ENHANCING LIPID PRODUCTION OF *CUTANEOTRICHOSPORON OLEAGINOSUS* THROUGH GENETIC INTERVENTION AND MEDIUM OPTIMIZATION

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Abstract

Background: There is an immense need for alternatives to palm oil that can meet consumer demands while ensuring a sustainable and ethical future. *C. oleaginosus* is an appealing alternative producer of microbial oil that can potentially replace palm oil. There are however certain bottlenecks related to the production costs, that could be resolved by enhancing lipid accumulation. Strategies reported to enhance lipid synthesis are through optimization of culture conditions, especially the C/N ratio, and genetic intervention. In this study, we performed C/N optimization experiments of WT and transformants *C. oleaginosus* on glycerol and urea as carbon and nitrogen sources. To study the combinatorial effect of overexpressing target genes, we built corresponding strains and tested them at C/N 30 and 175 g/g.

Results: Through regression analysis, we predicted optimal C/N ratios of *C. oleaginosus* transformants for fatty acid content: 188 g/g for ACL, 207 g/g for ACC, and 263 g/g for TS. Changing the C/N ratio and the transformant can steer the fatty acid composition, with saturated fatty acids decreasing with increasing C/N ratio. Designing double overexpressing strains resulted in a boost of 49% and 38% respectively in TS+ACC and ACL+ACC for lipid accumulation.

Conclusions: Here we provide optimal C/N ratios for WT, ACL, ACC, and TS to maximise their fatty acid production. Moreover, we deliver a tabloid of the fatty acid composition from overexpressing strains under different nitrogen limiting conditions. We also engineered double overexpressing strains with boosted lipid production. Our findings take *C. oleaginosus* oil production one step closer towards industrial feasibility.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's regulations and Code of Practice for Research Degree Programmes, and that it has not been submitted for any other academic awards. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in this dissertation are those of the author.

Signed: Elena Tsepani

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I. Introduction

Palm oil production is cheap and efficient, but its 'true cost' tells a different story. Indonesia, Malaysia, and Thailand which account for 88% of global production generate about US$60 billion profit annually(Kadandale et al., 2019; Statista, 2020). Being that palm oil is the most cost-effective vegetable oil, its demand is predicted to reach between 93 and 156 million metric tons by 2050(Braunwald et al., 2016). Nonetheless, the world suffers the consequences related to the unsustainable production of this commodity. One of the biggest issues is tropical forests being cut down and replaced by palm trees. Deforestation has led to loss of biodiversity, greenhouse gas emissions, and habitat destruction of already endangered species. This naturally interferes with ecosystems, worsening climate change. Furthermore, palm plantations are often carried out in illegal manners, violating indigenous land rights(Fund, 2010; Killeen et al., 2013; Murphy et al., 2021). Understandably, there is a need for alternatives to palm oil that can meet consumer demands while ensuring a sustainable and ethical future.

1.1 *C. oleaginosus:* a candidate for alternative palm oil production

An appealing alternative is oil production from oleaginous yeast, microorganisms that naturally produce oil of at least 20% (w/w) of their biomass(Beopoulos et al., 2009; Meijaard et al., 2020; Colin Ratledge & Wynn, 2002). These microorganisms exhibit advantages such as being independent of season or climate compared to plants, having a duplication time lower than 1 hour, growing on different sources, accumulating high amounts of lipids, and scalability(Abeln & Chuck, 2021; Adrio, 2017; Liu et al., 2015; Pham et al., 2021). One of the most efficient oleaginous yeasts is *Cutaneotrichosporon oleaginosus* ATCC 20509, found in the literature under different names such as *Candida curvata D*, *Apiotrichum curvatum*, *Cryptococcus curvatus*, *Trichosporon cutaneum*, or *Trichosporon oleaginosus*. To maintain consistency throughout our research, we will refer to it as *Cutaneotrichosporon oleaginosus*(Abeln & Chuck, 2021; Bracharz et al., 2017).

C.oleaginosus displays advantages over other oleaginous yeasts by accumulating higher lipid content, growing on a wide range of substrates, growing on high cell densities, and tolerating fermentation inhibitors(Abeln & Chuck, 2021). This yeast has been reported to accumulate lipids up to 60% (w/w) of its biomass dry weight depending on substrate and growth conditions(Awad et al., 2019; Beopoulos et al., 2011; Duman-Özdamar et al., 2022; Görner et al., 2016). More than 90% of the lipids produced are triacylglycerols, with a fatty acid composition similar to that of palm oil(Moon et al., n.d.; Papanikolaou et al., 2013). The microbial oil from *C. oleaginosus* has a typical profile of fatty acids of 25% palmitic acid (C16:0), 10% stearic acid (C18:0), 57% oleic acid (C18:1), and 7% linoleic acid (C18:2)(Ageitos et al., 2011; Bracharz et al., 2017; Hassan et al., 1994; Pham et al., 2021). *C.oleaginosus* utilizes different carbon sources for *de novo* lipid production, including glucose, galactose, xylose, n-acetylglucosamine, volatile fatty acids, cellobiose, sucrose, glycerol, and lactose, as well as various inexpensive waste streams such as from office paper production, crude glycerol from biodiesel production, whey permeate, wheat straw hydrolysate. food waste and municipal wastewater streams(Diliara et al., 2009; Evans & Ratledge, 1983; Görner et al., 2016; Gujjari et al., 2011; Hassan et al., 1994; Liu et al., 2015;

Meesters et al., 1996; Willis et al., 2014). This opens the door for future sustainable and circular production as well as reducing materials costs. Due to these attributes, *C.oleaginosus* has been identified as a promising cell factory for microbial oil.

Under nitrogen starvation, a high carbon-to-nitrogen ratio (C/N) in cultivation medium, growth slows down in *C.oleaginosus*, and instead, high amounts of lipids are accumulated(Beopoulos et al., 2011; Kourist et al., 2015; Papanikolaou et al., 2013; C. Ratledge, 2002; Ratledge & Wynn, 2002). The critical C/N ratio has been reported to be 11 g/g, where the excess carbon is redirected towards lipids accumulation. In a nutshell, lipid biosynthesis can be divided into three steps: fatty acid formation, triacylglyceride (TAG) synthesis, and phospholipid synthesis **(Figure 1)**.

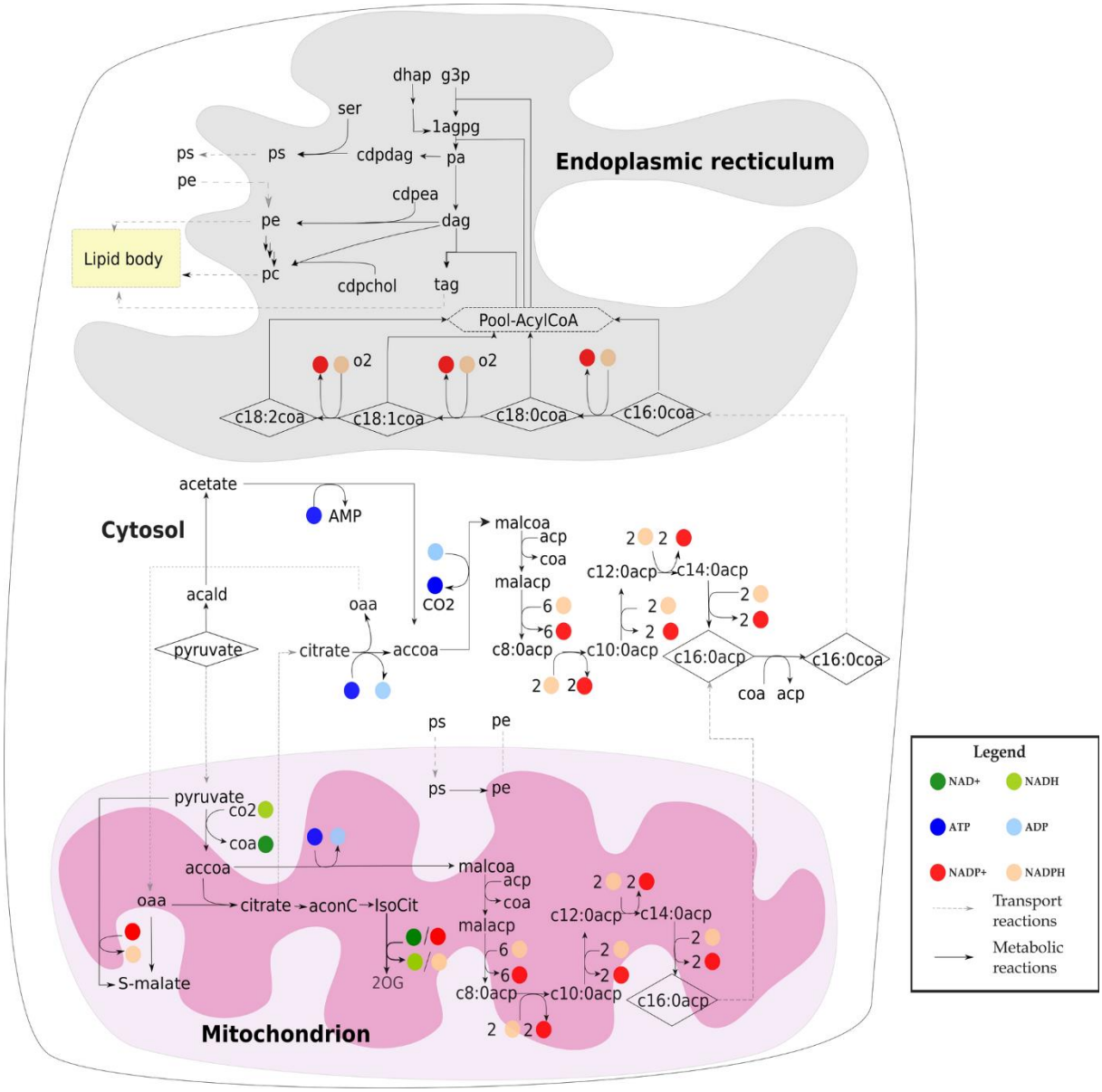

Figure 1. Lipid synthesis pathways in *C. oleaginosus* ATCC 20509. 1agpg-1-Acyl-sn: glycero-3-phosphoglycerol; 2OG-2: oxoglutarate; acald: acetaldehyde; accoa: acetyl-CoA; aconC: cis-aconitate; acp: acyl carrier protein; c8:0acp: octanoyl acyl carrier protein; c10:0acp: decanoyl acyl carrier protein; c12:0acp: dodecanoyl acyl carrier protein; c14:0acp: tetradecanoyl acyl carrier protein; c16:0acp: Hexadecanoyl acyl carrier protein; c16:0coa: Hexadecanoyl-coa; c18:0coa: Octadecanoyl-coa; c18:1coa: oleoyl-CoA; c18:2coa: linoleoyl coA; cdpdag: CDP-Diacylglycerol; cdpea: CDP-ethanolamine; cdpchol-: CDP-choline; coa: coenzyme A; dag: diacylglycerol; g3p: glyceraldehyde 3-phosphate; oaa: oxaloacetate; isoCit: isocitrate; malacp: malonyl acyl carrier protein; maloca: malonyl-CoA; pa: phosphatidate; pc: phosphatidylcholine; pe: phosphatidylethanolamine; ps: phosphatidylserine; ser: serine; tag: triacylglycerol. (Image from Pham et al., 2021).

The available carbon source in the media is metabolized through glycolysis and once pyruvate is in the mitochondria, it enters the tricarboxylic acid cycle (TCA) cycle as acetyl-CoA by pyruvate dehydrogenase. Under nitrogen-limiting conditions, isocitrate dehydrogenase is downregulated because of a decrease in the amount of intracellular AMP, cleaved to produce NH_4^+ ions needed for cell growth. This seizes the conversion of citrate to isocitrate, resulting in the accumulation of citrate in the mitochondria. Accumulated mitochondrial citrate is transported to the cytoplasm in exchange for malate, where it is hydrolyzed by ATP-citrate lyase (ACL) to produce acetyl-CoA and oxaloacetate. Another key enzyme, acetyl-CoA carboxylase (ACC), converts acetyl-CoA to malonyl-CoA, which serves as a building block for fatty acid synthesis by the fatty acid synthase complex (FAS) to produce acyl-CoA. Acyl-CoA and the glycerol-3-phosphate are the precursors for TAG synthesis which takes place in the endoplasmic reticulum (ER) and lipid bodies (LB) **(Figure 2)**. After storage of TAGs in LB, depending on the cellular requirements, they can be catalyzed for membrane formation or energy(Liu et al., 2015; Pham et al., 2021; Zhang et al., 2014).

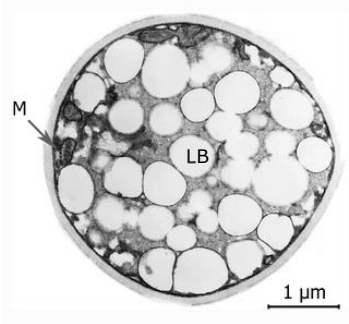

Figure 2. Lipid bodies of *C. oleaginosus* under nitrogen limiting conditions. LB: lipid bodies, M: mitochondrion. (Image from Holdsworth et al., 1988)

1.2. Microbial oil production bottlenecks and strategies to overcome them

Producing *C.oleaginosus* oil as an alternative to palm oil presents a challenge in today's capitalist economy. Palm oil is economically competitive as the most affordable vegetable oil (Papanikolaou et al., 2013). Koutinas et al., 2014 have estimated that lipid accumulation in *C.oleaginosus* should reach approximately 85% (w/w) for the process to become economically viable(Koutinas et al., 2014). At first sight, this number might seem unrealistic, however, process engineering, cultivation conditions optimization, and genetic engineering can improve the economic viability of the process.

From the process engineering front, there are means to decrease production costs by aiming for zero waste, as well as improve bioreactor design or efficiency of oil recovery and purification methods(Karlsson et al., 2016; Koutinas et al., 2014). Since substrate materials are a substantial contributor to costs, considering recycling inexpensive waste streams like raw glycerol that is found abundantly or lignocellulosic hydrolysates aids in lower production costs(Singh et al., 2016; Zhao et al., 2016). Additionally, due to its unique sugar composition, the spent *C. oleaginosus* cell wall after oil extraction is proposed as an animal feed additive to improve the techno-economic viability of the overall process(Awad et al., 2019; Masri et al., 2018).

Optimization of cultivation conditions is carried out to identify the factors that influence biomass and lipid formation(Singh et al., 2016). Applying Response Surface Methods (RSM) in *C. oleaginosus* cultivation led to an optimum C/N ratio of 120 g/g, achieving biomass of 18.4 ± 2.20 g/L and lipid yield of 9 ± 0.34 g/L, which corresponds to 49.74 ± 5.16% (g lipid weight/g dry yeast cell weight). (Awad et al., 2019). In a recent study conducted by Duman-Özdamar et al., 2022, RSM was applied to determine the optimal synergistic effect of temperature and C/N (variables) on lipid accumulation (response) in *C. oleaginosus*(Duman-Özdamar et al., 2022). Tested temperatures were 15, 25, 30, and 35 °C and C/N ratios 30, 60, 120, 240, and 300 g/g. Glycerol was selected as the carbon source, while urea served as the nitrogen source. Optimal synergistic effect of temperature and C/N ratio (variables) on lipid accumulation (response) of *C. oleaginosus* was explored. A second-order polynomial regression was fitted to this relationship, predicting optimal C/N of 175 g/g and temperature of 30 °C for lipid accumulation. Furthermore, the model predicted a C/N ratio of 185 and a temperature of 25 °C maximum biomass density. These values were validated in vitro, where lipid accumulation was measured at 51.17 ± 0.66%, w/w, slightly surpassing the in silico predictions, and biomass reached 5.35 ± 0.28 g/L. In addition, it was concluded that altering the temperature from higher to lower can shift the fatty acid profile from saturated to unsaturated and that higher temperatures leads to even longer saturated fatty acids.

Moreover, genetic engineering of oleaginous yeast strains is implemented to redirect metabolic fluxes toward lipogenic pathways(Leitner et al., 2004; Mlíčková et al., 2004). This is done by overexpressing or deleting genes involved in the fatty acid formation pathway. For instance, Koivuranta et al., 2018 pinpointed gene targets to create a complete mitochondrial pyruvate dehydrogenase (PDH) bypass, that was predicted to enhance lipid production in *C. oleaginosus* (Koivuranta et al., 2018). They successfully overexpressed the target genes in different

combinations and improved the yield of TAG at high C/N ratio with either glucose (0.233 g/g) or xylose (0.27 g/g) as substrates.

An important addition to guiding genetic engineering and cultivation conditions optimization in *C. oleaginosus* came from Pham et al., 2021, where they reannotated its genome and built the first GEM named iNP636_Coleaginosus_ATCC20509(Pham et al., 2021). Complementing cultivation conditions optimization, this model predicted an optimal C/N 120 g/g when inexpensive glycerol is used as a substrate. Moreover, it predicted ATP-citrate lyase ACL as a target to enhance lipid production, since it is the main source of cytosolic acetyl-CoA.

Duman-Özdamar et al., unpublished followed the design-build-test approach to further enhance lipid production in *C. oleaginosus*. Extensive in silico studies were conducted on the transcriptomics and metabolomics of *C. oleaginosus*, which led to the identification of four overexpression target genes (ACL, ACC, TS, and HMGS) and five potential media supplements (biotin, thiamine, threonine, serine, and aspartate). Preliminary experiments involving the overexpression of these genes and the addition of the identified supplements increased lipid content, reaching 56% (w/w). Furthermore, they established an effective electroporation method to facilitate co-transformation in *C. oleaginosus* to create overexpressing strains.

1.3. Research aim

Overall, the mentioned approaches, especially when complementing each other, can improve lipid production of *C. oleaginosus*, and allow for the customization of fatty acid profiles to meet specific industry requirements. However, the target lipid accumulation for industrial utilization is not yet achieved, necessitating further research. Standing on the shoulders of giants, the present research aims to combine medium optimization and genetic intervention techniques to further enhance the lipid content of *C. oleaginosus*. In more detail, our research will be conducted as follows **(Figure 3)**:

1) We will cultivate wild-type (WT) and the transformants ACL, ACC, and TS under 5 selected C/N ratios (30, 120, 175, 200, and 300) in order to:
- Predict the optimal C/N for fatty acid accumulation and dry cell weight in each of the strains.
- Recognize better performants for fatty acid accumulation and dry cell weight.
- Explore the fatty acid profile of the different under different C/N ratios.
2) We will build new *C. oleaginosus* strains that combine ACL, ACC, and TS targets.
3) We will cultivate new strains under C/N ratios 30 and 175 g/g in order to:
- Explore the combinatorial effect of target genes on lipid accumulation and dry cell weight.
- Recognize better performants for fatty acid accumulation and dry cell weight.

The outcomes of this study will pave the way for the commercial-scale utilization of *C. oleaginosus* as a sustainable and renewable resource for microbial oil production.

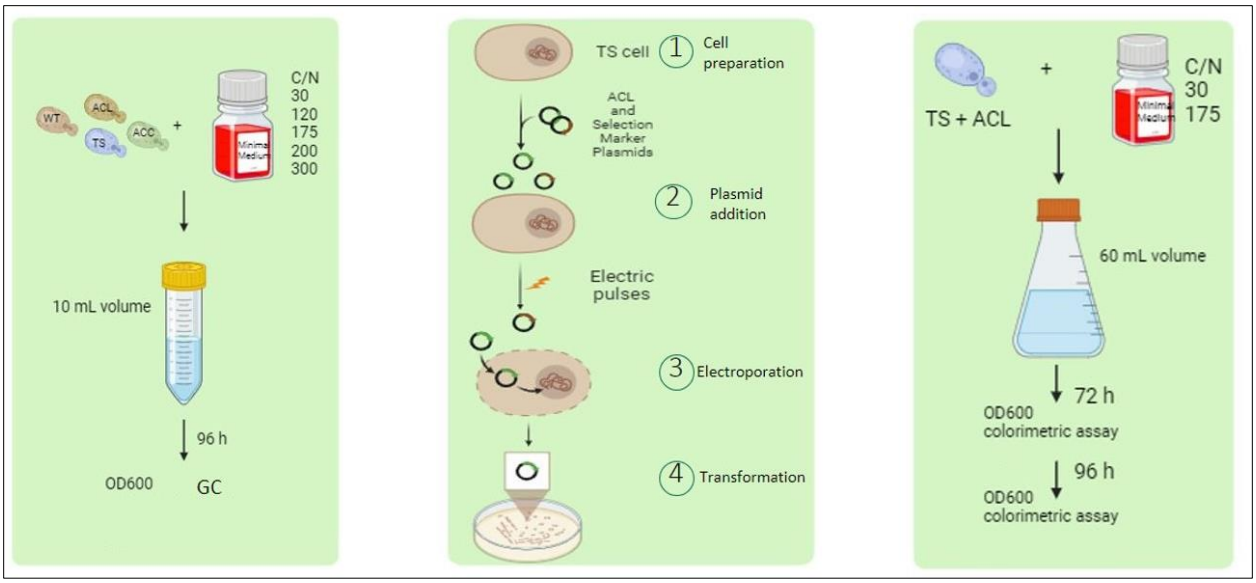

Figure 3. Experimental overview of this study. C/N: carbon-to-nitrogen ratio, ACL: ATP-citrate lyase, TS: threonine synthase GC: gas chromatography. Image created with Biorender.

II. Materials and Methods

2.1 Yeast strains, medium preparation, and cultivation

WT as well as transformed *C. oleaginosus* ATCC 20509 strains **(Table 1)**, stored as 20% (v/v) glycerol stocks, were plated on yeast extract peptone dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar). All plates were incubated at 30 ^{o}C for three days and then stored for up to 2 weeks at 4^{o}C. Pre-cultures were prepared by transferring a single colony into 10 mL of YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), incubated at 30 ^{o}C, 250 rotations-per-minute (rpm) for 16-18 hours.

Table 1. Previously generated *C. oleaginosus* strains and plasmids they were transformed with.

Transformed Strain	Plasmids Integrated	Genotype
ACL	pUC57-ACL + pCG6-NAT	TEF1p-ACL-TEF1t + GPDp-NAT-GPDt
ACC	pUC57-ACC + pCG6-NAT	TPI1p-ACC-TPI1t + GPDp-NAT-GPDt
TS	pUC57-TS + pCG6-NAT	YATp-TS-YATt + GPDp-NAT-GPDt

Minimal media, pH 5.5 (2.7 g/L KH_2PO_4, 1.79 g/L $Na_2HPO_4.7H_2O$, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L EDTA, 0.278 g/L urea) were prepared with different concentrations of glycerol, the carbon source, depending on the C/N ratio **(Table 2)**. Urea in the minimal media represents the nitrogen source. Trace elements solution (TES) 100x (4 g/L $CaCl_2.2H_2O$, 0.55 g/L $FeSO_4.7H_2O$, 0.48 g/L citric acid.H_2O, 0.10 g/L $ZnSO_4.7H_2O$, 0.08 g/L $MnSO_4$.H2O, 100 µL H_2SO_4) was added to the media in a final concentration of 10x.

Table 2. C/N ratio of minimal media and corresponding glycerol concentration.

C/N (g/g)	Glycerol (g/L)
30	9.97
120	40.90
175	58.48
200	66.45
300	102.24

For culture preparation, the overnight precultures were centrifuged at room temperature, 3500 rpm for 5 minutes. Pelleted cells were resuspended with 2 mL of the corresponding minimal medium and centrifuged again under the same conditions. After removing the supernatant, cells were resuspended using a minimal medium with the corresponding C/N. Resuspended cells were used to inoculate volumes of 11 mL (corresponding minimal medium containing TES 10x) in the case of filtered-cap tube experiments or 60 mL (corresponding minimal medium containing TES 10x) for the shake flask experiments until a 600nm optical density (OD_{600}) around 0.5 was reached. Following inoculation, tubes and flasks were incubated at 30 ^{o}C, 250 rpm for 96 hours. Three biological replicates were used for all culturing conditions.

2.2 Genetic modification of *C. oleaginosus*

Plasmid isolation, linearization, and elution

20% (v/v) glycerol stocks of *E. coli* with relevant plasmids were used to inoculate 10 mL Luria Bertani (LB) Broth Low Salt containing the corresponding antibiotic **(Table 3)**. The tubes were incubated overnight at 37 ºC and 250 rpm. The GeneJET Plasmid Miniprep Kit (#K0503) was used to isolate the plasmids. 4 mL from the overnight culture were centrifuged at 21300 rpm for 5 minutes. Plasmid isolation was performed as described by the manufacturer. The concentration and purity of isolated plasmids were measured using Nanodrop (Thermo Fisher Scientific), after which they were stored at -20 ºC for further analysis.

Table 3. List of plasmid stocks with the corresponding antibiotics to which they confer resistance as well as restriction enzyme (New England BioLabs) to be used for digestion.

Plasmid Stock Name	Antibiotics	Restriction enzyme
E.coli ZYMO 10B pUC57_ACL	Ampicillin/Carbenicillin (100 mg/mL)	EcoRI
E.coli ZYMO 10B pUC57_ACC	Ampicillin/Carbenicillin (100 mg/mL)	EcoRI
E.coli ZYMO 10B pUC57_TS	Ampicillin/Carbenicillin (100 mg/mL)	EcoRI
E.coli ZYMO 10B pUC57_HMGS	Ampicillin/Carbenicillin (100 mg/mL)	EcoRI
E.coli ZYMO 10B GG_ACL_TS	Kanamycin (50 mg/mL)	HindIII
E.coli ZYMO 10B pUC57_HPT	Ampicillin/Carbenicillin (100 mg/mL)	EcoRI

Plasmid linearization was done by mixing 17 µL isolated plasmid, 2 µL restriction enzyme CutSmart buffer (New England BioLabs,) and 1 µL Restriction enzyme (New England BioLabs) **(Table 3)**. Reaction tubes were put in a 37 ºC water bath for at least one hour. After that, cut plasmids with 6x loading dye (New England BioLabs) were run at 72V for 75 minutes on a 1% agarose gel to confirm linearization.
Linearized plasmids were eluted using the GeneJet Gel Extraction kit (#K0692) to clean out the restriction enzymes and impurities. After adding 33 µL of sterile MilliQ water to bring the volume of the linearized plasmid to 50 µL, instructions provided by the manufacturer were followed.

Preparation of electrocompetent *C. oleaginosus* cells

A single colony from the required strain was transferred to 10 mL YPD broth, put in a 30 ºC incubator, and shaking at 250 rpm for 16-18 hours. 250 mL Erlenmeyer flasks containing 50 mL YPD broth were inoculated with 2mL overnight preculture and incubated for 3-4 hours at 30ºC, 250 rpm until OD_{600} was 0.8-1. Culture was transferred into 50mL falcon tubes and centrifuged at 3500rpm for 15 minutes at 4 °C. After medium discard, the pellet was resuspended with 10mL cold 50 mM sodium phosphate buffer pH 7.5 with 25mM Dithiothreitol (DTT, filter sterilized with a 0.2µm filter). The formed solution was incubated for 12 minutes at 37 °C. After incubation, the solution was centrifuged at 3500rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 10mL cold sterile 270mM sucrose solution (10mM Tris/HCl pH 7.6, 1mM $MgCl_2$) and washed twice. In the end, electrocompetent cells were obtained by resuspending the pellet in 0.5 mL sucrose solution.

Electroporation and election for transformants

Before electroporation, 50 µL of prepared electrocompetent cells were transferred into a precooled 2mm electroporation cuvette. To that, approximately 1 µg per plasmid DNA (total volume of 5 µl) was added and the cuvettes were incubated for 5 minutes on ice. Negative controls were prepared by mixing MilliQ water with 50 µL electrocompetent cells. Cuvettes were set on the electroporation device (BioRad) and pulsed at 0.8 kVolt, 1000Ohm, 25 µFarad. Immediately after pulsing, 1mL YPD broth was added to the cuvette, and the total mixture was transferred into 2 mL Eppendorf tubes. The tubes were then incubated for 2.5 hours at 30 °C, and gently mixed by inversion every 30 minutes. 100 µL from prepared 1x, 10x, and 100x transformation culture dilutions were spread onto YPD agar plates containing 200 and 250 µg/mL hygromycin B for primary selection. To check the viability, transformation cultures were also plated on YPD agar without antibiotics. Plates were incubated at 30 °C for 2-3 days. After observing colony growth, colonies were picked to streak new YPD agar plates with 300 µg/mL hygromycin B for secondary selection. Ultimately, transformants of grown colonies from the secondary selection plates were confirmed via colony PCR. For that, DreamTaq Green PCR Mix (2x) in a reaction with specific primers **(Table 4)** was used following the instructions from the supplier (Thermo Fisher Scientific #K1081).

Table 4. List of primers used for colony PCR throughout this study.

Primer Name	Primer Sequence
GPD-Fw	GTCATCATCTCGGCCCCTTC
GPD-Rv	CAAGGTCAACGACCGAAACG
TEFp-FwN	GGTGCCACTTGCACTTGGCAG
ACL-intRv	ACTTCTGGATGTGGTGACCAC
TEFt-Rv	GCAGGACCAGTGCTAAGCCTGAC
ACL-int Fw	ACTCATGTGGCCGCAAGACTC
ACL-Fw1	CTGTTCGTTCTCGGTCGCTCGATG
Hpt-syn-int-Fw	TGACCTATTGCATCTCCCGC
Hpt-syn-int-reverse	CTACACAGCCATCGGTCCAG
TPLp-Fw	GGGTGTGGCAGCGAGTGTGGC
ACC-intRv	CAGGTGACTCACCGATGAATTG
ACC-int2 Rv	CTCCTGCTGAGCGATCTCAA
TPLt-Rv	GGTAGCCACGAGCTGGCCATC
ACC-int2 Fw	CGTTCTCGACACCCTTGAGA
YATF-R	CCTCTCCACCCTCTTGTTGG
YATt-Rv	TGGACCGGGTTTTGAACGC

2.3 Analytical methods

Determination of biomass, lipids, and fatty acid profile

Growth of *C oleaginosus* cultures was monitored at 0, 72 (only cultures in shake flasks), and 96 hours based on measured OD_{600}. Obtained values were converted into dry cell weight as described in Duman-Özdamar et al., 2022.

For the filtered-cap tube experiments, cultures were spun down at 4200 rpm for 15 minutes, washed twice with 10 mL demi water, and freeze-dried. The freeze-dried samples were used to quantify total fatty acid content via gas chromatography (GC), with 7830B GC systems (Aligent) as described in Duman-Özdamar et al., 2022. In the last step, chloroform was evaporated with nitrogen, followed by dissolving the remaining lipid with hexane.
As for the shake flasks experiments, the total lipids of each culture were quantified via colorimetric lipid assay. During the colorimetric assay, the absorbance at 530 nm of each culture was measured. These values were then converted into total lipids based on a calibration curve from standard samples **(Appendix B)**. Protocols for preparing standard samples and vanillin stock as well as the analysis procedure are reported in **Appendix A**.

Regression analysis

R software (version 4.3.1) was used to perform regression analysis on the relationship between the C/N ratio (variable) with the dry cell weight, fatty acid content for the filtered-cap cultures, and lipid content for the flasks cultures (responses). Student t-test was performed to test for significant differences in the results between different cultivation conditions and strains.

III. Results

This study aims to further enhance lipid production in *C. oleaginosus* since the process is yet to be deemed industrially viable. Additional lipid enhancement is necessary to establish this yeast as a palm oil alternative producer. In that regard, we directed our research towards characterizing the WT as well as previously built strains overexpressing ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and threonine synthase (TS) *C. oleaginosus* in filtered-cap tube experiments. Afterwards, we sought to build new strains to test the combinatorial effect of these overexpression targets on lipid accumulation. Lastly, the lipid production of the new strains was evaluated through shake flask experiments.

3.1 Cultivation of WT, ACL, ACC, and TS in filtered-cap tubes

The goal of this experiment was to further optimize the C/N ratio of WT and *C. oleaginosus* ACL, ACC, and TS transformants (Duman-Özdamar et al., unpublished). In light of this, C/N ratios (g/g) of 30, 120, 175, 200, and 300 were selected to fit a parabolic behavior, starting at a low C/N value (30) towards a higher C/N value (300). Under these nitrogen-limiting conditions, strains were characterized in terms of growth (g dry cell weight/L), fatty acid content (%, w/w), and fatty acid composition (%). In addition to that, regression analysis was performed to predict the optimum C/N ratio for dry cell weight and fatty acid content of each strain.

3.1.1 Growth profile and fatty acid content of WT, ACL, ACC, and TS

The C/N ratio is an important factor in the growth profile and fatty acid content of *C. oleaginosus* as it induces lipid accumulation. The TS strain stood out as the best performant regarding dry cell weight by reaching its maximum of 8.68 ± 0.00 g/L at C/N 120 g/g, about a 23% increase from WT under the same conditions **(Table 5)** (**Figure 4)**. Moreover, it had a significant increase in DCW when compared to WT under C/N (g/g) 30 and 300 with 6.23 ± 0.21 and 7.14 ± 0.14 g/L respectively. ACL reached, in general, higher values than WT in dry cell weight. These two strains seemed to follow the same pattern of maintaining a stable growth between C/N (g/g) 120 to 175 before dropping at 300 **(Table 5)** (**Figure 4).** The highest value was 7.46 ± 0.08 g/L at C/N 200 g/g, but without significant increase to WT. A significant increase was observed however at C/N (g/g) 30 and 300 with 5.21 ± 0.08 and 6.41 ± 0.08 g/L. As for ACC, it followed the same pattern as WT until C/N 175 g/g, then maintained the same growth until C/N 300 g/g. A significant 1.2-fold increase was observed at C/N 300 g/g **(Table 5)** (**Figure 4)**.

Fatty acid accumulation data of the different strains were obtained via gas chromatography. Fatty acids accumulated ranged from 24.95 ± 0.89 %, w/w (ACC at C/N 30 g/g) and 50.54 ± 0.58 %, w/w (TS at C/N 200 g/g). Out of all the strains, TS exhibited a peculiar behavior. Apart from reaching the highest content, a significant increase to WT (C/N 200 g/g) of 12%, it also maintained

a somewhat stable production throughout the increasing C/N ratios. This stableness was due to having high production even at C/N ratios 30 and 300 g/g, with 43.12 ± 1.82 %, w/w and 49.20 ± 1.36 %, w/w **(Table 5) (Figure 4)**. ACL and ACC also reached high lipid content, with ACL starting with a significantly increased value of 34.49 ± 0.98 %, w/w already at C/N 30 g/g. It reached a peak between C/N (g/g) 175 and 200 at about 48 %, w/w, before dropping to 38.97 ± 1.50 %, w/w (C/N 300 g/g) (**Table 5) (Figure 4)**. ACC on the other hand started with about the same amount as WT at CN/30 g/g with 24.95 ± 0.89 %, w/w, then followed a similar pattern to ACL. It had a significant increase to WT only at C/N 200 with an amount of 48.44 ± 1.00 %, w/w fatty acids **(Table 5) (Figure 4)**. Overall, fatty acid production was boosted around C/N 200 at all overexpressing transformants.

Table 5. Fatty acid content and dry cell weight of WT, ACL, ACC, and TS *C. oleaginosus* under different C/N ratio minimal medium. The level of significant increase between overexpressing transformants and WT is represented via Asterix (*) based on calculated p-values. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

C/N 30 (g/g) Minimal Medium				
Strain	**Fatty Acid Content (%, w/w)**		**Dry Cell Weight (g/L)**	
WT	26.79 ± 1.20		4.10 ± 0.08	
ACL	34.49 ± 0.98	**	5.21 ± 0.08	****
ACC	24.95 ± 0.89		4.27 ± 0.16	
TS	43.12 ± 1.82	***	6.23 ± 0.21	***
C/N 120 (g/g) Minimal Medium				
WT	42.83 ± 0.40		7.06 ± 0.08	
ACL	40.00 ± 1.07		7.39 ± 0.41	
ACC	40.71 ± 1.41		7.25 ± 0.32	
TS	46.51 ± 3.60		8.68 ± 0.00	***
C/N 175 (g/g) Minimal Medium				
WT	46.64 ± 0,20		7.05 ± 0.44	
ACL	48.06 ± 2.68		7.26 ± 0.21	
ACC	47.73 ± 0.95		7.10 ± 0.39	
TS	47.86 ± 0.89		7.44 ± 0.15	
C/N 200 (g/g) Minimal Medium				
WT	45.08 ± 0.26		7.43 ± 0.21	
ACL	48.23 ± 1.17	*	7.46 ± 0.08	
ACC	48.44 ± 1.00	*	7.28 ± 0.08	
TS	50.54 ± 0.58	***	7.60 ± 0.39	
C/N 300 (g/g) Minimal Medium				
WT	41.43 ± 1.99		5.90 ± 0.08	
ACL	38.97 ± 1.50		6.41 ± 0.08	**
ACC	41.11 ± 2.26		6.97 ± 0.23	**
TS	49.20 ± 1.36	**	7.14 ± 0.14	***

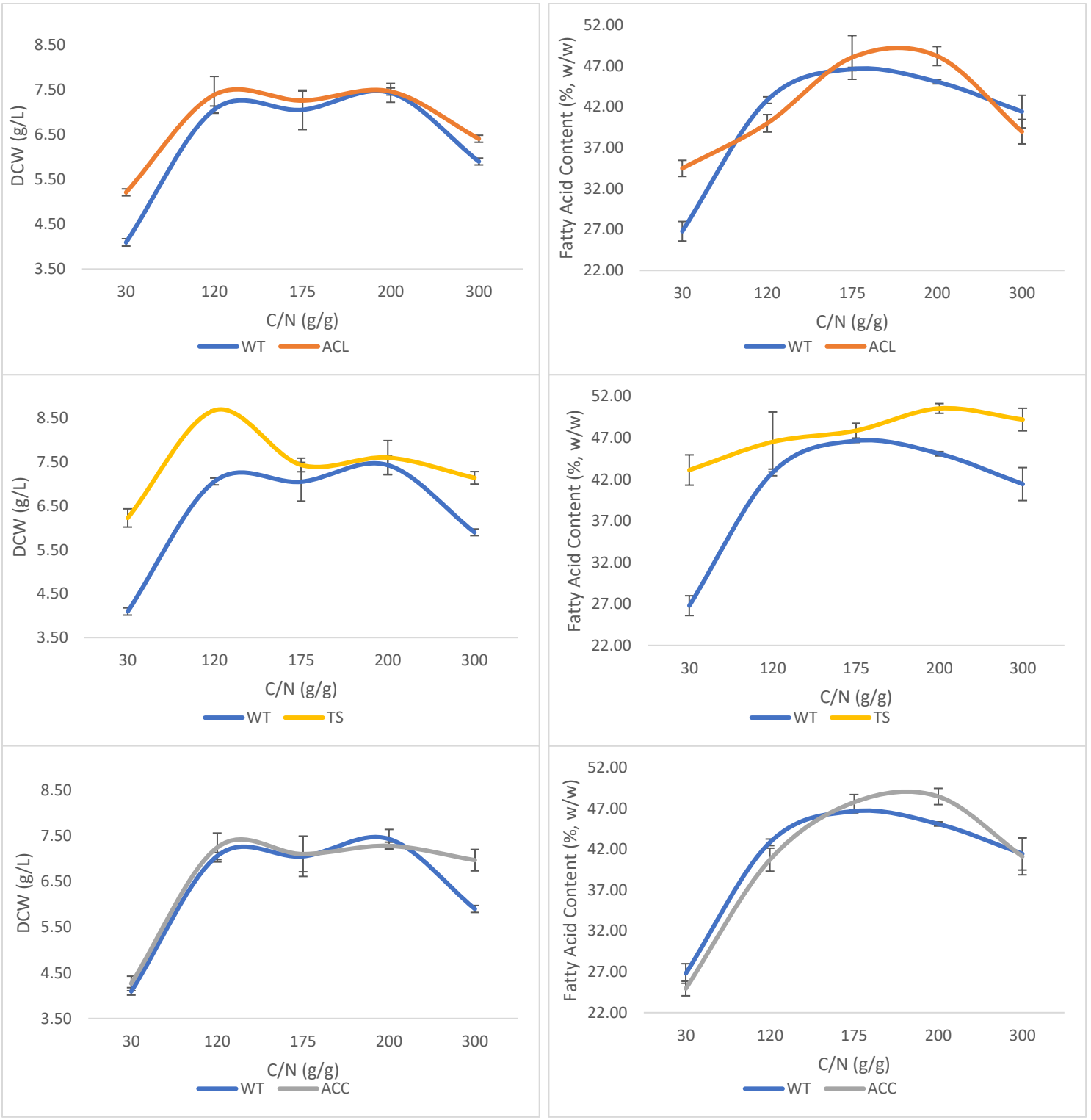

Figure 4. Dry cell weight (left) and fatty acid content (right) plots of WT compared to the overexpressing transformants ACL, ACC, and TS *C. oleaginosus* under the selected C/N ratios. DCW: dry cell weight, C/N: carbon-to-nitrogen ratio, ACL: ATP-citrate lyase, ACC: acetyl-CoA carboxylase, TS: threonine synthase.

3.1.2 Identification of optimal C/N ratios

Regression analysis was performed to predict the optimal C/N ratio for each strain when it comes to cell growth and fatty acid accumulation. The experimentally obtained data **(Table 5)** were fitted to a second-order polynomial equation **(Figure 5/6)**. The equations obtained were then used to calculate the optimal C/N ratios. The quality of relationships represented by the equations was assessed based on the adjusted coefficient of determination R^2, and p-value. R^2 values range between 0 and 1 and the closer to 1 the more adequate the model, hence the more accurate values it predicts. As for the p-value, it should be lower than 0.05 for the model to be regarded as significant. In our case, all regression analyses were at p value lower than 0.05, but looking at the adjusted R^2, only the DCW and fatty acid content models for WT as well as the ACC quadratic model for fatty acid content describe the experimental data accurately. Predicted optimal C/N ratios varied from strain to strain and from DCW to fatty acid content. The DCW and fatty acid content optimal values for ACL (187 and 188) and ACC (215 and 207) were quite close. For WT, DCW optimal C/N was at 190, whereas for fatty acid accumulation at 206. The predictions for TS were at 178 for DCW and 263 for fatty acid accumulation.

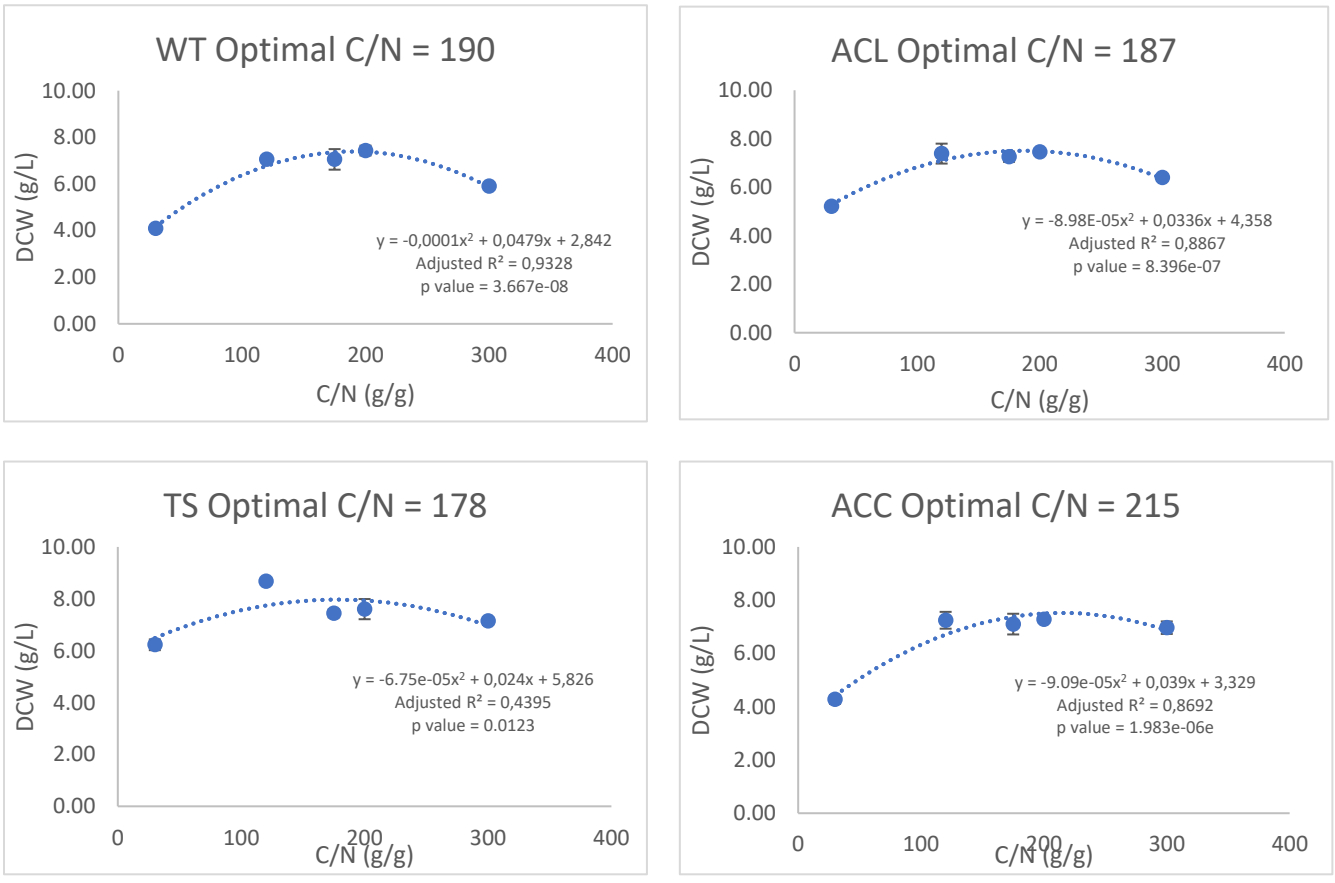

Figure 5. Regression analysis between C/N ratio and DCW in WT, ACL, ACC, and TS. DCW: dry cell weight, C/N: carbon-to-nitrogen ratio, ACL: ATP-citrate lyase, ACC: acetyl-Coa carboxylase, TS: threonine synthase.

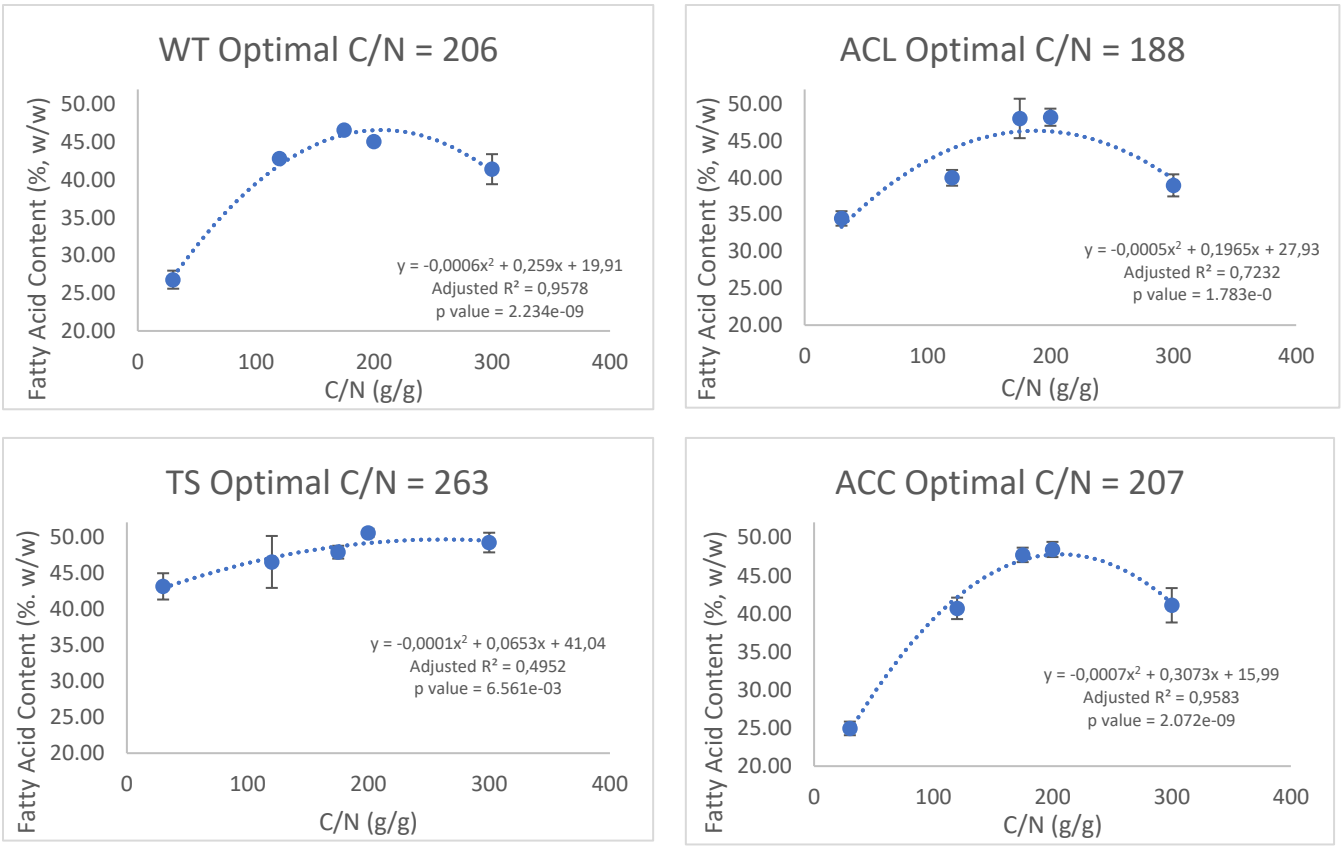

Figure 6. Regression analysis between C/N ratio and fatty acid content in WT and mutants. C/N: carbon-to-nitrogen ratio, ACL: ATP-citrate lyase, ACC: acetyl-CoA carboxylase, TS: threonine synthase

3.1.3 Analysis of fatty acid profile

Apart from characterizing the strains in terms of growth and fatty acid accumulation, we also evaluated the fatty acid profile under the selected C/N ratios **(Table 6)**. In detail, we compared the difference in the composition 1) with changing C/N ratio in each strain and 2) in each C/N ratio across all the strains **(Figure 7)**.

Overall, WT and all transformants contained higher unsaturated fatty acids than saturated (SAFA) ones across all the tested C/N ratios. There was an increase in unsaturated fatty acids with increasing C/N ratios; at C/N 30 g/g they range between 55.61% (TS) - 58.17% (ACC) and at C/N 300 g/g between 60.66% (ACL) - 63.49% (ACC). In WT, ACL, and ACC the jump in unsaturated fatty acids occurred at C/N (g/g) 120 and in TS at 175. After this 'jump', the amount of unsaturated fatty acids remained stable at around 60%, apart from in ACC where it increased to 63.49% at C/N 300 g/g. Zooming in on the unsaturated fatty acids, we observed an overall pattern of increasing monounsaturated ones from C/N (g/g) 30 to 300, whereas polyunsaturated fatty acids displayed similar portions in C/N (g/g) 30 and 300. As for the very long chain fatty acids (VLCFA), there was a general pattern of decrease from C/N (g/g) 30 to 300, but with fluctuations. The values ranged from 0.85% (ACC C/N 300 g/g) to 2.38% (ACL C/N 30g/g). Compared to the rest, TS

showed less of a decrease going from 2.25% at C/N 30 g/g to 1.91% at C/N 300 g/g, only dropping to 1.61% at C/N 175 g/g. VLCFAs across all strains were the lowest in ACC. When it comes to SAFAs, WT, and ACL exhibited similar amounts under the same C/N ratio, from 39.01% to 43.36%. Between C/N (g/g) 120 and 300, values remained around 39-40%. TS accounted for the highest values of all C/Ns, whereas ACC had the lowest.

Figure 7. Fatty acid profile of WT, ACL, ACC, and TS under different C/N ratios, categorized into SAFA, MUFA, and PUFA. SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Table 6. Calculated fatty acid profile of WT, ACL, ACC, and TS under different C/N ratios. SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, VLCFA: very long chain fatty acids.

C/N (g/g)	Fatty Acid Profile (%)												
	C14:0	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C20:0**	**C22:0**	**C24:0**	**SAFA**	**MUFA**	**PUFA**	**VLCFA**
							WT						
30	0.41 ± 0.02	22.26 ± 0.13	1.35 ± 0.09	18.07 ± 1.05	43.82 ± 0.56	11.31 ± 1.10	0.33 ± 0.23	0.30 ± 0.22	1.99 ± 0.35	43.36 ± 1.01	45.16 ± 0.49	11.48 ± 1.22	2.29 ± 0.32
120	0.29 ± 0.1	22.69 ± 0.28	1.44 ± 0.08	14.52 ± 0.39	49.59 ± 0.12	8.53 ± 0.19	1.10 ± 0.04	0.55 ± 0.02	1.12 ± 0.09	40.27 ± 0.15	51.03 ± 0.04	8.70 ± 0.19	1.67 ± 0.10
175	0.33 ± 0.1	26.91 ± 0.46	1.91 ± 0.05	11.08 ± 0.20	48.70 ± 0.35	8.83 ± 0.04	0.64 ± 0.02	0.28 ± 0.02	1.08 ± 0.06	40.32 ± 0.29	50.61 ± 0.29	9.07 ± 0.04	1.36 ± 0.05
200	0.33 ± 0.2	26.73 ± 1.42	1.82 ± 0.25	10.76 ± 1.78	49.12 ± 0.29	9.28 ± 0.75	0.51 ± 0.14	0.26 ± 0.10	0.93 ± 0.20	39.52 ± 0.79	51.02 ± 0.14	9.54 ± 0.78	1.19 ± 0.30
300	0.36 ± 0.1	27.07 ± 0.78	2.06 ± 0.12	9.87 ± 0.39	47.70 ± 0.30	10.89 ± 0.22	0.35 ± 0.03	0.13 ± 0.09	1.25 ± 0.35	39.03 ± 0.11	49.76 ± 0.21	11.21 ± 0.23	1.38 ± 0.35
							ACL						
30	0.35 ± 0.01	21.49 ± 0.33	1.25 ± 0.05	17.93 ± 0.65	45.59 ± 0.32	10.17 ± 0.70	0.64 ± 0.04	0.48 ± 0.04	1.90 ± 0.47	42.78 ± 1.01	46.84 ± 0.31	10.38 ± 0.86	2.38 ± 0.50
120	0.34 ± 0.01	26.67 ± 0.57	2.13 ± 0.10	10.90 ± 0.53	48.75 ± 0.22	8.86 ± 0.25	0.80 ± 0.06	0.33 ± 0.02	0.99 ± 0.03	40.03 ± 0.12	50.88 ± 0.12	9.08 ± 0.21	1.33 ± 0.05
175	0.36 ± 0.03	26.75 ± 0.24	2.01± 0.08	10.24 ± 0.98	48.68 ± 0.66	10.00 ± 1.20	0.51 ± 0.17	0.24 ± 0.07	0.91 ± 0.13	39.01 ± 1.01	50.69 ± 0.61	10.30 ± 1.22	1.15 ± 0.09
200	0.27 ± 0.01	21.59 ± 0.68	1.10 ± 0.06	16.13 ± 0.47	50.30 ± 0.04	7.98 ± 0.06	0.89 ± 0.03	0.44 ± 0.02	1.03 ± 0.18	40.36 ± 0.07	51.39 ± 0.06	8.25 ± 0.08	1.47 ± 0.19
300	0.30 ± 0.04	23.65 ± 2.49	1.52 ± 0.37	13.22 ± 2.84	49,45 ± 0.76	9.38 ± 0.88	0.66 ± 0.31	0.34 ± 0.16	1.17 ± 0.37	39.34 ± 0.84	50.97 ± 0.55	9.69 ± 0.90	1.51 ± 0.36
							ACC						
30	0.43 ± 0.02	23.08 ± 0.55	1.66 ± 0.02	15.99 ± 0.02	44.65 ± 0.06	11.87 ± 0.31	0.47 ± 0.01	0.26 ± 0.18	1.59 ± 0.17	41.82 ± 0.24	46.31 ± 0.08	11.87 ± 0.31	1.85 ± 0.35
120	0.35 ± 0.02	27.03 ± 1.03	2.16 ± 0.06	10.00 ± 0.58	49.08 ± 0.38	9.20 ± 0.01	0.70 ± 0.13	0.30 ± 0.06	0.91 ± 0.16	39.35 ± 0.30	51.24 ± 0.33	9.41 ± 0.03	1.21 ± 0.21
175	0.31 ± 0.02	25.17 ± 1.53	1.73 ± 0.17	12.12 ± 1.17	49.49 ± 0.60	8.60 ± 0.26	0.80 ± 0.18	0.41 ± 0.11	1.15 ± 0.13	39.95 ± 0.52	51.22 ± 0.49	8.82 ± 0.27	1.56 ± 0.21
200	0.29 ± 0.00	23.57 ± 0.34	1.43 ± 0.04	13.54 ± 0.25	50.64 ± 0.19	8.42 ± 0.03	0.73 ± 0.03	0.38 ± 0.01	0.79 ± 0.07	39.29 ± 0.18	52.07 ± 0.16	8.64 ± 0.03	1.16 ± 0.07
300	0.34 ± 0.02	25.98 ± 1.09	2.06 ± 0.23	9.02 ± 0.91	49.42 ± 0.77	11.67 ± 0.53	0.33 ± 0.07	0.16 ± 0.12	0.70 ± 0.14	36.51 ± 0.15	51.49 ± 0.60	12.00 ± 0.55	0.85 ± 0.22
							TS						
30	0.31 ± 0.00	18.68 ± 0.37	0.81 ± 0.05	22.28 ± 0.62	45.81 ± 0.23	8.55 ± 0.08	0.86 ± 0.02	0.60 ± 0.02	1.65 ± 0.04	44.38 ± 0.30	46.62 ± 0.27	8.99 ± 0.06	2.25 ± 0.05
120	0.30 ± 0.00	19.74 ± 0.40	0.71 ± 0.03	21.60 ± 0.23	46.86 ± 0.09	6.72 ± 0.06	1.22 ± 0.06	0.67 ± 0.02	1.46 ± 0.03	44.99 ± 0.10	47.57 ± 0.06	7.44 ± 0.06	2.13 ± 0.05
175	0.30 ± 0.02	23.05 ± 2.33	1.34 ± 0.40	15.20 ± 2.98	48.77 ± 0.08	8.46 ± 0.82	0.79 ± 0.12	0.42 ± 0.10	1.19 ± 0.17	40.95 ± 1.02	50.11 ± 0.32	8.94 ± 0.70	1.61 ± 0.27
200	0.27 ± 0.01	21.35 ± 1.03	1.04 ± 0.23	17.08 ± 1.66	48.68 ± 0.32	8.10 ± 0.38	0.85 ± 0.11	0.52 ± 0.05	1.47 ± 0.15	41.54 ± 0.83	49.72 ± 0.54	8.74 ± 0.28	1.99 ± 0.18
300	0.30 ± 0.00	23.05 ± 0.07	1.21 ± 0.03	13.22 ± 0.34	48.64 ± 0.08	10.11 ± 0.18	0.72 ± 0.01	0.47 ± 0.01	1.43 ± 0.11	39.19 ± 0.29	49.85 ± 0.09	10.95 ± 0.19	1.91 ± 0.11

3.2 Construction of double-overexpressing transformants

As overexpression of ACL, ACC, and TS increased lipid accumulation in *C. oleaginosus*, in this part, we focused on the effect of these target genes in a combination of two and three. To test this, we first sought to build these overexpressing strains by deploying the co-transformation method and random integration of the genes into the *C. oleaginosus* genome.

There were different options for creating the strains ACL+TS, TS+ACL, TS+ACC, and ACL+ACC. We used ACL and TS as background strains for transformation **(Table 7)**. We used the hygromycin B phosphotransferase (HPT) gene that confers resistance to the antibiotic hygromycin B as a selection marker. To validate correct gene integration, we performed colony PCR with corresponding primers for each gene **(Table 4)**. Based on the 1% gel electrophoresis visualization of the colony PCR products, colonies that were positive for all required genes were stored for downstream processes. One of the successful integrations was co-transforming ACL with ACC and HPT gene-containing plasmids, the second colony was selected, as it was positive for all required genes **(Figure 8)**.

Table 7. Overview of the new build strains with the background strains and integrated plasmids.

Background Yeast	Plasmids Integrated	Generated Strain
ACL	TS + HPT	ACL + TS
ACL	ACC + HPT	ACL+ACC
TS	ACL + HPT	TS+ACL
TS	ACC + HPT	TS+ACL
ACC	TS_ACL + HPT	-
ACC	ACL + HPT	-
ACC	TS + HPT	-

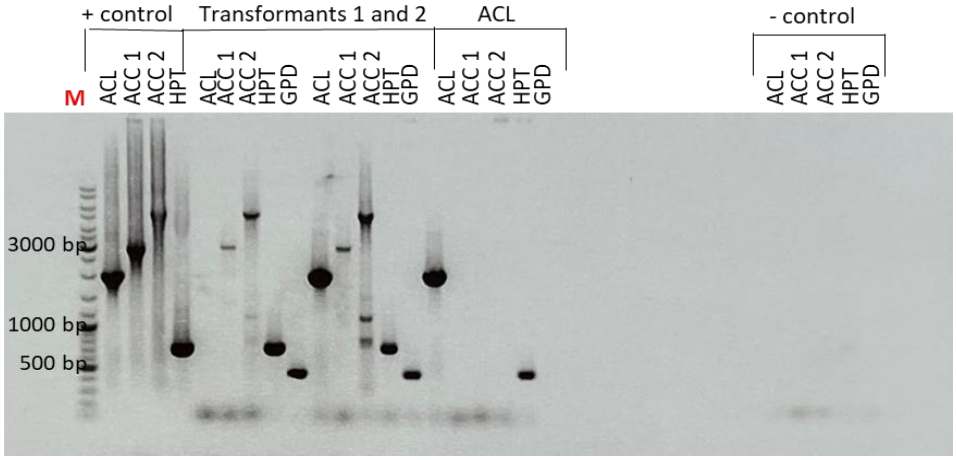

Figure 8. Gel image of colony PCR products from co-transforming ACL with the ACC and HPT gene-containing plasmids. Positive and negative controls were prepared by adding respectively plasmids and sterile MilliQ water instead of genomic DNA. The GPD gene was used as an internal PCR control, to assess the quality of genomic DNA. + control: positive control, - control: negative control, M: marker, ACL: part of ATP-citrate lyase gene (1969 base pairs bp), ACC1: part of acetyl-Coa carboxylase gene (2808 bp), ACC2: part of acetyl-Coa carboxylase gene (4964 bp), HPT: part of hygromycin phosphotransferase gene (708 bp), GPD, part of glyceraldehyde-3-phosphate dehydrogenase gene (447 bp).

In addition to the mentioned strains, we attempted to build combinations of ACC+TS+ACL, ACC+TS, and ACC+ACL, using ACC as the background for transformation. Following colony PCR, there was successful amplification of the target integrated genes from the genomic DNA **(Figure 9, colonies 1-4)**. Unfortunately, in all the attempted PCRs, no successful amplicon of the ACC gene could be detected. Therefore, we could not be confident in obtaining the desired strains when ACC was transformed.

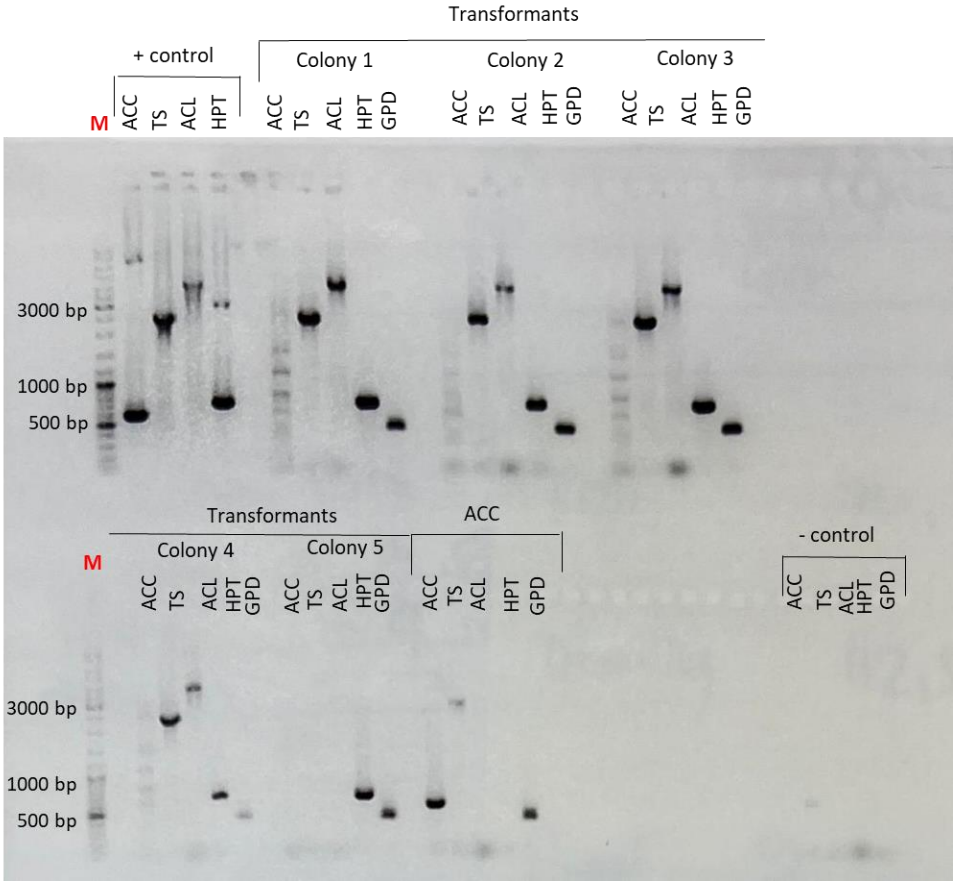

Figure 9. Gel image of colony PCR products from co-transforming ACC with the TS_ACL and HPT gene-containing plasmids. Positive controls were prepared by adding corresponding plasmids instead of genomic DNA. Negative controls were prepared by adding sterile MilliQ water. The GPD gene was used as an internal PCR control, to assess the quality of genomic DNA. + control: positive control, - control: negative control, M: marker, ACL: part of ATP-citrate lyase gene (1969 base pairs bp), ACC: part of acetyl-Coa carboxylase gene (596 bp), HPT: part of hygromycin phosphotransferase gene (708 bp), GPD, part of glyceraldehyde-3-phosphate dehydrogenase gene (447 bp).

3.3 Shake flask cultivation of the double gene overexpressing strains

The growth profile and lipid accumulation of the newly designed strains were tested under two C/N conditions, namely C/N 30 g/g and C/N 175 g/g. Lipid accumulation data were obtained via colorimetric lipid assay. Their biomass and lipids accumulated were compared to their background strains, ACL, and TS **(Table 8) (Figure 10).**

Generally, there was a significant increase in lipid content across all newly engineered strains, with values ranging from 36.90 ± 3.91 %, w/w (ACL+ACC, C/N 30, 72h) to 70.91 ± 3.73 %, w/w (ACL+ACC, C/N 175, 96h). Two high-performing strains stood out, TS+ACC and ACL+ACC. Under C/N 175, they both accumulated lipids of about 70 % of their biomass, a respective boost of 49% and 38% when compared to TS and ACL. Looking at the lipid weight at C/N 30, ACL+TS had the highest boost, 1.4 times more than ACL. Looking at the lipid weight at C/N 175, TS+ACL had the highest boost, 1.5-fold to TS. ACL+ACC, however, has the highest weight in lipids at 4.66 ± 0.24 g/L. It was also interesting to observe the TS+ACL and ACL+TS strains, since they contained the same genetic information. Nonetheless, it was intriguing to see that these strains do behave differently when characterized under C/N 30 and 175, from 72 to 96 hours post-inoculation. Either of them accumulated significantly more lipids than their backgrounds TS and ACL, about 1.3-fold for TS+ACL and 1.12 for ACL+TS. Performing the student's t-test between these two strains showed no significant difference. Another interesting observation was that under the C/N 30 condition, the new strains demonstrated rapid lipid accumulation. After this rapid accumulation, lipid production seemed stable until the 96-hour mark, except for TS+ACC and ACL+ACC. When looking at the lipid weight, this stableness was translated to all the strains at C/N 30.

Table 8. Characterization of new strains under C/N ratios 30 and 175. Values significantly higher than the controls ACL and TS are indicated with Asterix (∗) based on calculated p-values. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

		Lipid Content (%, w/w)		Dry Cell Weight (g/L)	
Strain	**Time (h)**	**C/N 30 (g/g)**	**CN/175 (g/g)**	**CN/30 (g/g)**	**CN/175 (g/g)**
WT	**72**	23.18 ± 1.00	33.4 ± 3.27	3.83 ± 0.12	5.20 ± 0.24
	96	34.32 ± 0.89	46.99 ± 3.38	4.16 ± 0.23	5.54 ± 0.34
ACL	**72**	25.46 ± 2.00	34.82 ± 0.94	4.30 ± 0.15	5.47 ± 0.08
	96	37.81 ± 1.26	51.45 ± 1.40	4.57 ± 0.08	5.64 ± 0.08
TS	**72**	34.45 ± 1.18	38.99 ± 1.71	5.11 ± 0.41	5.80 ± 0.31
	96	40.60 ± 0.77	47.38 ± 0.31	5.06 ± 0.13	6.03 ± 0.33
ACC	**72**	28.64 ± 2.91	36.41 ± 1.99	4.29 ± 0.21	5.36 ± 0.00
	96	38.92 ± 1.61	51.73 ± 1.12	4.41 ± 0.16	5.58 ± 0.08
TS+ACL	**72**	44.48 ± 1.45 ***	57.64 ± 4.39 **	5.36 ± 0.24	5.52 ± 0.14
	96	42.57 ± 1.28	61.35 ± 1.63 **	5.75 ± 0.28 *	7.03 ± 0.00 *
ACL+TS	**72**	39.22 ± 2.53 **	49.14 ± 3.45 *	5.51 ± 0.13 ***	5.30 ± 0.07 *
	96	40.35 ± 1.66	57.64 ± 2.64 *	5.84 ± 0.23 **	6.24 ± 0.21 *
TS+ACC	**72**	37.83 ± 2.25	63.06 ± 3.28 **	5.89 ± 0.21 *	4.95 ± 0.28 *
	96	45.57 ± 2.51 *	70.61 ± 3.66 **	4.95 ± 0.21	5.68 ± 0.47
ACL+ACC	**72**	36.90 ± 3.91 *	49.14 ± 3.27 **	6.07 ± 0.21 ***	6.80 ± 0.08 ***
	96	45.54 ± 2.05 **	70.91 ± 3.73 **	5.12 ± 0.08 **	6.41 ± 0.16 **

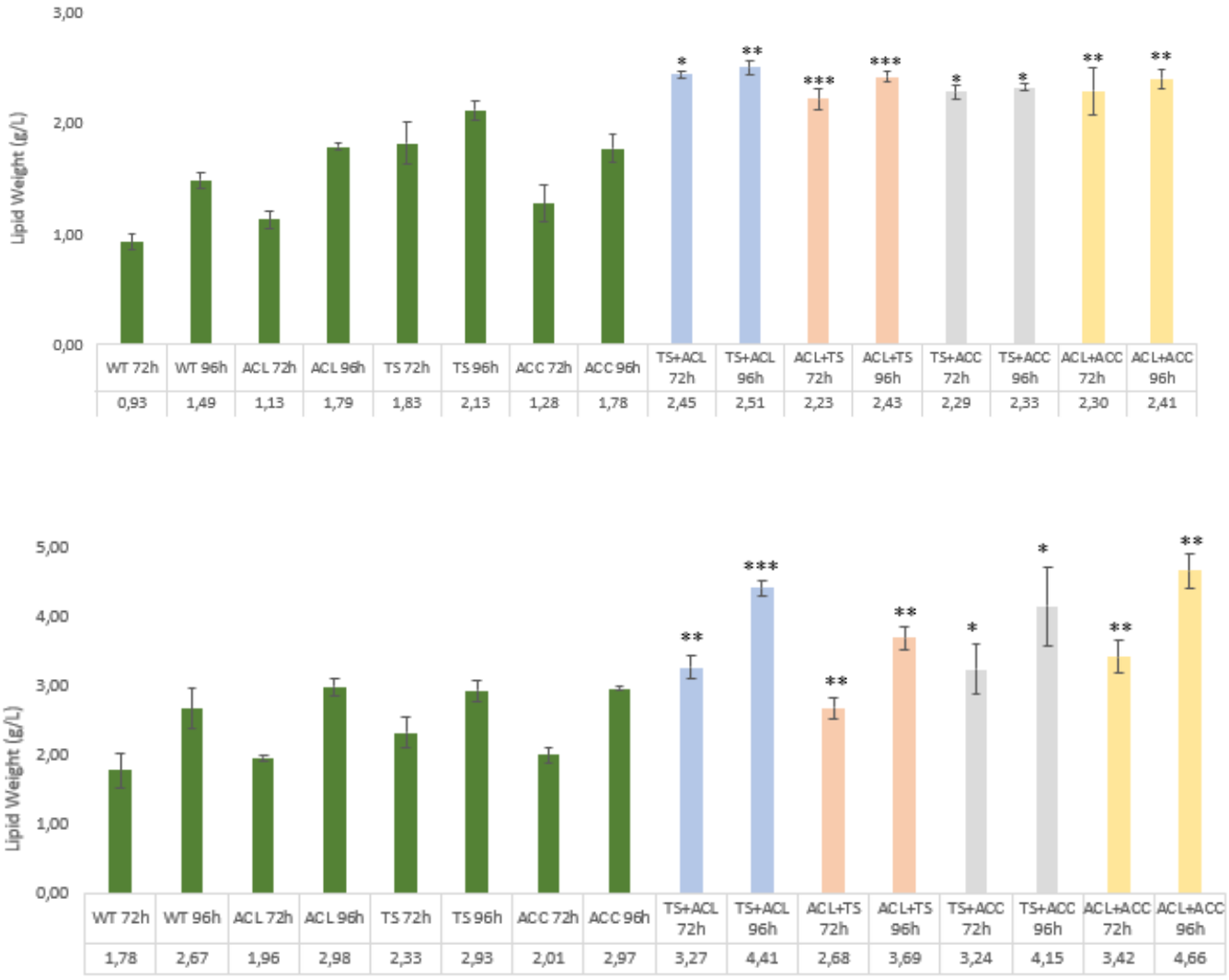

Figure 10. Lipid weight of the new strains under C/N 30 (up) and 175 (down). Values significantly higher than the controls ACL and TS are indicated with Asterix (∗) based on calculated p-values. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

IV. Discussion

C. oleaginosus is a robust yeast able to grow on a wide range of substrates, pH, and temperature, as well as tolerate fermentation inhibitors while accumulating high amounts of lipids with a similar constitution to palm oil(Abeln & Chuck, 2021; Bracharz et al., 2017). Due to these assets, it has been flagged as a cell factory candidate to produce sustainable alternatives to palm oil. Nevertheless, the production process is still not economically feasible, stimulating extensive research on enhancing lipid accumulation. It has been previously reported that carbon to nitrogen C/N ratio significantly improves lipid production. To that end, we evaluated the growth, fatty acid accumulation, and fatty acid composition of WT, ACL, ACC, and TS *C. oleaginosus* at a C/N ratio ranging from 30 to 300 g/g. Regression analysis was performed to determine the optimum C/N ratio for fatty acid content and growth in said strains. Moreover, we created transformants expressing the target genes in combinations of two and tested them under a C/N ratio of 30 and 175 g/g.

Previously, *C. oleaginosus* has been tested under different C/N ratios, resulting in an optimal C/N ratio of 175 when glycerol and urea are used as carbon and nitrogen sources. Based on tested experimental conditions as well as the in silico predictions, we selected a range from low to high C/N ratio, 30 to 300 g/g. We tested not only WT but also transformants previously built. Under such conditions, TS outperforms the rest of the strains in terms of biomass (8.68 g dry cell weight/L at C/N 120) and fatty acid content 50.54 0.58 %, w/w at C/N 200). ACL and ACC have a significant increase when compared to WT at C/N 200 g/g at about 48%, w/w. We observe a peculiar behavior of TS as it stably produces high amounts of lipids from C/N 30 to 300 g/g. This adds to its robustness and could be beneficial when using waste streams that do not have a defined medium. Comparing ACL and ACC to each other, they follow similar trends in both cell growth and lipid accumulation, not surprising since both these enzymes are part of the same pathway one after the other **(Figure 1)**. The WT and overexpressing strains ACL, ACC, and TS have been previously tested under C/N ratios 30 and 175 g/g, where higher lipid contents were reported. On the other hand, our tested conditions generate more biomass. These experiments have been executed the same way, apart from the volume and container. In our case the cultivation volume is 10 mL and in filtered-cap tubes, differently from the reported 60 mL volume in 300 mL shake flasks from Duman-Özdamar et al., unpublished. We speculate that these differences affect factors like aeration, oxygen transfer, mixing efficiency, source availability, or shear stress. For example, in the tubes, there is gentler mixing, leading to less shear stress, hence better cell growth. However, this can also lead to less efficient mixing that would impact lipid production. On the other hand, aeration differences, hence different oxygen levels, might have impacted metabolic pathways that also lead to less lipid synthesis. At this stage we can only speculate, as a more comprehensive approach needs to be undertaken to draw conclusions.

Duman-Özdamar et al., 2022 applied RSM, as an efficient and less laborious method, and concluded that the optimal C/N ratio (g/g) for lipid production in *C. oleaginosus* is 175 whereas for growth, 185. Here we performed regression analysis by implementing the data experimentally obtained with tubes from C/N 30 to 300. Comparing optimal C/N of WT between the two experiments, there is a small shift of about 2.5 % for biomass, whereas for lipid accumulation this shift increases by about 17.5%. As aforementioned, this shift could be related to the difference in cultivation between tubes and shake flasks. As for the overexpressing strains, this is the first study to optimize their C/N ratio as far as we know. There is variance in the reported values, which adds to these strains exhibiting somewhat different characteristics. To be considered is also the quality of the models built. Based on R^2

and p-value, we have a good representation of WT (biomass and lipid accumulation), ACL (lipid accumulation), and ACC (biomass and lipid accumulation). The cases with lower quality values are explained by the fact that tested C/N ratios were picked based on research with the WT. Moreover, the TS strain produces stable amounts of lipids under all tested C/N ratios, which does not fit the nature of a second-order polynomial equation. Nevertheless, the newly predicted optimal C/N ratios act as a starting point for future optimization studies. Noteworthy to mention, cell growth and lipid production are both phenomena affected by multiple factors and their interactions (temperature, pH), which were not considered in our preliminary models.

Another important aspect of utilizing *C. oleaginosus* as a candidate cell factory for alternative palm oil is the composition of fatty acids of the microbial oil produced. Palm oil is found in about 50% of supermarket goods and the application of it to these products is highly dependent on its constitution. Saturation level and chain length impact the melting point of fatty acid, which is an important factor related to the consistency of the oil and the product it could be applied to. Unsaturated and shorter chain-length fatty acids display lower melting temperatures and the other way around (Duman-Özdamar et al., 2022; Fasina et al., 2008). Taking that into account, we need to look into what happens to the fatty acids of the *C. oleaginosus* oil from different transformed strains as well as under different nitrogen-limiting conditions. From the conducted analysis of the fatty acids profile, we observed an increase in unsaturated fatty acids with increasing C/N ratio. As for the saturated ones, there is a decrease with an increasing C/N ratio. TS shows high levels of SAFAs and VLCFAs and the lowest in unsaturated fatty acids. ACC exhibits the opposite characteristics. Based on our observations, we further established that by changing the C/N ratio and transformed strain we can steer the chain length and saturation level of the fatty acids produced. Data from our research can aid in selecting between a range of C/N ratios and strains, based on the desired application of the microbial oil. Moreover, future studies with these transformed strains could complement our observations by employing another important factor in tailoring fatty acid composition, temperature.

When it comes to genetically modifying *C. oleaginosus*, it has been successfully transformed before with means of random integration or agrobacterium-mediated gene transfer(Görner et al., 2016; Stellner et al., 2023). Despite being challenging, integrating more than one gene at the same time has been successfully reported. In our case, we were triumphant in transforming TS and ACL cells, but not ACC. Since we could not amplify the ACC gene in any of the transformants post-integration, we speculate there might be an affinity for the ACC locus by the new plasmids, for reasons unbeknownst to us. Quantitative PCR might be used to shed light on this matter. An alternative to creating the triple overexpressing strain would be to use TS or ACL as background strains since we report successful integration of all required genes in these strains.

Despite the transformation bottlenecks, we obtained 4 new strains expressing the ACL, ACC, and TS target genes in different combinations of two. These transformants underwent early characterization under C/N 30 and 175 g/g. Based on the calculated lipid accumulation, a range from 57.64 ± 2.64 %, w/w (ALC+TS) to 70.91 ± 3.73 %, w/w (ALC+ACC) under C/N 175 was reached. These values show a significant increase when compared to the background strains ACL and TS. These values however account for the total lipid accumulation of the cells, and gas chromatography should be performed to evaluate concentrations of fatty acids produced. Looking into the combinatorial effect of the target genes we can confidently say that together they have a greater boost to lipid accumulation than the controls WT, ACL, ACC, or TS. At C/N 175, lipid production in TS+ACL is boosted by 30%, ACL+TS by 12%, TS+ACC by 49%,

and in ACL+ACC by 38%. The addition of either ACL or ACC gives a higher boost since these genes are directly involved in the lipid synthesis pathways. TS, on the other hand, might enhance lipid formation by indirectly entering the TCA cycle or is involved in pathways affecting cell growth. At this stage, this is only the beginning of experimenting with these strains. Future studies might reveal characteristics that can later be tailored to the production requirements.

V. Conclusions and Future Recommendations

Altogether, in this study, we explored strategies for further enhancement of lipids via medium optimization and genetic interventions. Through a series of experiments, we explored the growth, fatty acid content, and fatty acid composition of WT, ACL, ACC, and TS *C. oleaginosus* under C/N ratios from 30 to 300 g/g. The TS strain stood out in both growth (8.68 ± 0.00 g/L) and fatty acid accumulation (50.54 ± 0.58 %, w/w). It also revealed stable, high amounts of fatty acids through the C/N ratios. ACL and ACC also demonstrated increased lipid content, with ACL showing a peak at C/N 175-200 g/g and ACC reaching higher levels at C/N 200 g/g. Furthermore, following the fatty acid composition we conclude that manipulating genes expressed in *C. oleaginosus* as well as the C/N ratio allows tailoring of the fatty acid profile production of specific types of microbial oils with applications in various industries. Genetically modifying this yeast with target genes in combinations of two led to two outstanding lipid production (about 70 %, w/w) strains TS+ACC and ACL+ACC, a respective boost of 49% and 38% compared to TS and ACL. This study demonstrates the successful enhancement of lipid production of *C. oleaginosus* and takes the process one step further toward economic feasibility. We hope that these insights will contribute to replacing palm oil with microbial oil, ensuring a sustainable future.

A continuation of this research would be to test the newly designed strains under additional C/N ratios, especially at 200 g/g. Moreover, the fatty acid content and fatty acid composition should be evaluated. To complete the aim of studying the combinatorial effect of the target genes, the triple overexpressing strains should be achieved and tested under different C/N ratios. Especially interesting would be to test the TS+ACC strain supplemented with biotin and threonine, since they have previously significantly enhanced lipid accumulation. Furthermore, efforts should be made to transition from lab to industrial scale and even explore the strain's productivity when using side streams. Lastly, the stability over long production times of the new strains should be established.

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Appendices

Appendix A: Supplementary protocols

Colorimetric lipid assay: standard sample preparation

20 µL of *C oleoginosus* oil was added to 3980 µL chloroform. From this mixture, 15, 30, 60, 90, and 120 µL were transferred to empty glass centrifuge tubes. Chloroform was evaporated under a nitrogen gas stream with a sample concentrator. Then 100 µL MilliQ water was added to each tube.

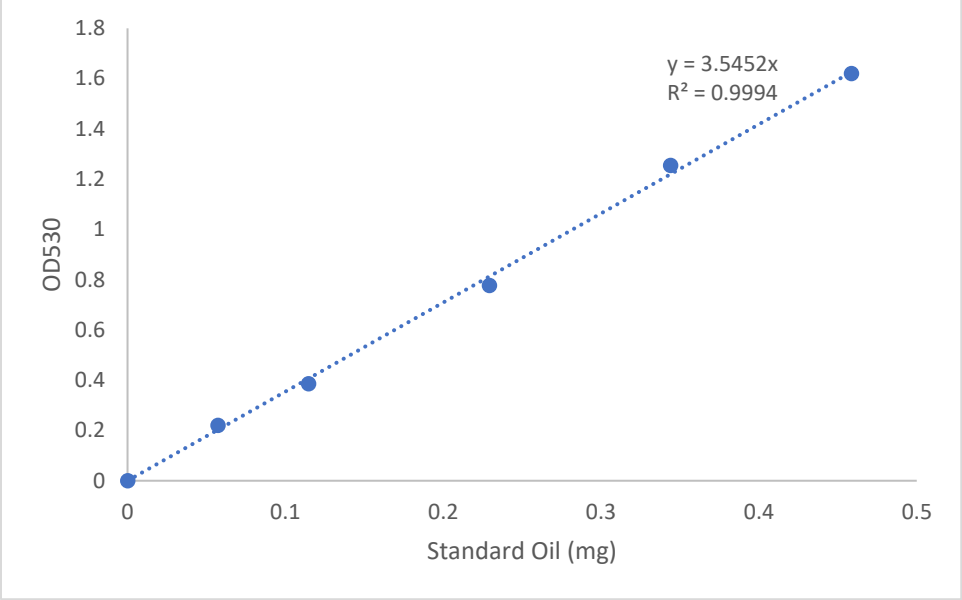
Colorimetric lipid assay: vanillin stock preparation

In 500 mL bottles, 0.3 g vanillin was dissolved with 5 mL pure ethanol and 45 mL MilliQ water, continuously stirring. To that 200mL 85% phosphoric acid was added, followed by mixing well. Vanillin stock was kept in the dark.

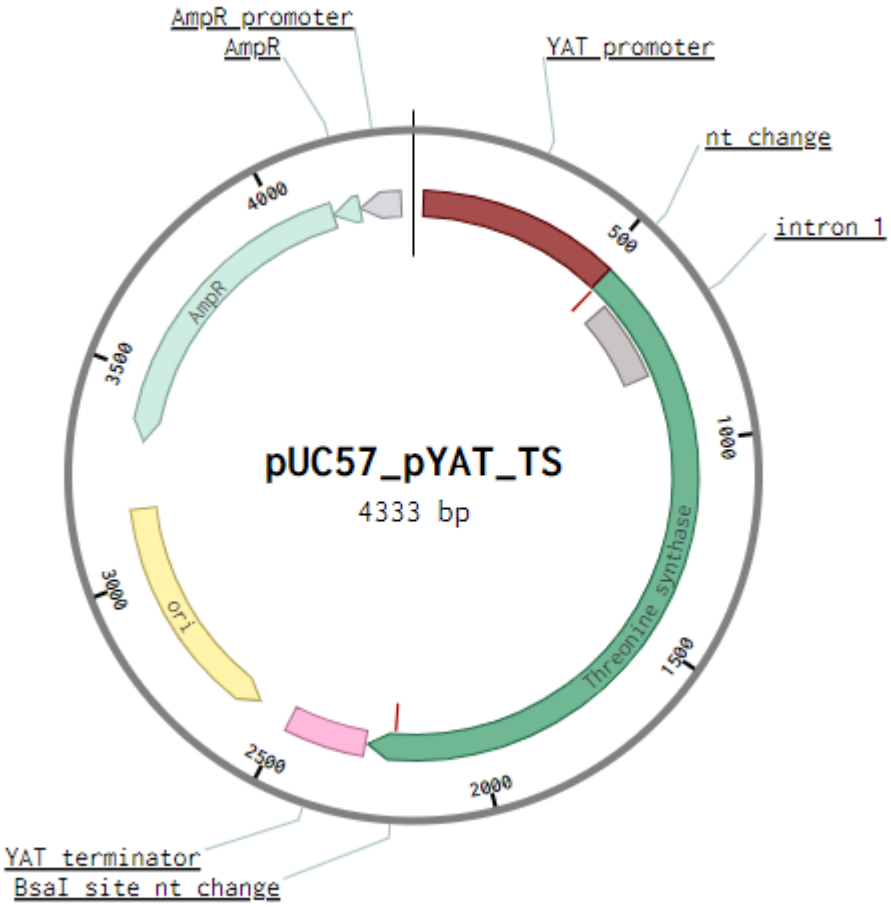
Colorimetric lipid assay: culture samples analysis

Collected samples from each culture condition were dissolved in 100 µL MilliQ water and transferred to empty glass centrifuge tubes. 2 mL 95-98% sulphuric acid was added to the tubes, followed by mixing and heating for 10 minutes at 100 ºC. After that, samples were cooled for 5 minutes in ice water. 5 mL of prepared vanillin stock was added to each tube and mixed well. Tubes were incubated at 37 ºC in a water bath while mixing every 5 minutes. OD at 530 nm was measured.

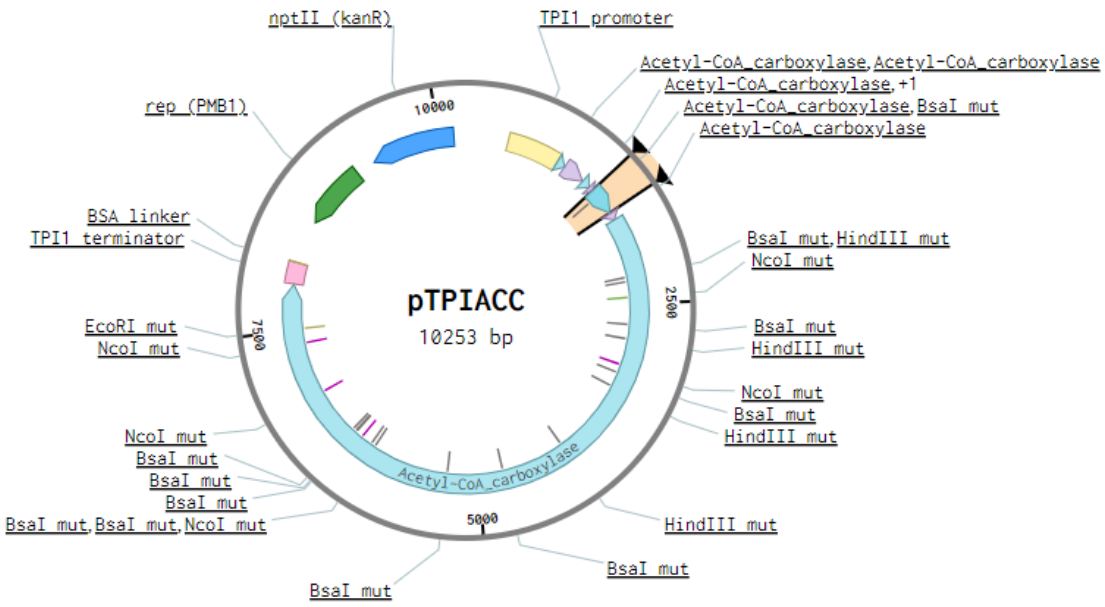
Appendix B: Supplementary figures



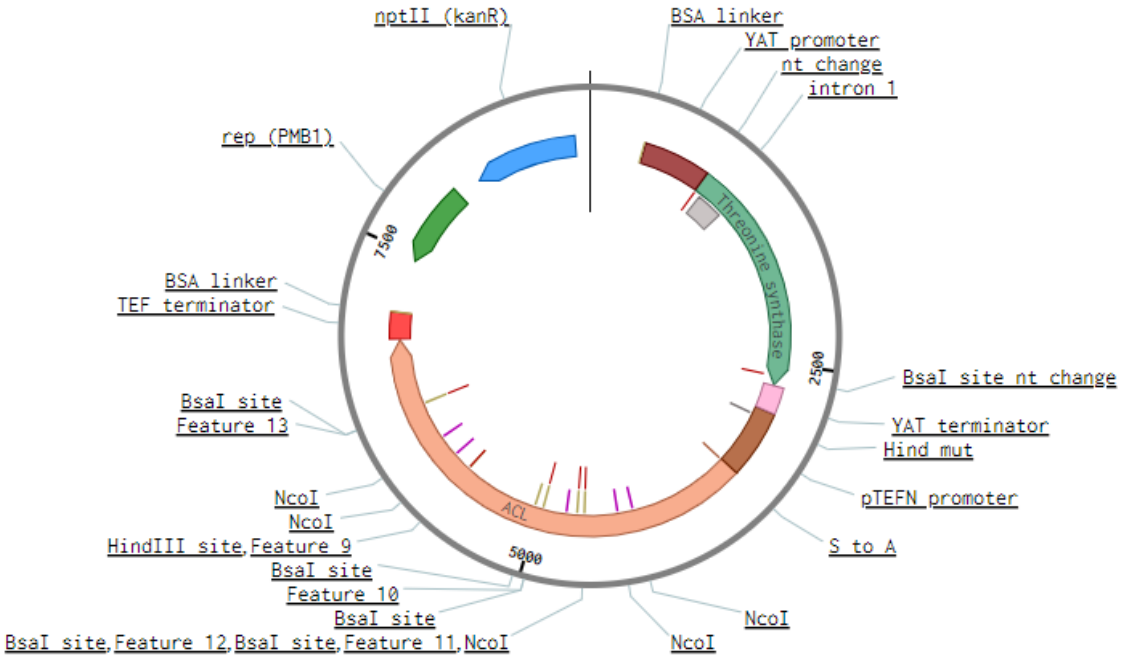
Supplementary Figure 1. Standard calibration curve to quantify lipids via colorimetric lipid assay. OD: optical density.



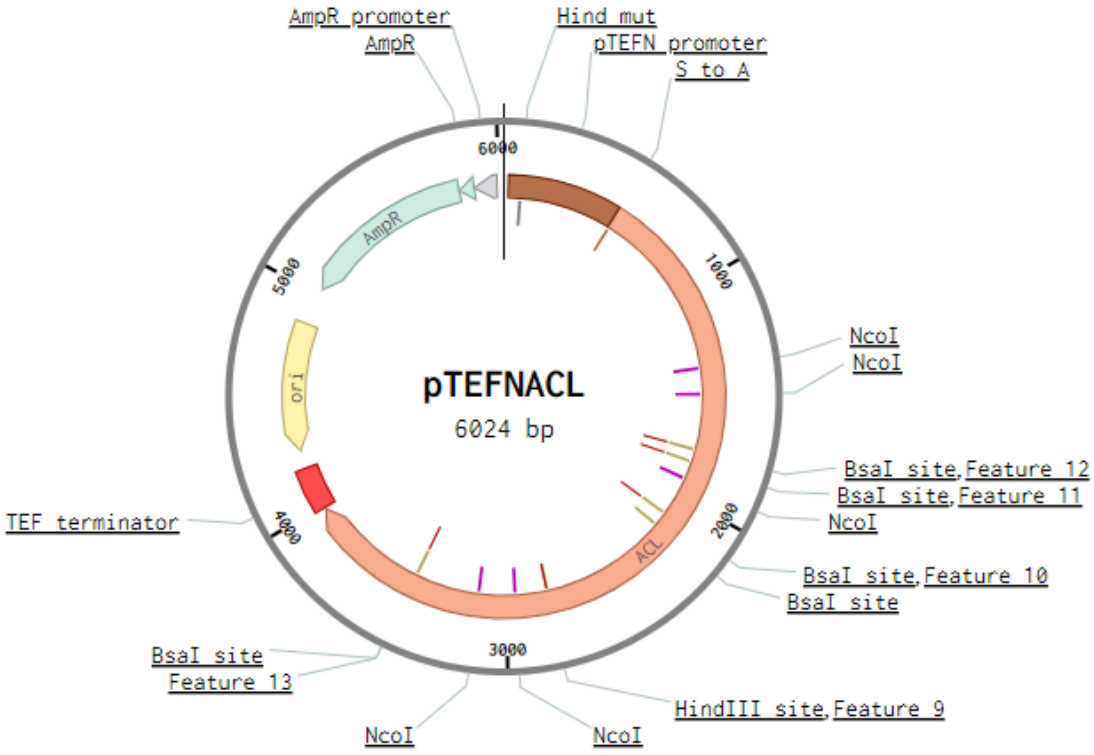
Supplementary Figure 2. Plasmid map containing the TS gene.



Supplementary Figure 3. Plasmid map containing the ACC gene.



Supplementary Figure 4. Plasmid map containing the TS and ACL genes.



Supplementary Figure 5. Plasmid map containing the TS and ACL genes.