, while, the initial steps of fatty acid synthesis are limiting to achieve a higher content of lipid accumulation with *C. oleaginosus*. A vast amount of work has been done in glucose-based medium with *Y. lipolytica* (Gajdoš, Nicaud, and Čertík 2017; Wang et al. 2020). These results represent the potential of *Y. lipolytica* in the glycerol-based medium. Altogether, we believe the strain developed and the findings in this study have remarkable potential, especially for the conversion of glycerol-containing side streams, that is, crude glycerol derived from biodiesel production, fat saponification due to producing lipids with higher yields (André et al. 2009; Dobrowolski et al. 2016; Tsirigka et al. 2023).

5 | Conclusion

In this study, we demonstrated a comprehensive and systematic approach to enhance lipid production in *Y. lipolytica* using the DBTL methodology. By integrating genetic intervention predictions from the GEM with experimental validations and finally fitting a second-order polynomial model, we achieved significant improvements in lipid accumulation. Our work highlighted the crucial roles of *ACC*, *ACL*, *TS* and *DGA1* and the interaction of these genes with increased intracellular citrate availability in lipid biosynthesis. Furthermore, we observed the positive effects of amino acid supplementation on total lipid and lipid yield on glycerol. Notably, overexpression of *DGA1* in *Δmfe* and *Δmfe_Δcex* backgrounds led to a remarkable 2.7-fold and 2.8-fold increase in lipid content, respectively. The combination of *TS* and *DGA1* overexpression in *Δmfe_Δcex* background resulted in a 200% increase in lipid content, a 3.35-fold improvement in total lipid, and a 230% increase in $Y_{P/S}$. These results underscore the potential of *Y. lipolytica* as a sustainable alternative for fatty acid production. The insights gained from this study not only advance our understanding of lipid metabolism in oleaginous yeasts but also pave the way for industrial applications, particularly in utilising glycerol-containing by-products for bio-based lipid production. Our findings contribute to the ongoing efforts to develop environmentally friendly and economically viable microbial oil production platforms, addressing the pressing need for sustainable alternatives to palm oil.

Author Contributions

Zeynep Efsun Duman-Özdamar: investigation, writing – original draft, writing – review and editing, methodology, conceptualization. **Mattijs K. Julsing:** supervision, project administration. **Vitor A. P. Martins dos Santos:** funding acquisition, writing – review and editing, supervision. **Jeroen Hugenholtz:** conceptualization, funding acquisition, writing – review and editing, supervision. **Maria Suarez-Diez:** conceptualization, writing – review and editing, funding acquisition, software, supervision.

Conflicts of Interest

J.H. has interests in NoPalm Ingredients BV and VAPMdS has interests in LifeGlimmer GmbH. The other authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GitLab at https://gitlab.com/wurssb/Modelling/sampling-tools.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.