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Combinatorial Engineering of *Yarrowia lipolytica* as a Promising Cell Biorefinery Platform for the *de novo* Production of Multi-Purpose Long Chain Dicarboxylic Acids

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Abstract: This proof-of-concept study establishes *Yarrowia lipolytica* (*Y. lipolytica*) as a whole cell factory for the *de novo* production of long chain dicarboxylic acid (LCDCA-16 and 18) using glycerol as the sole source of carbon. Modification of the fatty acid metabolism pathway enabled creating a pool of fatty acids in a β-oxidation deficient strain. We then selectively upregulated the native fatty acid ω-oxidation pathway for the enhanced terminal oxidation of the endogenous fatty acid precursors. Nitrogen-limiting conditions and leucine supplementation were employed to induce fatty acid biosynthesis in an engineered Leu$^-$ modified strain. Our genetic engineering strategy allowed a minimum production of 330 mg/L LCDCAs in shake flask. Scale up to a 1-L bioreactor increased the titer to 3.49 g/L. Our engineered yeast also produced citric acid as a major by-product at a titer of 39.2 g/L. These results provide basis for developing *Y. lipolytica* as a safe biorefinery platform for the sustainable production of high-value LCDCAs from non-oily feedstock.

Keywords: *Yarrowia lipolytica*; long chain dicarboxylic acid; building blocks; citric acid; glycerol; genetic and metabolic engineering; fermentation; bioconversion

1. Introduction

Growing demand for petroleum dependent chemicals, in addition to surging environmental concerns, has inspired increased use of renewable resources. The sustainable production of monomers and polymers is of particular interest for reducing petroleum feedstock dependency and CO_2 emissions. The environmental cost of using petrochemical polymers can be lowered by replacing them with bio-based polymers, produced through fermentation using biomass or byproducts as feedstock [1]. Glycerol is the main byproduct of biodiesel production process, and its efficient valorization would help offset biodiesel production costs. Such bioconversion also decreases the environmental impact of waste streams. Despite various technological successes, it is still challenging to establish an integrated bioprocess, at commercial scale, for the valorization of such hydrophilic substrates into desirable compounds [2]. Nonetheless, the development of high productive strains and high value co-products advance the commercialization of such bioprocesses.

Bulk monomers of high value are commonly targeted co-products for industrial biotechnology. LCDCA monomers have a much higher value than regular free fatty acids. These building block monomers are used to make various polyamides, polyester and, polyurethanes [3] in the bio-plastic and coating industries. Their use enables the synthesis of various novel polymers with enhanced

hydrolytic stability, solvent resistance, optical clarity, and flexibility properties [4]. They can also be used in the automotive, food, medical, and chemical industries as adhesives, corrosion inhibitors, lubricants, fragrances, surfactants, antiseptics, and personal care ingredients [5]. Non-renewable petroleum-derived alkanes or plant-derived fatty acids that compete with food production are currently the main hydrophobic feedstock for the production of these multi-purpose building blocks [6–8].

A chemical-based approach for the production of these monomers from hydrophobic substrates has already been established. For instance, the chemical degradation of plant oil fatty acids to LCDCAs via self-metathesis reaction is one of the major chemical-based production routes [9]. The chemical-based production of LCDCA is an energy-intensive and environmentally unfriendly process, which requires expensive catalysts and cost intensive purification steps due to the generation of various by-products. Contrary to chemical approaches, the bio-based approach offers higher selectivity and sustainability [10]. The microbial production of short to medium chain DCAs has been reviewed and partly industrialized [10–13]. In terms of LCDCAs, the major producing strain is a pathogenic yeast, *Candida tropicalis* [14].

The biosynthetic pathway to LCDCA monomers is mediated by the P450 system. In general, cytochrome P450 monooxygenases (P450 or CYP) systems are involved in the oxygenation of various hydrophobic compounds. For instance, the CYP52 family performs terminal ω-oxidation of alkanes, alcohols, and fatty acids of various chain lengths [15]. This family belongs to yeast species, including *Candida* and *Yarrowia*, and is comprised of members whose products have different specificities for alkanes versus fatty acids [16,17]. The yeast CYP52 has higher productivity than the bacterial ω-hydroxylases [18]. Terminal oxidation by P450 is carried out at high regioselectivity and stereoselectivity. This can result in the synthesis of high value compounds whose production is nearly impossible through chemical approaches due to the instability of double bonds or the generation of unwanted byproducts [19]. Several examples of cytochrome P450 monooxygenases with terminal ω-oxidation activity toward fatty acids have been reported [20–27]. Some are also capable of fatty acid over-oxidation to their corresponding dicarboxylic acids [23,26,28]. The mechanism of a P450 mediated ω-oxidation process has been discussed [29], and is briefly presented here.

This nicotinamide adenine dinucleotide phosphate (NADPH) dependent reaction takes place under mild conditions and requires molecular oxygen. In summary, an active intermediate is formed when the heme group of P450 monoxygenase is coupled to molecular oxygen. As a result, oxygen is added to the attached substrate, the C-H bond of fatty acid. Subsequently, another atom of oxygen is released in the form of a water molecule, and a hydrogen atom is transferred from the substrate to the heme group, leading to the generation of hydroxylated substrates. During this process, the P450-diflavin reductase partner (CPR) is bound to the NADPH cofactor to derive electrons from that while positioning the redox active nicotinamide ring and flavin adenine dinucleotide (FAD) at a close distance. The FAD accepts hydride ions from the NADPH and transfers electrons to the flavin mononucleotide (FMN) to reduce the heme group of the P450 monooxygenase [30]. This complex enzymatic system has some limitations, such as low activity and stability, dependency on cofactor and redox partner proteins, and low product yields for broad industrial usage [19,31]. Moreover, eukaryotic CYPs are often associated with ER membranes [32] and have lower solubility and stability compared to bacterial ones. High-density cultivation of P450-carrying cells or modification of the P450 system for higher expression and stability can help overcome these limitations in a bioreactor application [15].

Yarrowia lipolytica is an advantageous biorefinery platform for the production of fatty acid-based bioproducts [33] and for P450 catalytic bioconversion [34]. The P450-dependent hydroxylation of alkane, fatty alcohol, and fatty acid precursors is predominant in this yeast [35,36]. This assists with detoxification and degradation of these potentially toxic molecules. The overexpression of homologous and heterologous P450 has been employed in *Y. lipolytica* [37] resulting in a remarkable proliferation of ER [38]. There are at least 17 P450 genes and some electron transfer proteins in this oleaginous yeast [38]. Among them, twelve P450 Alk genes (*YlALK*) and a single NADPH P450 reductase gene,

YlCPR, have been isolated and characterized [32,36,38,39]. They have various substrate requirements with respect to length, degree of unsaturation, and functional groups [40]. For instance, *YlALK 3*, *YlALK 5*, and *YlALK 7* can catalyze the ω-oxidation of fatty acids [41]. The resulting ω-hydroxy fatty acids undergo further steps of oxidation that could also constitute rate limiting steps in this yeast. These steps can be carried out by fatty alcohol oxidase (*FAO1*; YALI0B14014g) and by the alcohol dehydrogenase genes (*FADHs*) in the ER and peroxisome [42,43]. The fatty acid precursors and their corresponding oxygenated fatty acid monomers are susceptible to the effective peroxisomal β-oxidation degradation in *Y. lipolytica*, resulting in the generation of energy, water, and CO_2. The *POX* genes are involved in the degradation of these monomers [44,45]. The disruption of the β-oxidation pathway via deletion of *POX* genes is commonly employed to prevent fatty acid and LCDCA degradation and unwanted chain modification [14,46,47]. In this yeast, the overproduction of fatty acids can be efficiently achieved in the form of free fatty acids rather than fatty acyl-CoAs, since the production of the latters are highly regulated by feedback inhibition [48,49]. In fact, the ω-oxidation of free fatty acids can favor their degradation and alleviate their toxicity. The ω-oxidation pathway acts as a rescue route in the peroxisome-deficient cell by generating oxygenated fatty acids of higher solubility in water [50,51].

Engineering *Y. lipolytica* for the *de novo* production of LCDCAs enables utilization of various unrelated feedstock, including sugars, glycerol, and short chain volatile fatty acids, for this process. *Y. lipolytica* has a preference for using glycerol as a carbon source [32]. Glycerol and glucose have a transcription-repressive effect, with various degrees of strength, on background P450 activities in this yeast [52]. Glycerol has a strong repressor effect, so the overexpression of selected P450 members in the presence of this substrate can enable higher selectivity through the minimization of background ω-oxidation activity. *Y. lipolytica* accumulates a larger proportion of endogenous unsaturated long chain fatty acids when grown on glucose [53]. The preservation of double bonds through the terminal ω-oxidation results in the generation of multi-functional unsaturated LCDCAs. These double bonds enable the attachment of additional functional groups [54] and the generation of cross-linked degradable polymer networks with adjustable properties [55].

In this study, we engineered *Y. lipolytica*, a "safe-to-use" yeast [56], for the *de novo* production of high value LCDCA monomers. This involved a one-step bioprocess, using glycerol as the sole source of carbon. This was done by inactivating the endogenous cytosolic fatty acyl-CoA synthetase, *YlFAA1* YALI0D17864g, and redirecting the resulting pool of free fatty acids toward an engineered ω-oxidation pathway, in a deficient β-oxidation background. The ω-oxidation pathway was engineered by overexpressing P450 reductase (YALI0D04422g), fatty alcohol oxidase *YlFAO1*, and a selected member of P450 monoxygenases (*YlALK5*, YALI0B13838g). After preliminary screening and shake flask cultivations, we used a 1-L bioreactor for the P450-based biocatalytic system and achieved a productivity of 0.04 g/L·h, which is more than the process productivity of 0.001 g/L·h acceptable for P450-based biocatalytic production of pharmaceuticals [19]. Our results demonstrated the promise of *Y. lipolytica* for use as an oleaginous yeast cell factory for LCDCA production. The safe status of this yeast enhances the suitability of the resulting LCDCA monomers for food and pharmaceutical uses, where high security standards should be met. To the best of our knowledge, this is the first reported study on the biosynthesis of LCDCAs from glycerol by engineered *Y. lipolytica*. The findings of this work present a unique biosynthetic route that is expected to advance *Yarrowia* platform for the sustainable production of these multi-purpose long chain building blocks.

2. Materials and Methods

2.1. Strains and Culture Conditions

Escherichia coli top 10 was used for plasmid DNA construction and propagation. According to the standard protocols, Luria-Bertani (LB) broth or agar medium was made and supplemented with ampicillin at the concentration of 100 $\mu g \cdot mL^{-1}$ [57]. The recombinant *Y. lipolytica* strains of this study

were all derived from *Y. lipolytica* H222 (a potential citric acid-producer wild-type German strain) [42] and are presented in Table 1

Table 1. *Y. lipolytica* strains used in this study.

***Y. lipolytica* Strains Names**	**Strain Genotypes**	**Gene Configurations**	**Reference**
H222 (W) [wild type]	*MatA*		[42]
H222ΔP (HP-U) [*leu*$^+$, *ura*$^-$]	*MatA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6*		[42]
H222ΔPΔLΔSΔF (F) [*leu*$^-$, *ura*$^+$]	Same as HP-U, +Δ*leu2 Δsnf1 Δfaa1 Δsnf1::URA3*	*loxR-URA3-loxP* flanked by *SNF1* upstream and downstream	This study
H222ΔPΔLΔSΔF ΔL +ALK5 YlCPR YlFAO1 (P) [*leu*$^-$, *ura*$^+$]	Same as F, +Δ*leu2::URA3 YlALK5 YlCPR YFAO1*	*loxR-URA3-loxP* flanked by *SNF1* upstream and downstream	This study
H222ΔPΔLΔSΔF ΔL +ALK5 YlCPR YlFAO1 ++ALK5 (M) [*leu*$^-$, *ura*$^+$]	Same as P, +*YlALK5*	Multiple-copy integration of *YlALK5* using zeta based integrative vector pINA1291(this strain was selected after screening of 10–20 transformants for their growth and production capacities)	This study

The yeast nitrogen base (YNB) medium with following composition was used to select auxotrophic Ura$^-$ and Leu$^-$ transformants and to prepare the seed cultures: 6.7 g/L yeast nitrogen base (without amino acids w/ammonium sulfate) (Becton, Dickinson and Company, Sparks, MD, USA), 20 g/L glucose, and 1.92 g/L synthetic mix minus uracil (YNB-Ura) or 1.62 g/L synthetic mix minus leucine (YNB-Leu) (US Biological, Salem, MA, USA). YNB-Leu was also used to grow the strain carrying the plasmid with the *LEU2* marker and *CRE* gene to restore the uracil auxotrophic derivatives of the F and P strains. Rich medium (YPD) containing 20 g/L glucose, 20 g/L bacto peptone (BD), and 10 g/L bacto yeast extract (BD) was used for non-selective propagation of strains. Agar (US Biological, Salem, MA, USA) was added at a concentration of 20 g/L for preparing plates.

Production media in shake flasks and bioreactor were performed in minimal media with the following defined composition: 1.7 g/L Yeast Nitrogen Base (without amino acids and without ammonium sulphate) (BD), 1.5 g/L $MgSO_4$. $7H_2O$, and1.92 g/L drop-out synthetic mix minus uracil. The $(NH4)_2SO_4$ was used as the major source of nitrogen at a concentration of 1.9 g/L. The production media formulations contained 52 g/L glycerol, to reach the target carbon to nitrogen (C/N) ratio of 50. The $CaCO_3$ was added to the shake flasks at a concentration of 6 g/L to provide buffering conditions. This carbonate plays a role in maintaining a stable pH [58]. The shake flask cultivations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of the medium at an agitation rate of 180 ± 5 rpm and a temperature of 28 ± 1 °C. Fresh colonies from the YNB-Ura plates were precultured in YNB-Ura broth. Exponentially growing cells were harvested by centrifugation, washed with water, and then resuspended in water. The production media were inoculated with the resuspended cells to an initial optical density (OD_{600}) of 0.1.

2.2. Batch Fermentation

Batch cultivation was carried out in a 1-L benchtop fermenter BioFlo 110 (New Brunswick Scientific, Enfield, CT, USA). Cells from a 24 h-shake flask YNB-Ura culture were harvested, washed using water, and inoculated into 700 mL of the fermentation medium (with C/N of 50) to reach a minimum OD_{600} of 0.1. The temperature was kept at 28 °C. The pH was controlled and maintained at 6 during the biomass propagation (typically for 1 day) and then was increased to 8 during the production phase using 5 M NaOH. This pH adjustment was also beneficial to lessen the rate of citric

acid production. A minimum dissolved oxygen level of 50% was maintained by cascading with an agitation ranging from 250 to 800 rpm, and by supplying sterile air at a flow rate of 2 vvm. Samples with a volume of 15–25 mL were taken daily during 4 days of fermentation. An antifoam Y-30 emulsion (Sigma, St. Louis, MO, USA) solution of 10% was made and added at the beginning of each batch cultivation, as well as during the fermentation, when required.

2.3. Genetic Techniques

Standard molecular manipulation techniques were used to develop the vectors [57]. All plasmids and their functions are presented in Table 2.

Table 2. Vectors used in this study.

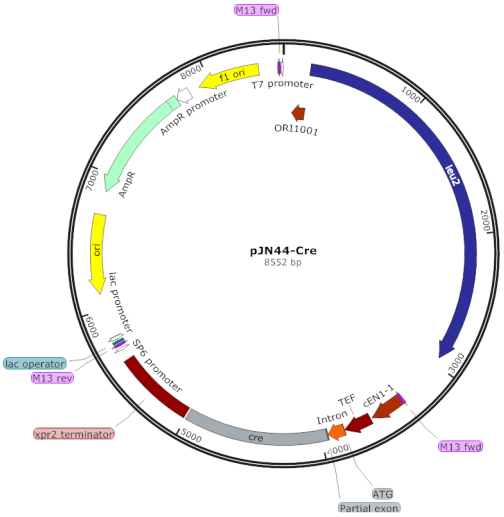
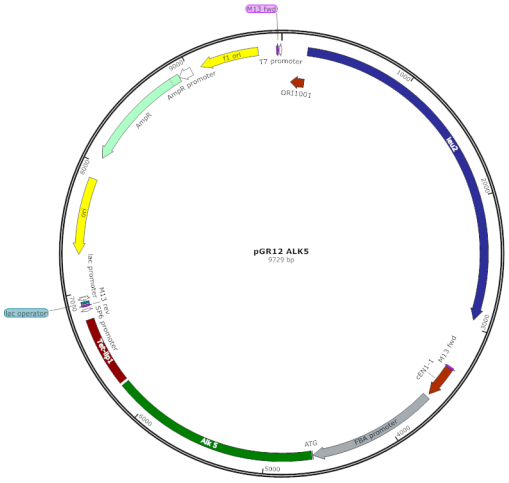
Vector Names	Map	Features
Cre (CR)		Cre recombinase flanked by TEFin promoter and Xpr2 terminator
pGR12 *YlALK5* (5)		Centromeric (CEN) replicative vector, low-copy CEN plasmids (1–2 copies/cell~1.6 plasmid copies/cell), *YlALK5* controlled by P_{FBA}-Tlip1, leucine selection marker

Table 2. *Cont.*

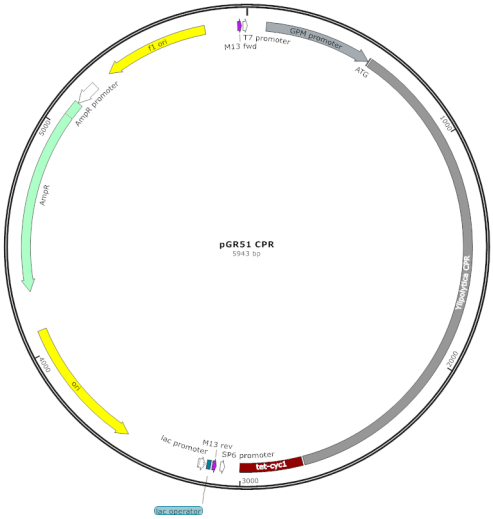
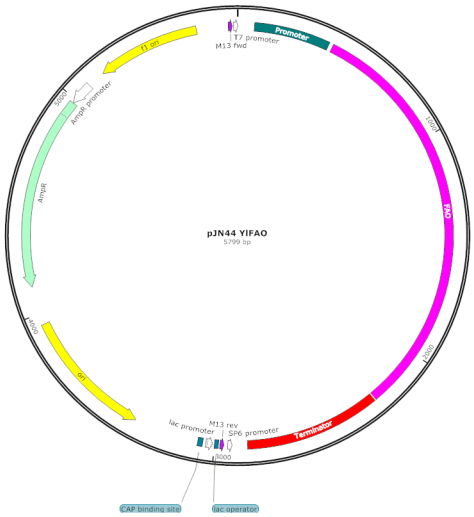
Vector Names	Map	Features
pGR51 *YlCPR* (C)		Centromeric (CEN) replicative vector, *YlCPR* controlled by P_{GPM}-Tcyc1
pJN44 *YlFAO* (F)		Centromeric (CEN) replicative vector, *YlFAO1* controlled by P_{TEFin}-Txpr2
ALK5 CPR FAO (5CF)		CEN replicative vector, P_{FBA}-*YlALK5* Tlip1 P_{GPM}-*YlCPR* Tcyc1 P_{TEFin}-*YlFAO1* Txpr2

Table 2. *Cont.*

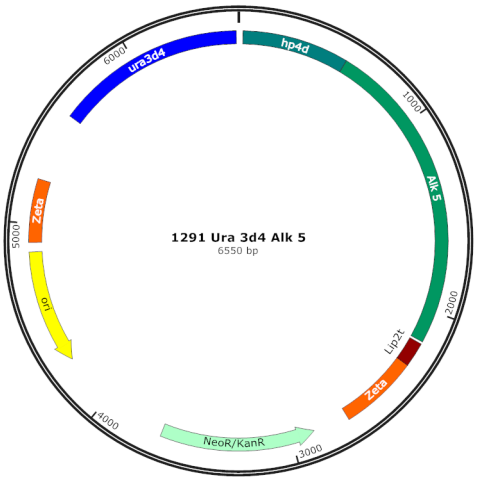
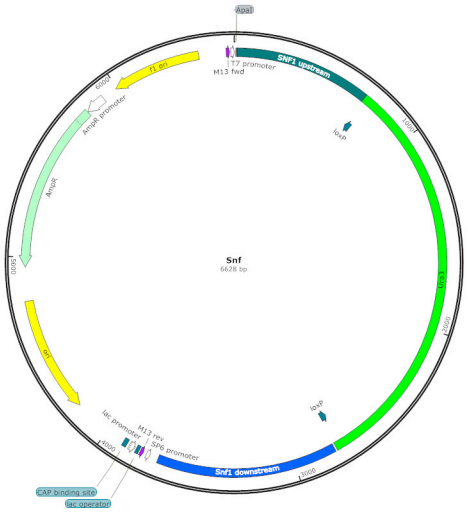
Vector Names	Map	Features
Leu 5cf (L5CF)		Uracil selection marker flanked by *LEU2* upstream and P_{FBA}-*YlALK5* Tlip1 P_{GPM}-*YlCPR* Tcyc1 P_{TEFin}-*YlFAO1* Txpr2 *LEU2* downstream sequences
pINA1291 ALK5 (Z5)		Integrative vector, hp4d- *YlALK5* Tlip2, ura3d4 defective marker, zeta region for multi-copy integration
LEU (LU)		Uracil selection marker flanked by *LEU2* upstream and downstream sequences

Table 2. *Cont.*

Vector Names	Map	Features
SNF (SU)		Uracil selection marker flanked by *SNF1* upstream and downstream sequences
FAA (FU)		Uracil selection marker flanked by *FAA1* upstream and downstream sequences

The construction of the triple gene expression cassette was carried out by the amplification of *Yarrowia lipolytica* cytochrome P450 *YlALK5*, NADPH-P450 reductase *YlCPR*, and fatty alcohol oxidase *FAO1* gene segments. Cloning inserts were obtained from polymerase chain reaction (PCR) using Q5 high fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), the gDNA of Po1f (ATCC MYA-2613) as the template, along with the primers presented in Table 3. The amplicons were individually digested and inserted into *Y. lipolytica* plasmid pGR12 (P_{FBA}-T_{lip1}), pJN44 (P_{TEFin}-T_{xpr2}), and pGR51 (P_{GPM1}-Tcyc1). These are the constitutive promoters with the following orders of strength: FBA1 > GPM1~TEF [59]. The segments of P_{GPM}-*YlCPR* Tcyc1 and P_{TEFin}-*FAO1*-T_{xpr2} were separated from the non-shuttle vectors by digestion with XbaI and SpeI. For a more distinguishable separation of the plasmid digestion products on the gel, the unique sites of XmnI and ScaI were also used in a triple digestion process. The target gel recovered fragments were consecutively inserted into the SpeI-digested and Fast Alkaline Phosphatase treated *ALK5*–pGR12 plasmid. Gene knock-out was carried out using the uracil maker containing vectors. The marker was surrounded by LoxP sites. For knock-out plasmid constructions, the 0.6–1.1 kb 5′- and 3′-flanking regions of the *Y. lipolytica LEU2*, *SNF1*, and *FAA1* genes were amplified with the primers listed in Table 3. The amplicons were inserted into the sites upstream and downstream of uracil gene upon digestion and purification. The triple gene expression cassette segment was double digested using XbaI and SpeI and purified on gel for subsequent insertion into the SpeI-digested and Fast Alkaline Phosphatase treated *LEU2* knock-out

vector. The triple gene cassette was successfully integrated into the genomic *LEU2* locus, in a one step site-specific gene knock in/out. This was carried out to reach long-term genetic stability. *YlALK5* gene segment was also inserted into a vector under the control of the growth phase-dependent hp4d promoter. This promoter shows high quasi-constitutive activity [60] at the start of the stationary phase, and is relatively independent of medium composition and pH [61]. Application of the hp4d growth dependent promoter lessens the metabolic burden of *YlALK5* overexpression during the growth phase. The corresponding vector contains zeta sequences (Ylt1 retrotransposon LTRs), enabling its multiple random integration into the genome of strains devoid of Ylt1 retrotransposon, such as the P strain. This zeta-based vector bears the defective marker, ura3d4 allele, thus allowing a random ectopic integration of about ten copies into all H222 derivatives, which are devoid of Ylt1 retrotransposon [62].

Table 3. PCR primers used in this study.

No.	Name	Sequence (5′→3′, Underlined Restriction Site)
1	*Alk 5* F HindIII	GAGCGAAAGCTTATGCTACAACTCTTTGGCGTCC
2	*Alk 5* R PstI	CTTAGA CTGCAG CTACGCCTTCTCACCCTTATACA
3	*Alk 5* Zeta F A	AATGCTACAACTCTTTGGCGTCC
4	Alk 5 Zeta KpnI R	TTGCAAGGTACCCTACGCCTTCTCACCCTTATACATCT
5	*YlCPR* F HindIII	GAGCGAAAGCTT ATGGCTCTACTCGAC TCTC
6	*YlCPR* R SmaI	GTTAT CCCGGG CTACCACACATCTTCCTGG
7	*FAO1*F NdeI	CCTCA CATATGATGTCTGACGACAAGCACACT
8	*FAO1*R SmaI	GTTAT CCCGGG AGGATCTCCGACCTCGAATC
9	*LEU2* up F ApaI	CTATAGGGCCC ACCGGCAAGATCTCGTTAAGACAC
10	*LEU2* up R XbaI	GATCCTCTAGATGTGTGTGGTTGTATGTGTGATGTGG
11	*LEU2* down F SpeI	CTGGACTAGTCTCTATAAAAAGGGCCCAGCCCTG
12	*LEU2* down R NdeI	CCTCACATATG GACAGCCTTGACAACTTGGTTGTTG
13	*LEU2* F Ura	TACAGTTGTAACTATGGTGCTTATCTGGG
14	*LEU2* Ura R	CCTTGGGAACCACCACCGT
15	*LEU2* Ura F	ACTTCCTGGAGGCAGAAGAACTT
16	*LEU2* R Ura	ATAGCAAATTTAGTCGTCGAGAAAGGGTC
17	*SNF1* up F ApaI	CAATTGGGCCCGTGATCAAAGCATGAGATACTGTCAAGG
18	*SNF1* up R XbaI	GATCCTCTAGAGAGGTGGTGGAAGGAGTGGTATGTAGTC
19	*SNF1* down F SpeI	CTGGACTAGT TCATTAATACGTTTCCCTGGTG
20	*SNF1* down R NdeI	CCTCACATATGGGAATTCGTGCAGAAGAACA
21	*SNF1* F Ura	GCGGGAAATCAAGATTGAGA
22	*SNF1*Ura R	CGGTCCATTTCTCACCAACT
23	*SNF1* Ura F	CCTGGAGGCAGAAGAACTTG
24	*SNF1* R Ura	ACTACTGGCGGACTTTGTGG
25	*FAA1* up F ApaI	CAATT GGGCCC CCAGGTCTCAGTTGCACTTGC
26	*FAA1* up R XbaI	GATCC TCTAGA CAAATTATACCCCTCATCTCTCTAGGACA
27	*FAA1* down F SpeI	CT GGACTAGTTTGGTGAGCCCACCGC
28	*FAA1* down R NdeI	CCTCACATATGAACCTCCAGCAGACTAACTAGAACA
29	*FAA1* F Ura	ACTGTAGCTAGATGGGTGCC
30	*FAA1* Ura R	CGGTCCATTTCTCACCAACT
31	*FAA1* Ura F	CCTGGAGGCAGAAGAACTTG
32	*FAA1* R Ura	ACCAGCCCAGCCGG
33	*LEU2* up F Ura	TCATGTTCGTGGAGGGGAG
34	*LEU2* up Ura R	AAAACGCAGCTGTCAGACC
35	*LEU2* down F FAO	GGAGTCCAAGCCCTTCGA
36	*LEU2* down R	CACAAGACGTCAACTAAAGCGT

Site-specific gene knock in/out was achieved by transforming the linearized vectors containing homologous upstream and downstream sequences. *Y. lipolytica* was transformed with the linearized plasmids, consisting of NdeI-digested LU, ApaI-digested SU, NdeI-digested FU, and NsiI-digested L5CF. The zeta-containing vector was linearized using FD NotI enzyme. Transformation was performed using a Zymogen Frozen EZ yeast transformation kit II (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. We modified this protocol to enhance the efficiency of multi-copy integration transformation. The *loxR–URA3–loxP* modules were rescued for subsequent genetic modification by the LoxP-Cre system as previously reported [63]. Gene deletions and expression cassette insertion were confirmed by PCR using the primers listed in Table 3.

Construction of vectors was carried out using standard cloning methods, with FastDigest restriction enzymes (Thermo Fisher Scientific, Waltham, MA, USA). Yeast genomic DNA was isolated as described previously [64] and was used for PCR amplification and verification. A DNA Clean &

Concentrator-5 Kit (Zymo Research, Irvine, CA, USA) was used for the purification of DNA products from PCR and digestion reactions. A GeneJET Gel Extraction Kit (Thermo Fisher Scientific) was used for the recovery of nucleic acid fragments from agarose gels.

2.4. Analytical Methods

2.4.1. Dry Biomass

Seven milliliters of culture broth were collected daily for dry cell weight, extracellular metabolites, fatty acids, and LCDCAs measurements. Five milliliters were centrifuged for 5 min at 13,300 rpm. The cell pellet underwent two stages of washing with a saline solution (0.9% NaCl) and distilled water. The biomass yield was measured gravimetrically by drying samples at 105 °C to reach a consistent weight. This was presented in grams of dry cell weight per liter (g·DCW/L).

2.4.2. Glycerol and Citric Acid Concentrations

High performance liquid chromatography (HPLC) with an Aminex HPX-87H column was used to measure the concentration of glycerol and citric acid. After centrifugation, supernatants were filtered using 0.22 µm pore-size membranes and eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min, at 65 °C. Refractive index (RI) and UV (210 nm) detectors were employed for the detection of target signals. Standards were used for the identification and quantification of glycerol and citric acid. Our methods did not adequately separate citric acid from its isomer, iso-citric acid. Thus, the reported concentration corresponds to the sum of these isomers, expressed as citric acid.

2.4.3. Qualitative and Quantitative Analysis of Fatty Acids and LCDCAs

Fatty acid methyl esters (FAME) and dicarboxylic dimethyl esters were made in hexane after lipid extraction and transesterification, in compliance with the previously described method [65]. Analysis of esterified compounds was conducted using an Agilent 7890A gas chromatography instrument coupled with a flame-ionization detector (FID) and a FAMEWAX column (30 m × 320 µm × 0.25 µm) (Restek Corporation, Bellefonte, PA, USA). The injection temperature and volume were set at 250 °C and 1 µL, respectively. The injection was performed with a split mode (ratio 20:1). The initial temperature of oven was 190 °C and progressed to 240 °C at a rate of 5 °C/min. The final temperature was maintained for 20 min. The FID temperature was 250 °C. FAME standards were used to identify the fatty acid peaks in the chromatograms. Quantification of fatty acids and LCDCAs were conducted by comparison with tridecanoic acid (C13:0) (0.5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) as internal standard and by the calculation of response factor. The supernatant was filtered and used for possible extracellular metabolite extraction and quantification. Results show that the GC-FID method did not sufficiently separate C16 and C16:1 fatty acids, C18 and C18:1 fatty acids, LCDCA16 and LCDCA16:1, and LCDCA 18 and LCDCA 18:1. Therefore, the reported concentration represents the sum of these acid pairs, expressed as total fatty acid 16, total fatty acid 18, total LCDCA 16, and total LCDCA 18. LCDCA18:2 was also quantified and added to the category of LCDCA 18. An Agilent GC-MS was also used for the qualitative analysis of other dicarboxylic acids (DCAs), DCA12 and DCA14. The initial temperature of 170 °C with a holding time of 1 min, was increased to 250 °C at a rate of 3 °C/min, and held at the final temperature for 20 min. The split ratio was 30:1.

3. Results

3.1. Screening Process

We developed several single, double, and triple gene expression vectors with different promoters and terminators carrying homologous P450 monoxygenase (*YlAlk 2*, *3*, *5*, *7*, and *10*), P450 reductase *YlCPR*, fatty alcohol oxidase *YlFAO1*, and fatty aldehyde dehydrogenase *YlHFD1* gene segments. We tested these vectors for their performance in the ω-oxidation of endogenous fatty acids to

the corresponding LCDCA monomers. YlAlk gene products catalyze the rate-limiting step of the ω-oxidation pathway. Therefore, it is important to use strong FBA promoter for their overexpression when replicative vectors with low-copy numbers are used. Among YlALK 2, 3, 5, 7 and 10, we noted that *YlALK3*, *YlALK* 5, and *YlALK* 7 each have an effect on the production of LCDCA-16 with the following order of strength: YlALK5 > YlALK7 > YlALK3. We also observed that co-expression of *YlALK5* and *YlCPR* led to the generation of ω-hydroxy fatty acids of 16 carbons in the F strain. We selected *YlALK5* for single as well as multiple integration because it exhibited the highest activity toward the ω-oxidation of accumulated fatty acids among the tested monoxygenases. We constructed the F strain by deleting *YlLEU2*, *YlSNF1*, and *YlFAA1* in the HP-U (*POX*-deleted) strain. Then, the P strain was constructed by performing the site specific single-copy integration of the *YlALK5*, *YlCPR*, and *YlFAO1* expression cassette into the locus of the *LEU2* gene. It is notable that this single-copy integration improved the growth behavior of the resulting strain over the F strain in the minimal YNB-Ura media. Then, the M strain was constructed by random multiple integration of *YlALK5* into the P strain. Ten to twenty colonies were screened for their growth on selective YNB-Ura plate and LCDCA production. All of the F, P, and M strains were uracil prototrophic and leucine auxotrophic. The YNB-Ura culture of the M strain was additionally supplemented with an additional 400 mg/L leucine to study the possible stimulatory effect of this amino acid on the metabolite production by the M strain. This was labeled as the ML strain/treatment. Preliminary shake flask studies were conducted to find the best strain for a fermentation scale-up. However the production of LCDCAs in shake flask cultures is generally challenging due to insufficient aeration, agitation, and poor control over pH. The next section presents the data from the 6-day shake flask cultivations.

3.2. Preliminary Shake Flask Experiments for Fatty Acid and LCDCA Production

We tested four strains (wild type W and engineered F, P, and M) for fatty acids and monomer production in shake flask cultures. Glycerol was added to YNB-Ura at a concentration of 52 g/L to reach a C/N ratio of 50. This medium contained about 0.38 g/L of leucine and was supplemented with an additional 400 mg/L of leucine for the ML strain/treatment. Results of biomass production in the 6-day shake flask culture are summarized in Figure 1.

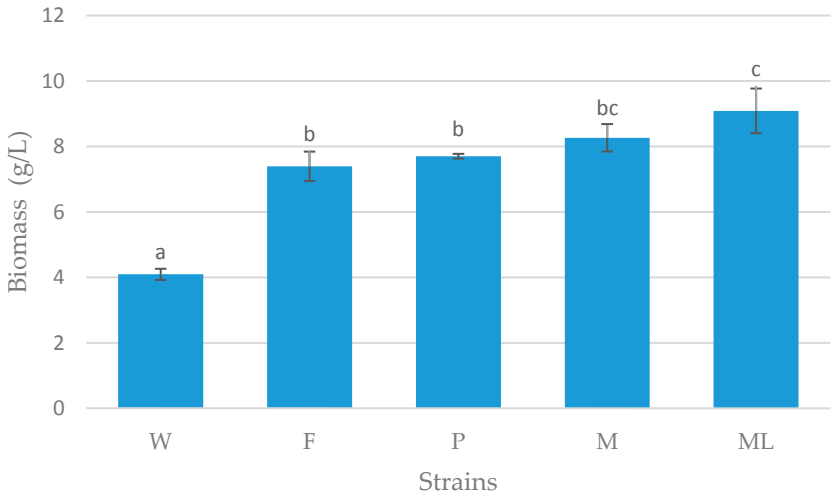


Figure 1. Study of 6-day shake flask cultures for biomass production by the W, F, P, M and ML strains/treatment. Glycerol based media under nitrogen-limiting conditions (C/N = 50). Error bars represent standard deviation for $n = 3$. W: H222 wild type strain, F: Δ*POX1*-6 Δ*LEU2* Δ*SNF1* Δ*FAA1* Δ*SNF1::URA3*, P: F + Δ *LEU2:: URA3 YlALK5 YlCPR YlFAO1*, M: P + *YlALK5:: URA3*, ML: M + 400 mg/L leucine. abc Columns with dissimilar letters at the top are significantly different ($p < 0.05$).

The data present a significant ($p < 0.05$) difference between the wild type (W) strain and all mutant strains. This can be explained in part by the final pH of media, which can affect the $CaCO_3$ solubility. Although all shake flask media contained an equal amount of calcium carbonate (6 g/L), their final pH varied depending on the strain. For example, the final pH of the W strain was above 4.1, while it was in the range of 3.2 to 3.9 for other strains because of a higher citric acid production. The pH of media can affect $CaCO_3$ solubility and this interferes with dry biomass measurements. In addition to this explanation, we also noted that glycerol was almost completely consumed by the end of the 6-day period for all mutant strains. However, we observed a residual glycerol level of 15 g/L for the W strain. The integration of the fatty acid-oxidizing genes, did not significantly ($P > 0.05$) enhance the final biomass level under nitrogen limiting conditions. However, the supplementation of the YNB-Ura medium with an additional leucine caused the M strain to produce a significantly higher biomass level than F and P strains.

We also assessed the capability of the strains for citric acid production. Our methods could not distinguish between citric acid and isocitric acid, so the reported data in Figure 2 is the sum of these two isomers.

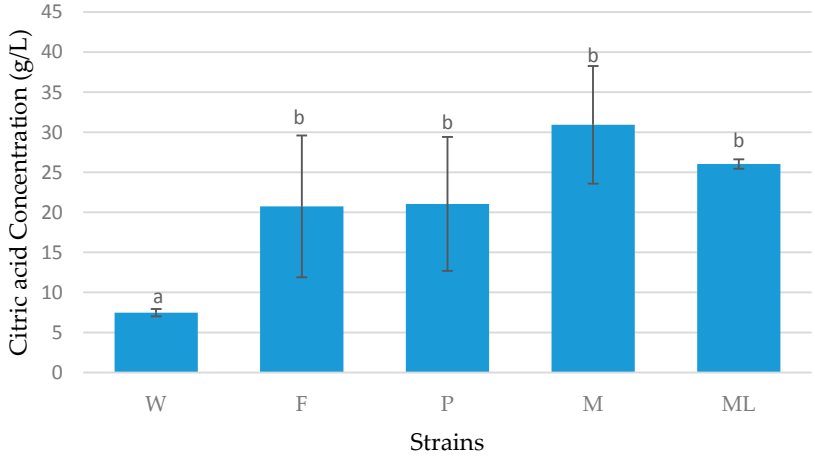


Figure 2. Study of 6-day shake flask cultures for citric acid production by the H, F, P, M and ML strains/treatment. Glycerol based media under nitrogen-limiting conditions (C/N = 50). Error bars represent a standard deviation for $n = 3$. W: H222 wild type strain, F: Δ*POX1*-6 Δ*LEU2* Δ*SNF1* Δ*FAA1* Δ*SNF1::URA3*, P: F + Δ *LEU2:: URA3 YlALK5 YlCPR YlFAO1*, M: P + *YlALK5:: URA3*, ML: M + 400 mg/L leucine. ab Columns with dissimilar letters at the top are significantly different ($p < 0.05$).

All mutant strains produced a significantly ($p < 0.05$) higher level of citric acid than the W strain, in the range of 21 to 31 g/L. A small amount of the detected acid can be in the form of its isomer, iso-citric acid because this yeast is able to produce citric and iso-citric acid and their proportion is mainly influenced by feedstocks and strains [35]. Although the M strain produced the highest level of citric acid, there was no significant ($p > 0.05$) difference among the mutant strains. Moreover, the leucine supplementation did not have a significant effect on citric acid production. Citric acid is the major by-product of the lipid biosynthesis in *Y. lipolytica* under nitrogen-limiting conditions [66]. Higher re-direction of carbon flux from citric acid to fatty acid can be achieved by upregulating the first committed step of fatty acid biosynthesis, controlled by *ACC1* [67], and by removing the feedback inhibition effect of endogenous fatty acyl-CoA through thioesterase overexpression or acyl-CoA synthetase inactivation [49].

Figure 3 presents the total fatty acid and LCDCA monomer production by the strains from 6-day shake flask cultivation.

The wild-type W strain accumulated fatty acids at a low concentration of 340 mg/L with oleic acid (FA18:1) as the main constituent. The concentration of total fatty acids in the mutant strains

was significantly ($p < 0.05$) higher than in the W strain. The deletion of *YlFAA1* in the F strain significantly ($p < 0.05$) increased the TFA level to 3300 mg/L in the F strain. These endogenous fatty acids were precursors for the LCDCA production. The LCDCAs were not produced by the W strain. The production of these monomers was initiated by the deletion of *YlFAA1* in the F strain. This shows the involvement of background ω-oxidation activity in LCDCA-16 and 18 production. This contribution was higher toward the end of 6-day shake flask cultivations. Single and multiple copy integration of the fatty acid-oxidizing genes significantly ($p < 0.05$) increased LCDCA production. This LCDCA level was similar for the P, M, and ML strains/treatment. Generally, LCDCA production in shake flasks is not stable and faces several challenges, including inadequate aeration, agitation, and pH control. Oxygen limitations are typical for shake flask cultures. Moreover, addition of 6 g/L $CaCO_3$ cannot sufficiently maintain the stability of pH value in shake flask cultures. These limitations undermine the effect of genetic or cultivation modification on LCDCA production. For instance, we observed the positive effect of leucine supplementation on biomass and LCDCA production by the M strain in bioreactor fermentations. However, application of similar treatment did not significantly change the LCDCA production level of the M strain in shake flask cultivations. Additionally, an extensive post-screening for choosing the best LCDCA-producing strain is beneficial to reach the strongest effect of multiple-integration. The preliminary shake flask studies were carried out, nonetheless, to select the appropriate strain and conditions for bioreactor fermentation. We obtained a LCDCA titer of 330 to 430 mg/L from the P, M and ML strains/treatment under nitrogen-limiting conditions. Chromatograms from the F and P culture indicate that a negligible amount of FA-17 was also produced by these strains.

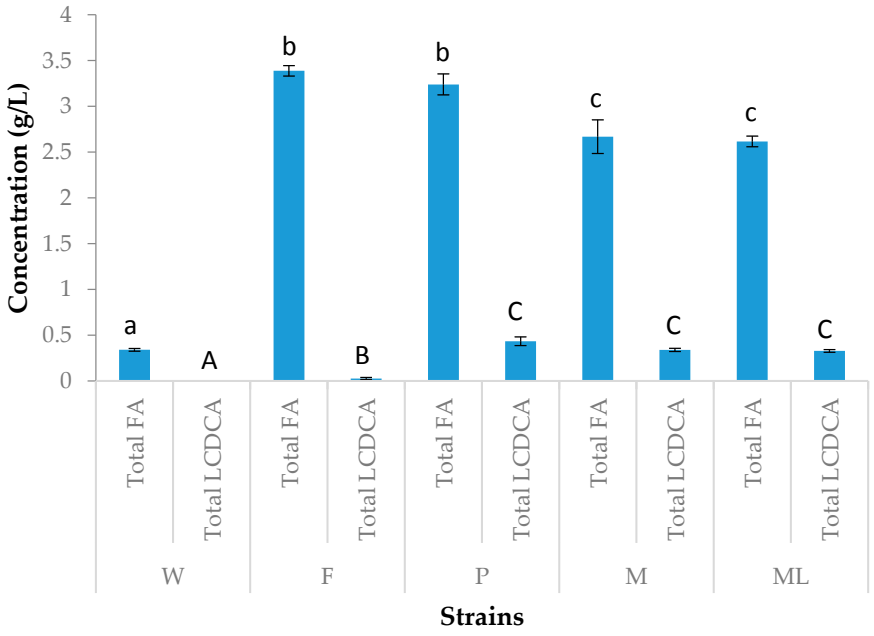


Figure 3. Study of 6-day shake flask cultures for fatty acid and LCDCA production by the H, F, P, M, and ML strains/treatment. Glycerol based media under nitrogen-limiting conditions (C/N = 50). Error bars a represent standard deviation of $n = 3$. W: H222 wild type strain, F: Δ*POX1-6* Δ*LEU2* Δ*SNF1* Δ*FAA1* Δ*SNF1*::*URA3*, P: F + Δ *LEU2*:: *URA3 YlALK5 YlCPR YlFAO1*, M: P + *YlALK5*:: *URA3*, ML: M + 400 mg/L leucine. Total FA: total fatty acids of 16 and 18 carbons, Total LCDCA: total long chain dicarboxylic acids of 16 and 18 carbons. ABC/abc Columns with dissimilar letters at the top are significantly different ($p < 0.05$).

In general, the deletion of *POX* genes prevents the degradation of fatty acid precursors and monomers. Additionally, the inactivation of *YlFAA1* promotes the generation of free fatty acids,

which do not have the feedback inhibitory effect of acyl-CoA on the fatty acid biosynthesis pathway. The combination of these genetic modifications with the deletion of *YlSNF1* can promote the re-direction of the carbon flux towards malonyl-CoA and eventually free fatty acid accumulation. In addition to background ω-oxidation activity, the upregulation of the ω-oxidation pathway can enhance the transformation of free fatty acid pool into the monomers of less toxicity. Therefore, our genetic engineering served the dual purpose of enhancing fatty acid accumulation and producing LCDCA monomers. The ML strain and treatment was selected for further bioreactor fermentation experiments.

3.3. Batch Fermentation

The batch bioreactor fermentation was carried out using YNB-Ura medium with a C/N of 50. Glycerol was added to the medium at a concentration of 52 g/L. The production medium was also supplemented with 400 mg/L leucine. The time course study of biomass and citric acid production as well as glycerol utilization during the 4-day fermentation is presented in Figure 4.

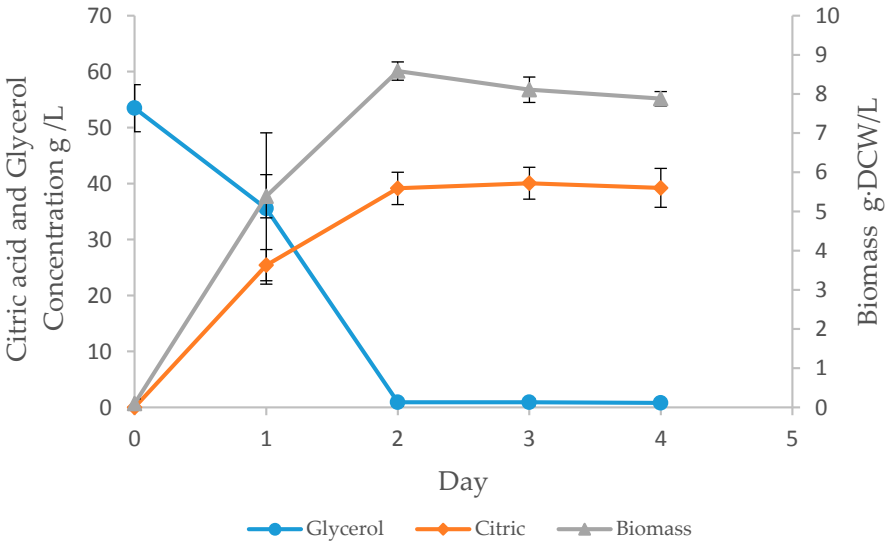


Figure 4. Time course study of glycerol utilization, biomass proliferation, and citric acid production by the ML strain/treatment. Glycerol based YNB-Ura media under nitrogen-limiting conditions (C/N = 50). Data from 2 rounds of batch fermentation. *ML: ΔPOX1-6 ΔLEU2 ΔSNF1 ΔFAA1 + Δ LEU2:: YlALK5 YlCPR YlFAO1 + YlALK5:: URA3* + 400 mg/L leucine.

Glycerol was consumed rapidly within 2 days of fermentation. During this period, the citric acid and biomass reached their peak at about 39 and 8.6 g/L, respectively. Afterwards, there was a slight reduction in the concentration of biomass, possibly due to some cell attachment to the fermenter vessel. The ML strain and treatment produced citric acid at a yield of more than 0.75 g/g of consumed glycerol. During the last two days of fermentation, the citric acid concentration did not undergo a marked change. It is notable that the mutant strains of this study are derived from H222 stain, which has a strong potential for citric acid production. Additionally, nitrogen-limiting conditions reroute carbon flux to lipid accumulation for storage and to citric acid for secretion. Both citric acid and fatty acids compete for the building units of acetyl-CoA [68]. To gain insight into monomers production conditions, we analyzed the concentration of fatty acids and monomers during the 4-day fermentation. Results are shown in Figure 5.

Batch fermentation in the 1-L bioreactor significantly enhanced the titer of LCDCA from 0.33 g/L, which was obtained in the shake flask culture, to 3.49 g/L for the ML strain and treatment. This corresponds to a minimum yield of 0.067 g/g of consumed glycerol and to a productivity of 0.04 g/L·h. This significant improvement was mainly due to adequate aeration, agitation, and precise control over

the pH. The increase in LCDCA level continued until the last day of the 4-day fermentation at the expense of fatty acid pool.

The LCDCAs are more soluble than their fatty acid counterparts. This feature facilitates their transportation across the cell. We also analyzed the concentration of extracellular LCDCA monomers in cell-free production media to find more about the spatial distribution of LCDCAs. Figure 6 presents the results from this analysis.

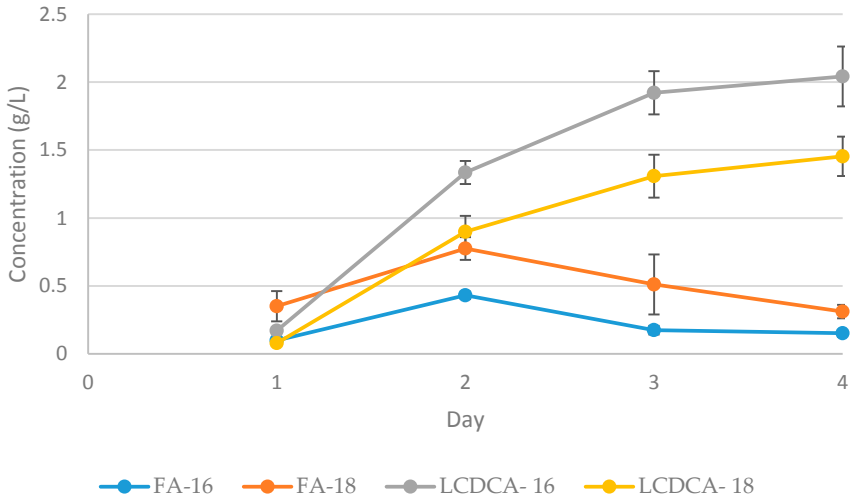


Figure 5. Time course study of fatty acids and LCDCA production by the ML strain/treatment. Glycerol based media under nitrogen-limiting conditions (C/N = 50). ML: Δ*POX1*-6 Δ*LEU2* Δ*SNF1* Δ*FAA1* Δ*LEU2*:: *YlALK5 YlCPR YlFAO1* + *YlALK5*:: *URA3* + 400 mg/L leucine. FA-16: fatty acid of 16 carbons, FA-18: fatty acid of 18 carbons, LCDCA-16: long chain dicarboxylic acids of 16 carbons, LCDCA-18: long chain dicarboxylic acids of 18 carbons. Data from two rounds of batch fermentation.

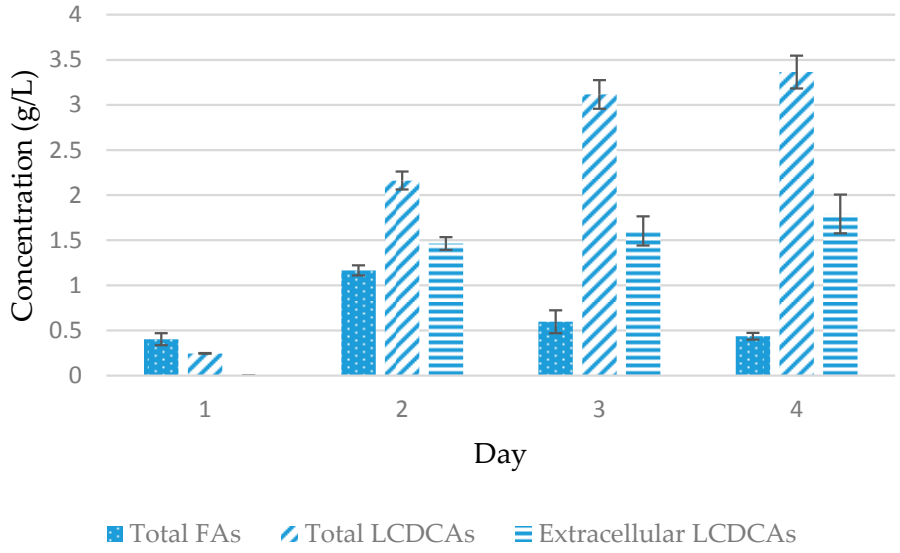


Figure 6. Time course study of total fatty acid, total LCDCA, and extracellular LCDCA production by the ML strains/treatment. Glycerol based media under nitrogen-limiting conditions (C/N = 50). Data from 2 rounds of batch fermentation. ML: Δ*POX1*-6 Δ*LEU2* Δ*SNF1* Δ*FAA1* Δ*LEU2*::*YlALK5 YlCPR YlFAO1* +*YlALK5*:: *URA3* + 400 mg/L leucine. Total FAs: fatty acids of 16 and 18 carbons, Total LCDCAs: long chain dicarboxylic acids of 16 and 18 carbons. Data from 2 rounds of batch fermentation.

According to these results, more than half of the LCDCAs were present in the extracellular medium while the fatty acids were intracellularly used as precursors for the monomer production. Figure 6 also indicates that the availability of intracellular free fatty acids is the rate limiting step for *de novo* LCDCA production in the 1-L bioreactor fermentation. It should be noted that a small amount of dicarboxylic acids of 12 and 14 carbons was also found in the extracellular medium (less than 0.2 g/L). This can be due to the activity of *YlALK5* that was integrated into the genomes at multiple copies. The composition of metabolites was also determined. Results are presented in Figure 7.

Figure 7 shows that LCDCAs became predominant fatty acid-based metabolites at the end of the 4-day fermentation. Also, LCDCA-16 accounted for more than 50% of LCDCAs, meaning that the ω-oxidation rate of 16 carbon-fatty acid was higher than that of 18 carbon-fatty acids. This rate is influenced by the background ω-oxidation activity, as well as the activity of those genes that have been overexpressed. Table 4 presents the summary of data from biomass, citric acid, and LCDCAs production.

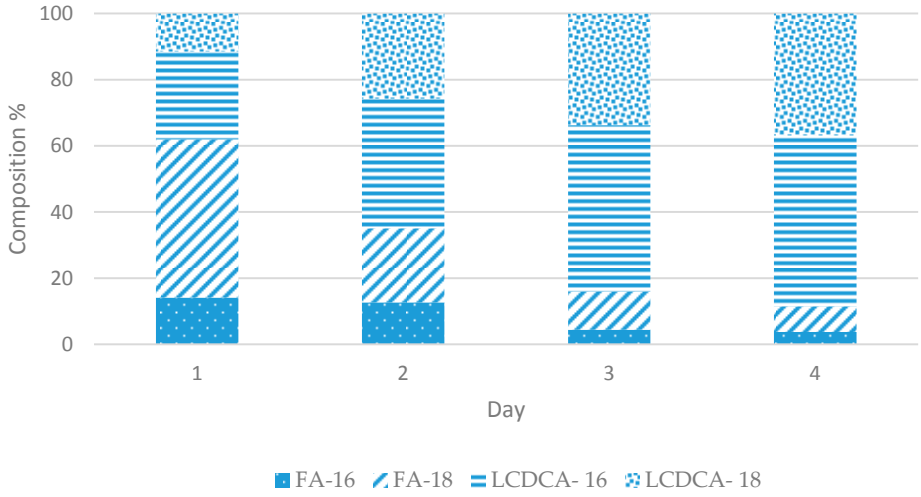


Figure 7. Percentage composition of fatty acids and LCDCAs produced by the ML strain/treatment. Glycerol based media under nitrogen-limiting conditions (C/N = 50). ML: Δ*POX1-6* Δ*LEU2* Δ*SNF1* Δ*FAA1* Δ*LEU2::YlALK5 YlCPR YlFAO1* +*YlALK5:: URA3* + 400 mg/L leucine. FA-16: fatty acid of 16 carbons, FA-18: fatty acid of 18 carbons, LCDCA-16: long chain dicarboxylic acids of 16 carbons, LCDCA-18: long chain dicarboxylic acids of 18 carbons. Data from 2 rounds of batch fermentation.

Table 4. Summary of the production level for biomass, citric acid, and LCDCAs.

DCW (g/L)	Citric Acid (g/L)	LCDCA Titer (g/L)	LCDCA Yield (g/g)	LCDCA Productivity (g/L·h)
8.58 ± 0.23	39.2 ± 3.5	3.49 ± 0.14	0.06	0.04

4. Discussion

The *de novo* production of fatty acid-derived monomeric compounds from unrelated feedstock has been previously demonstrated. For example, the overexpression of a plant thioesterase and of an engineered self-sufficient monooxygenase fusion protein in a β-oxidation deficient *E. coli* strain resulted in 234 μM ω-hydroxy octanoic acid production in a fed-batch fermentation [69]. In another study, *E. coli* was engineered for sugar-based hydroxyl fatty acid production [70]. That was achieved by the coexpression of acetyl-CoA carboxylase and acyl-CoA thioesterase in an inactivated acyl-CoA synthetase background, followed by the expression of a heterologous fatty acid hydroxylase. This resulted in the production of different hydroxyl fatty acids with chain lengths of 10 to 18 carbons at a final titer of 58.7 and 548 mg/L in culture broth and fed-batch fermentation, respectively. The engineered reversal of the β-oxidation cycle, in combination with the expression of heterologous

thioesterase and monooxygenase systems, has led to the biosynthesis of medium chain ω-hydroxy fatty acids and dicarboxylic acids at a titer of 0.8 and 0.5 g/L, respectively, from glycerol in *E. coli* [71]. This bacterial host has also been engineered for the biosynthesis of long chain hydroxylated fatty acids at a titer of 117 mg/L [72]. In that study, both thioesterase and fatty acid hydroxylase $P450_{BM3}$ were involved in the production of free fatty acids and in their conversion into oxygenated monomers. Bowen et al. (2015) also engineered *E. coli* for sugar-based C12 and C14 ω-hydroxy FAs and α,ω-DCAs production [6]. This was achieved by expressing high-specificity acyl ACP thioesterases (TEs) to detach free fatty acids from the fatty acid synthase (FAS II). The application of p450 (CYP) and alcohol/aldehyde dehydrogenases led to the generation of ω-hydroxy FAs and subsequently α,ω-DCAs at a titer and productivity of 600 mg/L and 0.026 g/L·h, respectively. Figure 8 presents a summary of aforementioned strategies.

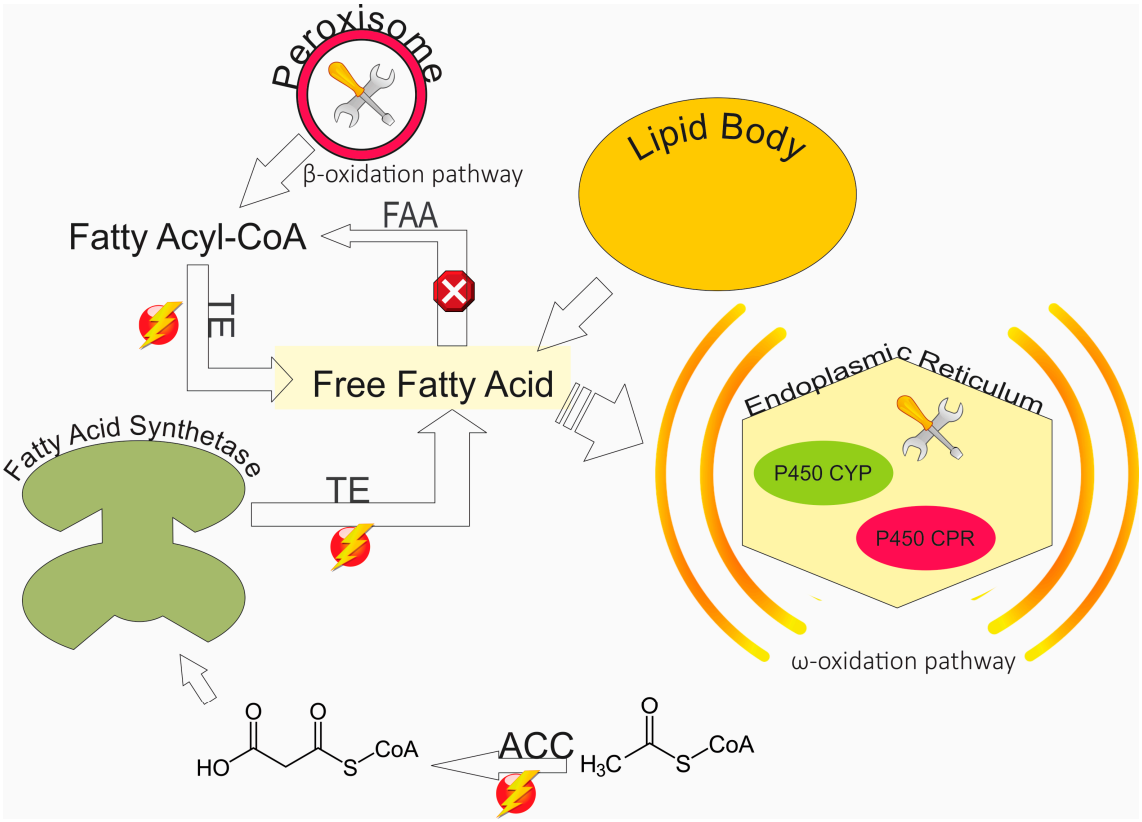


Figure 8. Genetic engineering strategies for the *de novo* production of oxygenated fatty acid monomers: overexpression of acetyl-CoA carboxylase (ACC) for higher carbon flux re-direction toward fatty acid biosynthesis pathway, overexpression of length specific thioesterases (TE) for the hydrolysis of acyl-CoA and consequently mitigation of their feedback inhibition effect, inactivation of the fatty acyl-CoA synthase (FAA) for the prevention of free fatty acid re-activation, inactivation or reversal of the β-Oxidation cycle for enhancing fatty acid pool and preventing degradation of fatty acid derivatives, and selective upregulation of the ω-oxidation pathway for the terminal oxidation of endogenous fatty acids to their oxygenated counterparts.

The above-mentioned yields and titers are notably less than those reported for whole-cell biotransformation of exogenously added long chain fatty acids to the corresponding monomers [8,73]. For instance, engineering *Y. lipolytica* for the production of LCDCA from C18 oil allowed to reach a titer of 16 g/L. This was accomplished through the blockage of β-oxidation and overexpression of *ALK1,2* together with the *CPR* gene under the control of p*POX2* [46].

This study reports the construction of *Y. lipolytica* as an oleaginous yeast cell platform for the *de novo* production of LCDCA monomers from renewable hydrophilic feedstock. Our LCDCA-producing platform is independent of oily feedstock whose utilization has potential disadvantages such as toxicity, low solubility, and weaker cellular uptake [7]. Instead, this sustainability-driven biosynthetic route utilizes hydrophilic substrates for the dual purpose of energy production and LCDCA biosynthesis under nitrogen-limiting conditions.

We developed our platform through a combinatorial genetic engineering strategy in a β-oxidation deficient strain. Our strategy involved the inactivation of *SNF1* for enhancing malonyl-CoA biosynthesis, disruption of the major cytosolic acyl-CoA synthetase (*FAA1*) for generating free fatty acid precursors, and upregulation of native ω-oxidation pathway for improving oxidation of the endogenous free fatty acid precursors into the corresponding long chain monomers. This strategy was developed based on the functions of the targeted genes.

In general, it is necessary to delete the *POX* genes in order to inactivate the β-oxidation pathway as the major degradation route. This prevents the degradation of fatty acid precursors and LCDCAs [45,47]. However, complete disruption of the peroxisome creates unwanted consequences. Also, defects in peroxisome biogenesis may have an inhibitory effect on the native P450 system [23,74], and may interfere with the oxidation of various metabolites [75]. *SNF2* deletion has been found to enhance lipid accumulation [76]. Inactivation of the *SNF1* gene led to an enhanced constitutive fatty acid accumulation and citric acid production in *Y. lipolytica* [66,77]. An alternative strategy to enhance carbon flux re-direction from citric acid production to fatty acid biosynthesis is to overexpress *ACC1* or use hyperactive Acc1p [67,78]. This strategy can prevent the possible unintended metabolic consequences resulting from *SNF1* deletion. Inactivation of *FAA1* can interfere with the re-activation of released fatty acids that could serve in energy production, membrane maintenance, or triglyceride (TAG) homeostasis [49,79]. Deletion of the *FAA1* and *FAA4* genes in *S. cerevisiae* have been found to interfere with fatty acid incorporation in phospholipids or TAG, while generating a free fatty acid-producing phenotype [80].

In *Y. lipolytica*, the free fatty acids that are activated by YlFaa1p are partially incorporated into the lipid bodies [81]. In this yeast, several gene products that differ in their substrate specificities are involved in the activation of fatty acids in different organelles [82]. Faa1p is the major cytosolic long chain fatty acid activator [83]. This gene plays a crucial role in the utilization of both *ex novo* fatty acids and those obtained from the metabolism of *n*-alkanes [84]. *FAA1* also plays a role in the storage of fatty acids in lipid bodies [81,83]. Besides YlFat1p, which is mainly involved in fatty acid remobilization, there are at least 10 *AAL* genes involved in the activation of fatty acids in the peroxisome and lipid bodies. The inactivation of these genes can prevent the cell from storing fatty acids in lipid bodies [82]. The deficiency of fatty acid activation systems can also negatively affect yeast growth in batch cultures with glucose [85], due to either the detergent-like properties of free fatty acids or metabolic disorder [86].

We observed some residual fatty acids at the end of the 4-day batch fermentation. Since these fatty acids may occur in the esterified form in lipid bodies, they do not act as substrates for the ω-oxidation reactions. Our genetic engineering did not involve the disruption of TAG biosynthesis. This is because the generation of free fatty acids, particularly unsaturated ones, causes toxicity in the cell with deficient lipid body formation [87]. This occurs for several reasons: the destabilizing effect of fatty acid overproduction on the cellular membrane [80,88], the harmful effects of oxidative species derived from the oxidation of unsaturated fatty acids [89], and a lack of protection against excess lipid intermediates [90].

Free fatty acid accumulation or starvation can also trigger the ω-hydroxylation of fatty acids [91,92]. The resulting hydroxylated fatty acid monomers may also interfere with the TAG homeostasis [93]. We propose that ω-oxidation serves as a rescue route to detoxify free acids in the β-oxidation-disrupted strains, especially when *FAA1* is inactivated. The stress relieving effect of fatty acid oxidizing enzyme has also been observed in plants [23]. In *Y. lipolytica*, *ALK* genes are involved

in the detoxification of fatty alcohol, dodecanol [36]. Thus, this background ω-oxidative activity processes free fatty acids to oxygenated monomers of higher solubility and less toxicity. This can explain the stimulatory effect of ω-oxidation upregulation on biomass proliferation in the F strain with deficient FAA1p activity. In fact, the natural enzymatic machinery of the host cell plays an influential role in fatty acid over-oxidation [18].

Results show that our combinatorial genetic engineering for the biosynthesis of free fatty acids and their terminal oxidation to the corresponding LCDCAs served multiple purposes. These included the reduction of free fatty acid toxicity, increase of product solubility, facilitation of product transportation across the membrane, and the creation of a pull for further fatty acid flux direction toward ω-oxidation.

We observed that free fatty acids, generated from the *FAA1* inactivation, are suitable substrates for the native ω-oxidation pathway. This accords with a previous study, which reported the hydroxylation of the free fatty acid pool by a fatty acid hydroxylase (CYP102A1) from *Bacillus megaterium* [70]. Free fatty acids do not have the feedback inhibition regulatory effect of fatty acyl-CoAs on some genes of lipid synthesis [48,86]. To obtain free fatty acids, the inactivation of native fatty acid activation systems or overexpression of heterologous/homologous thioesterases has been suggested in the literature. For example, inactivation of *FAA1* and *FAA4* genes in *S. cerevisiae* was found to mitigate the feedback inhibition of acyl-CoA on fatty acid synthesis and undermine the β-oxidation upregulating-effect of fatty acid overproduction [86]. Overexpression of thioesterases has also been employed to release the free fatty acids of specific chain lengths [6].

Studies show that *YlALK3*, *YlALK*5, and *YlALK7* exhibit ω-oxidation activity against dodecanoic acid [36,41]. However none could result in a detactable amount of products from ω-oxidation of hexadecanoic acid in a Δ*alk1-12* strain [36]. It was postulated that Alk3, 5, and 7p may prefer shorter fatty acids, while larger fatty acids are preferrably used for cell maintenance. In this study, the DCAs of 12 and 14 carbons were detected as minor products. Their production can be due to the overexpression of *YlALK5*. Additionally, the overexpression of this gene, using the replicative vector with the strong constitutive promoter, contributed toward LCDCA-16 and ω-hydroxy fatty acid-16 production in our preliminary shake flask cultivations. In fact, a high gene dosage is necessary to achieve sufficient P450 expression in *Y. lipolytica* [94]. This contribution was in addtiton to the background ω-oxidation activity in the F strain toward LCDCA-16 and 18 production. We also observed the contribution of *YlCPR* and *YlFAO1* to the multiple-step oxidation of endogenous long chain free fatty acids precursors to the monomers. In the same fashion, amplification of the P450 reductase gene was found to enhance LCDCA production from *C. tropicalis* [14]. Similarly, it was reported that the overexpression of *YlFAO1* improves LCDCA production from exogenous hydrophobic substrates [42]. This accords with the contribution of *FAO1* to diacid production observed in *C. tropicalis* [95]. Various fatty aldehyde dehydrogenases have been identified in *Y. lipolytica* that are involved in the detoxification of fatty acid aldehyde intermediates by oxidizing them to dicarboxylic acids [96–98]. These native genes likely contribute to the background ω-oxidation activity.

A bioreactor is much more efficient than a shake flask for DCA production [7]. Adequate aeration, agittion, and control over pH play major roles in the P450-based LCDCA production. The inner pH of the cell, as well as aeration, both affect the induction of yeast CYP monooxygenase and CYP reductase [10]. Studies show that a higher dissolved oxygen (DO) level can remarkably improve P450 activity [99,100]. This level was kept at 80% saturation for LCDCA production from *C. ropiclais* [14]. In fact, the native P450 sytem utilizes molecular oxygen that is abundant in a bioreactor fermentation. In our study, fermentation scale-up to 1-L bioreactor under the DO level of at least 50% significantly enhanced the rate of free fatty acid oxidation to LCDCAs. The majority of free fatty acids were converted to LCDCA during the four days of bioreactor fermentation, reaching a titer of 2 g/L for LCDCA-16 and 1.45 g/L for LCDCA-18. Adjustment of the pH is also required for the biosynthesis of LCDCAs. During the production phase, the pH was adjusted to 8 since the LCDCAs are completely diasscoiated at this level. For instance, the diassociation constant of DCA13 is less than 5.8. A higher extracellular pH level increases the solubility of LCDCAs, improves their transportation efficiency, and consequently mitigates the corresponding

product inhibition [101]. Adjustment of pH as well as metabolite production in the production media increases the demand for antifoam addition. Thus, the antfoam was also periodically added to the media.

Transport of LCDCAs out of the cell can improve P450 activity [100] and simplify recovery for an enhanced downstream process at a lower cost. Our engineered strain showed good secretion efficiency, since more than half of the synthetized monomers were found in the media [102]. In fact, DCA of 13 carbons has been found to undergo passive diffusion due to the concentration difference between the inside and outside cells [101]. At a low intracellular pH, the undissociated form of the carboxylic acid is expected, which can mitigate their transportation across the cell membrane [103]. In this study, we showed that LCDCAs are extracellular products. Secretion allows LCDCA production to exceed the lipid accumulation capacity of the cell, and promotes their recovery from media after cell harvest at a lower cost. This also can alleviate their inhibitory effect on P450-based hydroxylation reaction due to their competitive binding to P450 [104].

We supplemented the production media with 400 mg/L leucine to take the advantage of its possible stimulatory effect on the metabolite produciton, under nitrogen-limiting conditions. Supplementation of media with leucine had significant effect on biomass level in shake flask cutures. In addition to that, our preliminary bioreactor fermentation using YNB-Ura, without additional leucine supplementation, resulted in less biomass and LCDCA production from the M strain (Biomass up to 7 g/L and maximum LCDCA of 3 g/L). Similalrly, addition of leucine was reported to increase the growth and lipid accumulation in *S. cerevisiae* devoid of active *SNF2* [105].

Although nitrogen-limiting conditions hinder growth, they induce lipid and citric acid production. The resting cell production of LCDCAs has several advantages, including the dedication of a larger proportion of supplied oxygen to metabolism and the targeted P450 reaction [106], the repeated utilization of LCDCA-exporting cells, the use of high cell concentration, particularly for the multi-copy transformants [107], as well as more strain robustness against fermentation media. In fact, resting cells consume nearly half of what they typically utilize when they have access to a nitrogen source [106]. We used this strategy for the *de novo* production of free fatty acids. This strategy also caused our engineered cell factory to produce more than 36 g/L of citric acid as the major bioproduct which was nearly stable during the 4-day bioreactor fermentation. The production of this byproduct, which is extensively used in pharmaceutical industries [108] raises more revenue and offsets the total production cost.

This *Y. lipolytica* platform can be tailored to produce LCDCA of higher specificity with respect to chain length and saturation. This yeast accumulates a higher proportion of unsaturated fatty acids, oleic and linoleic acids, when it is grown on sugar, due to the strong expression of endogenous desaturases [53]. Overexpression of these native desaturase genes, including Δ-9 and Δ-12 desaturases, can increase the proportion of desaturated fatty acids in lipid bodies. Together with *FAA1* inactivation, this can result in a larger pool of unsaturated free fatty acids for higher unsaturated LCDCA production. In fact, *Y. lipolytica* predominantly accumulate fatty acids in the form of C16:0 and C18:1. These fatty acids are more susceptible to remobolization [109]. Disruption of their re-activation in the Δ*FAA1* background, enbales their availibiltiy for the ω-oxidation. Our study confirmed that this oxidation does not change the configuration of double bonds. As a result, LCDCA16 and LCDCA18:1 were the predominant LCDCA products of our platform. Moreover, according to the chromatogram of GC-FID, a smaller portion of LCDCA-18 had two double bonds (less than 10%). These bonds are dervied from the linolenic acid, FA 18:2, whose concntration was less than oleic acid. Regarding chain length specificity and selectivity, there is room for improvement. For example, manipulation of *YlYAS* genes [110], and selective overexpression of elongase, monooxygenase, and thioesterase can be employed to modulate background activity and enhance the length-specificity of the terminal oxidation.

Y. lipolytica has several advantages as a LCDCA-producing platform. This yeast platform has its own native P450 system that catalyzes terminal oxidation of alkanes and fatty acids [36]. Moreover,

this platform possesses an endogenous redox partner (cytochrome P450 reductase), has the capability for large free fatty acid accumulation [111], and can efficiently generates NADPH cofactor mainly through the pentose phosphate pathway (PPP) [112,113]. This pathway is not tightly regulated by nitrogen concentration [114]. Together with glycolysis, it can contribute significantly to the energy supply of resting cells [115]. At the stationary phase, a higher expression of cytochrome P450 monoxygenases [104] and lipid remobilization [40] are also expected. These features enable the large-scale application of engineered *Y. lipolytica* cells without the need for cofactor addition. Regeneration of NADPH is crucial in whole-cell P450-based biotransformation [116], particularly in the NADPH- and adenosine triphosphate (ATP)-consuming process of fatty acid biosynthesis and oxidation. Moreover, whole cells also provide a protected environment for the increased stability of the P450 enzymes [51]. A whole cell biocatalyst is a cheaper option than the isolated or immobilized enzyme [106]. The pathogenic yeast, *C. tropicalis*, has been commonly engineered for whole cell biotransformation of aliphatic feedstock to LCDCAs [14,25]. However, *Y. lipolytica* has safe status, and has been categorized as a GRAS microorganism by the FDA (Food and Drug Administration) [56,88]. This status, as well as its probiotic properties [117], can pave the way toward the biosynthesis of LCDCAs that meet the safety requirements of the food and pharmaceutical industries.

Despite some chemical based-production of LCDCA at large scale, the high price of the LCDCAs, and their limited commercial availability are still major constraints in expanding the spectrum of their application [25]. Therefore, researchers should use various renewable resources for the sustainable production of these multi-purpose building blocks. An alternative sustainable solution is to establish a P450-based biological approach. However, commercial application of a P450-based platform has also its own advantages and limitations. For example, the scalability of modified cytochrome P450 for multi-kilogram scale synthesis remains a challenge [118]. The majority of P450-based biocatalysts have not met a minimum space-time yield of 0.1 g/L·h in previous studies. A solution to this limitation would be the production of high-value pharmaceuticals. In this scenario, a minimum process productivity of 0.001 g/L·h can justify large-scale bioprocessing from an economic standpoint [19]. The LCDCAs have relatively high selling price, depending on their purity, and therefore are outstanding targets for white biotechnology [12]. Our study is the first report on engineering *Y. lipolytica* for the efficient *de novo* production of LCDCAs from glycerol. Results from this study showed a minimum productivity of 0.04 g/L·h, which is more than the foregoing required productivity. However, the provision of adequate agitation and oxygen transfer in larger fermenters will be challenging to maintain this minimum productivity. Our biological-based approach has several advantages over chemically-based approaches, including a broad range of renewable feedstock, milder process conditions, a more environmentally friendly process, and higher selectivity, particularly toward long chain unsaturated fatty acids [10,119].

These monomers have superior properties, promising future, and a large global market that is expected to reach USD $300.3 millions by 2025 [120]. These highly demanding monomers are used in manufacturing various high-performance polymeric compounds such as nylon and other polyamides. They have a wide scope of application in the industrial manufacture of automobiles, medicines, fragrances, adhesives, and macrolide antibiotics [10]. However, pharmaceutical applications exhibit the quickest growth in terms of revenue [120].

Compared to the shorter diacids, these building blocks and their LCDCA-based polymers have superior bio-based properties such as improved biodegradability, hydrolytic stability, crystallinity, melting temperature, optical transparency, dispersion, miscibility, material toughness, and flexibility; lower moisture uptake, dielectric constant, and surface tension [5]. These long chain monomers are also the building blocks of naturally occurring polyesters [121]. Additionally, the double bonds of unsaturated monomers, which are maintained during terminal oxidation, show promise for additional functional groups and linkages. These features also allow for the production of novel unsaturated LCDCAs [54] to synthesize cross-linked polymer networks with adjustable properties. Synthesizing

these unsaturated long chain monomers is difficult via chemical routes due to the reactivity and participation of the double bonds in unwanted reactions [122].

Our findings suggest that the tunable high-level expression of selected P450 genes, by high copy and stable integration or by high copy number expression plasmids, can foster LCDCA overproduction. Application of a lipid responsive strong promoter may provide additional benefit for P450 gene expression. The multiple-copy integration of a selected gene at random loci is influenced by the integration site underlining the need for an intensive screening afterwards [62,123]. We recommend an intensive screening process for choosing the most productive multiple-copy transformants. We selected the *YlALK5* for multiple-copy integration since it exhibited the highest ω-oxidation activity, among the tested *YlALKSs* toward the endogenously synthesized fatty acids of 16 carbons. The *YlALK5* gene product is a good candidate for protein engineering, allowing higher selectivity and turn-over rates for long chain fatty acids. The combination of the *YlALK5* and *YlFAO1* overexpression can create a push and pull of fatty acid precursors and oxygenated fatty acids in the ω-oxidation pathway. Further studies are recommended to identify the major native cytochome monoxygenese member involved in the temrinal oxidation of 18 carbon-fatty acids. We also suggest modulating the ratio of P450 monoxygenase/reductase overexpression for better performance. Our engineered cell factory can be further optimized to produce a higher proportion of LCDCAs with specific chain lengths or degrees of desaturation. This can be achieved in two ways: by introducing heterologous thioesterase-coding genes that have long chain fatty acid specificity (e.g., *Cocos nucifera* fatty acyl-ACP thioesterase CnFatB2 and *Iris germanica* acyl-ACP thioesterase AAG43857 [124]) or by manipulating the native desaturase and elongase systems. The *LEU2* gene was disrupted for enabling a successful site-specific multiple gene integration and testing various expression systems. However, we suggest targeting other sites for future multiple-gene integrations, due to the positive role of the *LEU2* gene on biomass and metabolite production. We used a standard minimal medium for production. However, the availability of rich nutrients, heme containing compounds, and low cost substrates, including lignocellulosic hydrolysates and crude glycerol, could enhance production level and economy of this bioprocess. The overexpression of key genes from PPP can promote NADPH availability [113], and this may serve the NADPH-consuming process of endogenous fatty acid and LCDCA production. We also recommend the application of immobilized cells and a biofilm reactor for integrated production and recovery and for efficient use of multi-copy transformants. The *de novo* production of LCDCA can be further improved by generating a larger pool of free fatty acids [125], which have stronger stimulatory effect on the induction of the background ω-oxidation activity. This could be achieved by overexpressing the upstream and downstream targets of the fatty acid biosynthesis pathway. Acetyl-CoA carboxylase (*ACC1*) and thioesterase (*TE*) are examples of these targets. Disruption of other acyl-CoA synthetases (e.g., *YlFat1*) [81] and overexpression of intracellular lipases (e.g., *TGL3* and *TGL4*) [126] may also contribute to a larger pool of free fatty acids. Microscopic examination of resting cells may reveal more information about the status of remaining fatty acids. Moreover, time course qualitative analysis of free fatty acids can also provide further insight into their threshold for the induction of background ω-oxidation activity. Despite the good LCDCA transport efficiency of our platform, the overexpression of potential heterologous transporters such as CmCDR1 [127], or homologous transporter like ABC2 [128] could improve LCDCA export to minimize product inhibition. Optimization of culture conditions was beyond the scope of this study. However, production titer and yield can be enhanced by optimizing production media and developing fed-batch or continuous bioprocess. For example, providing a reduced aeration rate [129] or nitrogen as co-feed [130] might lessen citric acid production in favor of more fatty acid accumulation. Summary of elements for further improvement is shown in Figure 9.

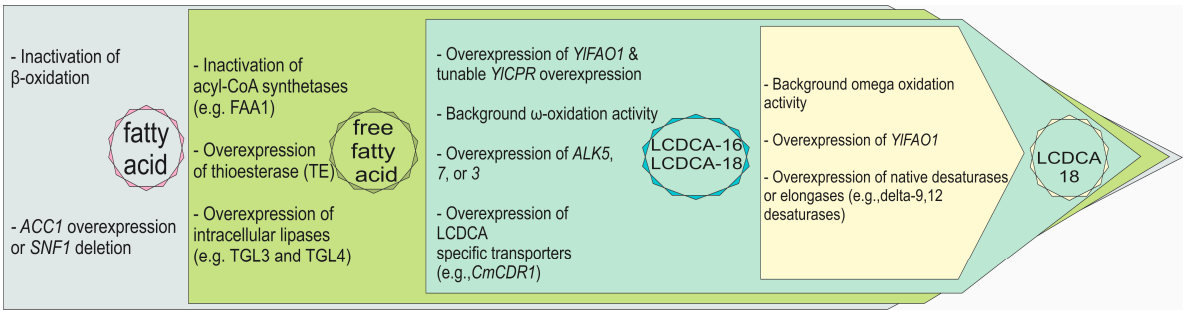


Figure 9. Summary of elements for further improvement.

5. Conclusions

Our study presents the construction of *Y. lipolytica* as a cell factory platform for the *de novo* production of LCDCAs from glycerol. We accomplished this by harnessing the native capabilities of this yeast for endogenous fatty acid accumulation and ω-oxidation. Our combinatorial genetic engineering mainly involved inactivating the peroxisomal fatty acid degradation, disrupting cytosolic fatty acid activation, and upregulating fatty acid ω-oxidation. Further enhancement was obtained through fermentation scale-up to the 1-L nitrogen-limited bioreactor culture. Our engineering strategies led *Y. lipolytica* strains to produce about 3.49 g/L LCDCA, with a yield of 0.06 g/g. To the best of our knowledge, this is the highest *de novo* production level ever reported for LCDCA monomers. We envision that this study constitutes a foundational work in developing *Y. lipolytica* as a safe oleaginous yeast platform to upgrade low-value, unrelated feedstock to high-value multi-purpose LCDCAs at a larger scale in the foreseeable future. This work represents a key advance in yeast strain manipulation and fermentation for the biosynthesis of LCDCAs from renewable non-oily feedstock. Last, this research paves the way for an industrial process that can supplement the global supply of LCDCA from a second source, independent of the non-renewable hydrophobic substrates such as crude oil.

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