

Article

# Engineering *Yarrowia lipolytica* for Enhanced Production of Lipid and Citric Acid

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**Abstract:** Increasing demand for plant oil for food, feed, and fuel production has led to food-fuel competition, higher plant lipid cost, and more need for agricultural land. On the other hand, the growing global production of biodiesel has increased the production of glycerol as a by-product. Efficient utilization of this by-product can reduce biodiesel production costs. We engineered *Yarrowia lipolytica* (*Y. lipolytica*) at various metabolic levels of lipid biosynthesis, degradation, and regulation for enhanced lipid and citric acid production. We used a one-step double gene knock-in and site-specific gene knock-out strategy. The resulting final strain combines the overexpression of homologous *DGA1* and *DGA2* in a *POX*-deleted background, and deletion of the *SNF1* lipid regulator. This increased lipid and citric acid production in the strain under nitrogen-limiting conditions (C/N molar ratio of 60). The engineered strain constitutively accumulated lipid at a titer of more than 4.8 g/L with a lipid content of 53% of dry cell weight (DCW). The secreted citric acid reached a yield of 0.75 g/g (up to ~45 g/L) from pure glycerol in 3 days of batch fermentation using a 1-L bioreactor. This yeast cell factory was capable of simultaneous lipid accumulation and citric acid secretion. It can be used in fed-batch or continuous bioprocessing for citric acid recovery from the supernatant, along with lipid extraction from the harvested biomass.

**Keywords:** *Yarrowia lipolytica*; microbial lipid; citric acid; glycerol; genetic and metabolic engineering; fermentation; leucine metabolism and biosynthesis; bioconversion

## 1. Introduction

Volatility of energy price and concerns over climate change have motivated efforts to explore alternative approaches for production of fuels and chemicals. Microbial fermentation of low-value biomass is a promising strategy for sustainable production of these compounds. Single-cell-oil (SCO), for example, is of great interest to the food, nutraceuticals, and biodiesel industries. Oleaginous organisms such as fungi, yeasts, and algae can accumulate oil beyond 20% of their biomass under appropriate cultivation conditions [1]. The application of oleaginous yeasts as a lipid-producing platform offers many advantages. These include feedstock flexibility, higher sustainability, shorter life cycles, easy cultivation and handling, robustness against contamination, seasonal independence, and lower net greenhouse gas emissions [1,2].

Industrial-scale production of SCO is challenging due to large volumes and low profit margins [3]. Technological and cellular-level improvements are required to reduce processing costs and achieve higher productivity with wider range of low-value substrates [4]. Prior to genetic modification, the lipid content of a wild-type *Y. lipolytica* strain rarely reaches 20% DCW [5]. Therefore, metabolic engineering is necessary to improve lipid productivity. Additionally, the production of other value-added co-products and exploration of zero-cost waste or by-product streams such as glycerol, as feedstock, for yeast SCO production is recommended [6].

Plant-based production of biodiesel is anticipated to reach  $30 \times 10^6$  t. in 2021. Since 1 kg glycerol is produced per 10 kg of biodiesel, this would generate  $3 \times 10^6$  t. glycerol as by-product [7]. Valorization of glycerol for producing SCO or other higher added-value compounds offsets the costs of biodiesel, reduces glycerol surplus, and favors the viability of SCO bioprocess.

Much research has focused on the oleaginous yeast *Y. lipolytica*, a known model non-conventional yeast, to produce and/or secrete various oleochemicals and recombinant proteins [8–10]. This platform is commonly considered for production of lipid, citric acid, as well as oleochemicals derived from acetyl-CoA and fatty acid [11,12]. Although *Y. lipolytica* and *Aspergillus niger* are major producers of citric acid [13], the former is more resistant to metals and offers more environmentally friendly process [14]. *Y. lipolytica* can release both citric acid, at higher concentration, and its isomer isocitric acid at lower concentration. This ratio depends on the feedstock [15]. For example, Morgunov et al., fed this yeast with pure and raw glycerol in a fed-batch cultivation for citric acid production. They reported a citric acid/isocitric acid ratio of 21 to 25, with isocitric acid represented up to 5% [16]. While citric acid is an extracellular metabolite and is secreted into the culture medium, lipid is intracellularly stored in the form of triglycerides (TAG) in this oleaginous yeast. TAG does not have lipotoxicity on the cells as free fatty acids do [17], and can accommodate essential and non-essential fatty acids and precursors for dynamic cell maintenance. *Y. lipolytica* has also shown promise in the bioconversion of glycerol as renewable feedstock to various compounds [18], including lipid [19–24] and citric acid [13,16]. This yeast can efficiently utilize glycerol and prefers it over many other carbon sources [25]. It also has a similar rate of lipid production when fed with pure or crude forms of glycerol [26]. Therefore, this yeast can play a dual role in upstream and downstream processes of biodiesel industries by producing microbial lipid and other valuable pharmaceuticals from glycerol [19].

In this study, we aimed to engineer *Y. lipolytica* to enhance lipid and citric acid production from pure glycerol. We took advantage of the one-step gene knock in/out for targeted integration and overexpression of key TAG synthesizing genes, followed by deletion of *SNF1* gene in the *POX* deleted strain. This strategy served constitutive diversion of carbon flux into the neutral lipid and citric acid in nitrogen-limited glycerol-based media supplemented with leucine. We also examined the effect of leucine supplementation or *LEU2* expression on metabolite production and biomass generation. We cultivated engineered *Y. lipolytica* strains in a shake flask and then performed batch cultivation in a 1-L bioreactor under well-controlled conditions to enhance lipid and citric acid productivity.

## 2. Materials and Methods

### 2.1. Strains and Culture Condition

Table 1 describes the recombinant *Y. lipolytica* strains that were derived from the citric acid producer strain H222 (wild-type German strain) [27]. *Escherichia coli* top 10 was used to develop vectors. Ampicillin was added to the Luria-Bertani (LB) broth medium at concentration of 100 µg/mL according to standard protocols [28].

**Table 1.** *Yarrowia* strains used in this study.

<i>Y. Lipolytica</i> Strain Names	Strain Genotypes	Gene Configurations	Reference
H222 (H)	MatA mating type		[27]
H222ΔP <i>leu</i> <sup>+</sup> <i>ura</i> <sup>-</sup> (HP-U)	<i>MATA ura3-302::SUC2 ΔPOX1-6</i>		[27]
H222ΔP <i>leu</i> <sup>+</sup> <i>ura</i> <sup>+</sup> (HP)	HP-U, Δ <i>POX3::URA3</i>	<i>loxR-URA3-loxP</i> flanked by <i>POX3</i> homologous up/down stream sequences	This study
H222ΔP ΔL + <i>DGA1 DGA2 leu</i> <sup>-</sup> <i>ura</i> <sup>+</sup> (HPDD)	HP, Δ <i>LEU2</i> + <i>DGA1</i> + <i>DGA2::URA3</i>	<i>loxR-URA3-loxP</i> flanked by <i>LEU2</i> homologous upstream and <i>pFBA-DGA1-tLip1 pTEF-DGA2-tXPR2 LEU2</i> homologous downstream sequences	This study
H222ΔP ΔL + <i>DGA1 DGA2 ΔSNF1 leu</i> <sup>-</sup> <i>ura</i> <sup>+</sup> (HPDDS)	HPDD, Δ <i>SNF1::URA3</i>	<i>loxR-URA3-loxP</i> flanked by <i>SNF1</i> up/down homologous stream sequences	This study

Synthetic defined media containing 6.7 g/L Yeast Nitrogen Base (YNB) w/ammonium sulfate w/o amino acids (Becton, Dickson, and company), 20 g/L glucose, and a drop-out synthetic mix minus uracil (-Ura) or minus leucine (-Leu) (US Biological) were used for the selection of knock out/in strains. The uracil auxotrophic strains were obtained by growing in YNB-Leu liquid medium with the expression of Cre recombinase. Seed culture preparation was carried out using the synthetic defined medium devoid of uracil (YNB-Ura). A rich medium (YPD) was prepared with 20 g/L glucose, 20 g/L bacto peptone (BD), and 10 g/L bacto yeast extract (BD), and was used for non-selective propagation of strains. The YNB-Ura and YNB-Leu media were buffered with a 50 mM sodium phosphate buffer, pH 6.8, to determine the effects of leucine supplementation and *LEU2* expression on biomass and metabolite production. For solid media, 20 g/L agar (US Biological, Swampscott, MA, USA) was added.

For lipid production in the shake flask and bioreactor, previous data on glycerol based fermentation media was taken into account, followed by some modifications [19,29]. The medium was formulated as follows: 1.5 g/L yeast extract (BD), 1.5 g/L  $MgSO_4 \cdot 7H_2O$ , 7 g/L  $KH_2PO_4$ , 2.5 g/L  $Na_2HPO_4$ , 0.15 g/L  $CaCl_2 \cdot 2H_2O$ , 0.15 g/L  $FeCl_3 \cdot 6H_2O$ , 0.02 g/L  $ZnSO_4 \cdot 7H_2O$ , 0.06 g/L  $MnSO_4 \cdot H_2O$ , 0.1 mg/L  $CoCl_2 \cdot 6H_2O$ , and 0.04 mg/L  $CuSO_4 \cdot 5H_2O$ . Prior research suggested for this yeast, glycerol concentration should range from 52 to 112 g/L for bioconversion of glycerol to biomass and lipid [21]. In the batch cultivations of this study, glycerol solution was separately sterilized and added to the flasks to reach an initial concentration of  $60 \pm 2$  g/L. The carbon to nitrogen ratio (C/N) was adjusted to 60 for all production media using pure glycerol (J.T. baker) and 1.1 g/L  $(NH_4)_2SO_4$  as major carbon and nitrogen sources, respectively. Leucine was added to production media in shake flask and bioreactor at a concentration of 100 mg/L (Teknova) to compensate for *LEU2* deletion in the HPDD and HPDDS strains. Shake flask cultivations were performed in 250 mL Erlenmeyer flasks containing 50 mL of the medium at an agitation rate of  $180 \pm 5$  rpm and temperature of  $28 \pm 1$  °C. Colonies from solid YNB-Ura plates were precultured in the selective defined media. Exponentially growing cells were harvested by centrifuge, washed and then resuspended in water. They were subsequently inoculated into the production medium to reach 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). A single colony of *Y. lipolytica* grown on DOB-Ura was transferred into the YNB-Ura broth. Cells from 100 mL 24 h shake flask pre-culture were harvested by centrifugation at 12,000 rpm, washed twice with water and inoculated into 700 mL of the fermentation medium (with C/N 60) to reach an initial  $OD_{600}$  of ~0.3. The temperature was kept at 28 °C, and the pH was controlled not to drop below 2.5, using 1 M NaOH. Dissolved oxygen was maintained at 25% until peak biomass was attained (from 48 h to ~72 h). This was achieved by cascading with agitation ranging from 250 to 800 rpm, and by supplying sterile, filtered air at flow rate of 2 vvm. The dissolved oxygen and airflow rate were later decreased to ~5% and 0.5 vvm, respectively, near the end of the 5-day fermentation. The fermenter experiments were performed in duplicate. Samples with the volume of 25 mL were taken daily. An antifoam Y-30 emulsion (Sigma-Aldrich, St. Louis, MO, USA) solution was prepared at a concentration of 5%, and was periodically added to control the foam level.

## 2.3. Genetic Techniques

Standard molecular biology techniques were used to construct the vectors [28]. Table 2 presents all plasmids and their functions (See Supplementary Materials for plasmid maps).

**Table 2.** Vectors used in this study.

Vector Names	Features
Cre-recombinase (CR)	Shuttle vector carrying leucine marker, Cre recombinase flanked by TEFin promoter and Xpr2 terminator
pGR12 (L)	Shuttle empty vector carrying leucine marker, FBA promoter and lip1 terminator, used for study of leucine biosynthesis
POX3 Ura (PU)	Uracil selection marker flanked by <i>POX3</i> upstream and downstream homologous sequences, used for construction of HP strain
LEU2 Ura (LU)	Uracil selection marker flanked by <i>LEU2</i> upstream and downstream homologous sequences, used for construction of LDD vector
SNF1 Ura (SU)	Uracil selection marker flanked by <i>SNF1</i> upstream and downstream homologous sequences, used for construction of HPDDS strain
pGR12 DGA1 (D1)	Single gene centromeric shuttle replicative vector with leucine selection marker, <i>DGA1</i> gene cloned between FBA promoter and lip1 terminator, used for double gene expression cassette construction
pJN44 DGA2 (D2)	Single gene centromeric shuttle replicative vector with leucine selection marker, <i>DGA2</i> gene cloned between TEFin promoter and xpr2 terminator, used for double gene expression cassette construction
DGA1 DGA2 (DD)	Double gene centromeric shuttle replicative vector with leucine selection marker, used for construction of LDD vector
LEU2 DGA1 DGA2 (LDD)	Uracil selection marker flanked by <i>LEU2</i> homologous upstream sequence and combination of double gene expression cassettes and <i>LEU2</i> homologous downstream sequence, used for construction of HPDD strain

Construction of the double gene expression cassette was carried out by amplification of diacylglycerol acyltransferases *DGA1* (YALI0E32769g) and *DGA2* (YALI0D07986g) gene segments using the Q5 high fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and gDNA from *Po1f* (ATCC MYA-2613) as the template with the primers listed in Table 3. The *DGA1* and *DGA2* amplicons were individually digested and inserted into *Y. lipolytica* plasmid pGR12 (PFBA-Tlip1) and pJN44 (PTEFin-Txpr2), respectively. The segment of PTEFin-*DGA2*-Txpr2 was obtained by digestion with XbaI and SpeI and then, recovered from the gel. Then it was inserted into SpeI and Fast Alkaline Phosphatase digested *DGA1*-pGR12 plasmid.

Plasmids for gene knock-out contained the uracil selection marker 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 *POX3* genes were amplified with the primers listed in Table 3. The amplicons were digested, purified, and inserted into the upstream and downstream of the uracil marker. The double gene expression cassette segment underwent double digestion with XbaI and SpeI followed by gel recovery for subsequent insertion into SpeI and Fast Alkaline Phosphatase digested *LEU2* knock-out plasmid (LU), which was used in one step knock in/out.

Targeted gene knock in/out was achieved by transformation of the linearized vectors containing homologous upstream and downstream sequences. The linearized vectors consisted of NdeI-digested PU, ApaI-digested SU, and NdeI-digested LDD plasmids. Transformation was performed using the Zymogen Frozen EZ yeast transformation kit II (Zymo Research, Irvine, CA, USA), in compliance with the manufacturer's protocol. The *loxR*-*URA3*-*loxP* modules were rescued for subsequent genetic modification by the LoxP-Cre system as previously reported [30]. Gene deletions and expression cassette insertion were confirmed by Polymerase chain reaction (PCR) using the primers listed in Table 3.

**Table 3.** PCR primers used in this study.

No.	Name	Sequence (5'→3', Underlined Restriction Site)
1	POX3 up F ApaI	CTATAGGGCCCCTGGGCTGTTCCGGTCGA
2	POX3 up R XbaI	GATCCTCTAGAAGGACGCACAACGCC
3	POX3 down F SpeI	CTGGACTAGTCGCTCCCATTGGAACTACGA
4	POX3 down R NdeI	CCTCACATATGTCTCTTCGCTGTGGTCTAGG
5	POX3 F Ura	GTCTCTACTTGTAGTTCTGTAGACAGACT
6	POX3 Ura R	GAAGAATGTATCGTCAAAGTGATCCAAG
7	POX3 Ura F	TGACTTGTGTATGACTTATTCTCAACTACA
8	POX3 R Ura	AGATGCGTGATAGATTACTTGGATTTAGT
9	DGA1 F HindIII	GAGCGAAAGCTTATGACTATCGACTCACAATACTACAAGT
10	DGA1 R SalI	GTTCAAGTCGACTTACTCAATCATTCCGGAAGCTCTGGG
11	DGA2 F HindIII	GCAAGGAAGCTTATGGAAGTCCGACGACGA
12	DGA2 R PstI	ATGCTACTGCAGCTACTGGTTCTGCTTGTAGTTGT
13	LEU2 up F ApaI	CTATAGGGCCC ACCGGCAAGATCTCGTTAAGACAC
14	LEU2 up R XbaI	GATCCTCTAGATGTGTGTGGTTGTATGTGTGATGTGG
15	LEU2 down F SpeI	CTGGACTAGTCTCTATAAAAAGGGCCCAGCCCTG
16	LEU2 down R NdeI	CCTCACATATG GACAGCCTTGACAACCTTGGTTGTTG
17	LEU2 F Ura	TACAGTTGTAACCTATGGTGCCTTATCTGGG
18	LEU2 Ura R	CCTTGGGAACCAACCCCGT
19	LEU2 Ura F	ACTTCCTGGAGGCAGAAGAAGCTT
20	LEU2 R Ura	ATAGCAAATTTAGTCGTCGAGAAAGGGTC
21	SNF1 up F ApaI	CAATTGGGCCCCGTGATCAAAGCATGAGATACTGTCAAGG
22	SNF1 up R XbaI	GATCCTCTAGAGAGGTGGTGGAAGGAGTGGTATGTAGTC
23	SNF1 down F SpeI	CTGGACTAGT TCATTAATACGTTTCCCTGGTG
24	SNF1 down R NdeI	CCTCACATATGGGAATTCGTGCAGAAGAACA
25	SNF1 F Ura	GCGGGAAATCAAGATTGAGA
26	SNF1 Ura R	CGGTCCATTTCTACCAACT
27	SNF1 Ura F	CCTGGAGGCAGAAGAAGCTTG
28	SNF1 R Ura	ACTACTGGCGGACTTTGTGG

The plasmids were constructed using standard restriction digestion cloning with FastDigest restriction enzymes (Thermo Fisher Scientific, Waltham, MA, USA). Yeast genomic DNA was prepared for PCR amplification and verification as described previously [31]. The DNA products of PCR and digestion were purified with the clean and concentrator-5 Kit (Zymo Research). DNA fragments were recovered from agarose gels with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific).

## 2.4. Analytical Methods

### 2.4.1. Dry Biomass

Seven-milliliter samples were collected daily. Five-milliliter samples were centrifuged for 5 min at 13,300 rpm. The cell pellet was washed first with saline (0.9% NaCl solution) and then with distilled water. The biomass yield was determined gravimetrically after the samples were dried at 105 °C until a consistent weight was reached. This was expressed in grams of dry cell weight per liter (g DCW/L).

### 2.4.2. Glycerol and Citric Acid Concentrations

Concentrations of glycerol and citric acid in fermentation broth were analyzed by varian Pro Star 230 high-performance liquid chromatography (HPLC) using an Aminex HPX- 87H column. Samples were centrifuged and supernatants were filtered using 0.22 µm pore-size membranes (Simsii, Inc., Irvine, CA, USA). Subsequently, they were eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL/min and 65 °C. Signals were detected by refractive index (RI) and UV (210 nm) detectors. Standards were used for identification and quantification of the glycerol and citric acid. This method was not able to distinguish between citric acid and its isomer isocitric acid. Thus, the sum of their concentrations was determined.

### 2.4.3. Qualitative and Quantitative Analysis of Lipids

Total lipid extraction and transesterification were carried out according to the procedure described previously by O'Fallon et al., 2007 [32]. Fatty acid methyl esters (FAME) were prepared in hexane and analyzed by gas chromatography (GC). This analysis was performed 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 was set at 250 °C and 1 μL, respectively. The injection was performed with a split mode (ratio 20:1). The oven was initially 190 °C, and was increased to 240 °C at a rate of 5 °C min<sup>-1</sup>. This was maintained at the final temperature for 20 min. The FID temperature was 250 °C. FAME standards were used to identify the fatty acid peaks in the chromatograms. The (0.5 mg/mL) tridecanoic acid (C13:0) (Sigma-Aldrich, St. Louis, MO, USA) solution in methanol was used as the internal standard to quantify the fatty acids. The total lipid titer and content was reported as g/L and percentage of the DCW, respectively. The supernatant was analyzed for possible extracellular lipid extraction.

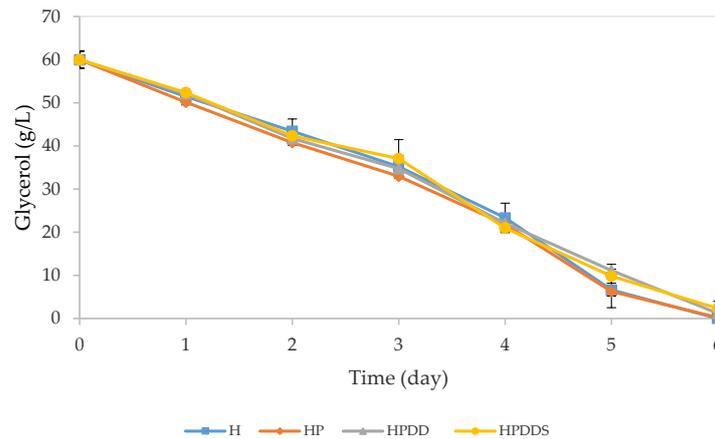
## 3. Results

### 3.1. Comparative Time-Course Study

In this research, we constructed several strains through overexpressing key TAG-synthesizing genes and deleting the key negative regulator of the *de novo* fatty acid biosynthesis pathway. Specifically, the double gene expression cassette of *DGA1* and *DGA2* was integrated into *LEU2* locus of the  $\Delta$ *POX1-6* HP strain to improve lipid synthesis and generate the HPDD strain. The deletion of *SNF1* was combined into  $\Delta$ *POX1-6*,  $\Delta$ *LEU2* *DGA1p* and *DGA1p* overexpression background to construct the HPDDS strain for creating potential synergy in carbon dedication to lipid and citric acid production. All of our strains were phototrophic for uracil. Pure glycerol was used as a carbon source at an initial concentration of 60 g/L under nitrogen-limiting conditions (C/N = 60). The following section presents data from the comparative time course study of feedstock consumption and the production of biomass, citric acid, and lipid by four strains. For this purpose, we collected samples at one-day intervals for six days. Although we present related data and previous findings from the literature, accurate comparison between the results and those of previous research is only possible when all variables are taken into account, including strain types, cultivation conditions, and genetic engineering strategies.

#### 3.1.1. Glycerol Consumption

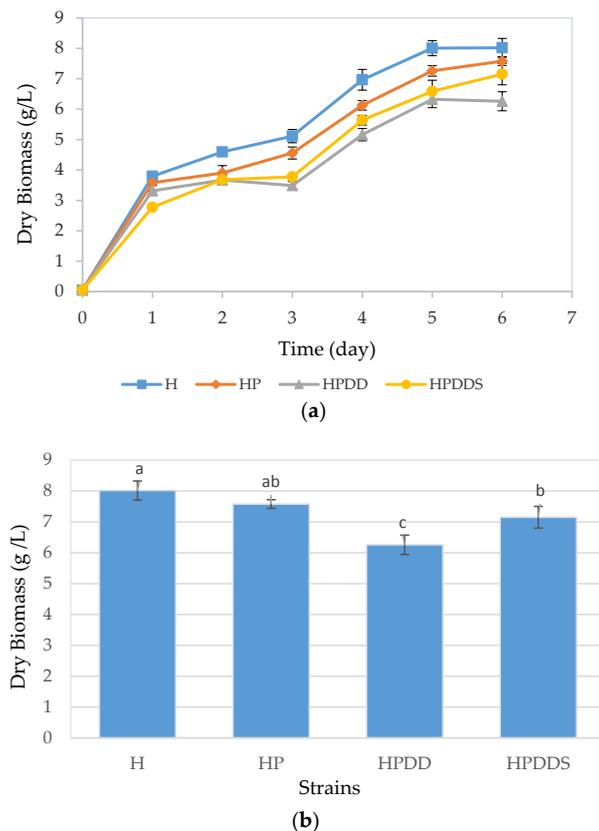
The comparative study of glycerol consumption by four strains during the 6-day fermentation was conducted. It can be seen in Figure 1 that the H and HP strains utilized almost all of the glycerol during the 6-day fermentation. However, 1–3 g/L glycerol remained from both the HPDD and HPDDS strains during the same period. The lower glycerol consumption rate may be due to a lower cell biomass level consuming the feedstock, lack of *LEU2* expression, and some metabolic perturbations caused by our genetic modification. It is noticeable that the genetic background of the strains affects the diversion of glycerol metabolism into specific pathways and outcomes. For instance, in the wild-type strain, the feedstock was used for more biomass production and corresponding cell maintenance, while in the HPDD and HPDDS strains, a higher portion of the feedstock was spent on lipid and citric acid production.



**Figure 1.** Comparative glycerol consumption by four strains during 6-day shake flask cultivation at  $28 \pm 1$  °C under nitrogen limiting conditions (C/N = 60). Error bars represent standard deviation of  $n = 3$ . H: H222 wild-type strain, HP: H222  $\Delta$ POX1-6, HPDD: H222  $\Delta$ POX1-6  $\Delta$ LEU2 +DGA1 DGA2, HPDDS: H222  $\Delta$ POX1-6  $\Delta$ LEU2 +DGA1 DGA2  $\Delta$ SNF1.

### 3.1.2. Biomass Production

The results of biomass production from 6-day shake flask cultivations for all four strains are summarized in Figure 2.

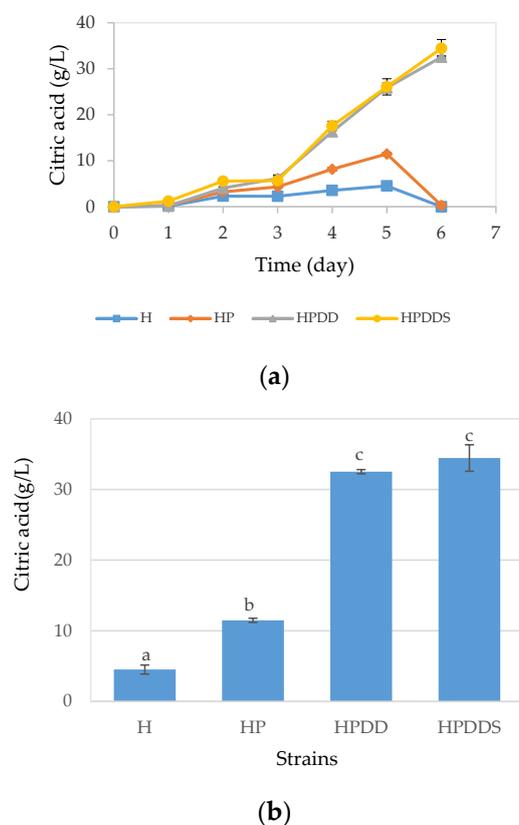


**Figure 2.** Comparative biomass production by four strains during 6-day shake flask cultivation (a) and on the last day (b) at  $28 \pm 1$  °C under nitrogen limiting conditions (C/N = 60). Error bars represent standard deviation of  $n = 3$ . H: H222 wild-type strain, HP: H222  $\Delta$ POX1-6, HPDD: H222  $\Delta$ POX1-6  $\Delta$ LEU2 + DGA1 DGA2, HPDDS: H222  $\Delta$ POX1-6  $\Delta$ LEU2 + DGA1 DGA2  $\Delta$ SNF1. abc columns with dissimilar letters at the top are significantly different ( $p < 0.05$ ).

The yeast biomass was produced by four strains during six days of shake flask cultivations. As shown in Figure 2, the wild-type strain produced the highest level of the dried yeast biomass (about 8 g/L) under the study conditions. The deletion of *POX* genes slightly affected biomass formation, while simultaneous *LEU2* deletion and *DGA1, 2* overexpression led to a significant ( $p < 0.05$ ) reduction of biomass to 6.3 g/L. This loss was recovered in part by a higher lipid accumulation caused by *SNF1* gene deletion. The engineered strain HPDDS formed 7.15 g/L of biomass during the six days of incubation.

### 3.1.3. Citric Acid Production

The results of the comparative time-course study performed on citric acid production from shake flask cultivations for all four strains are illustrated in Figure 3. Considering that citric acid was unstable in both the H and HP cultures, their maximum peaks were taken into account for statistical analysis.



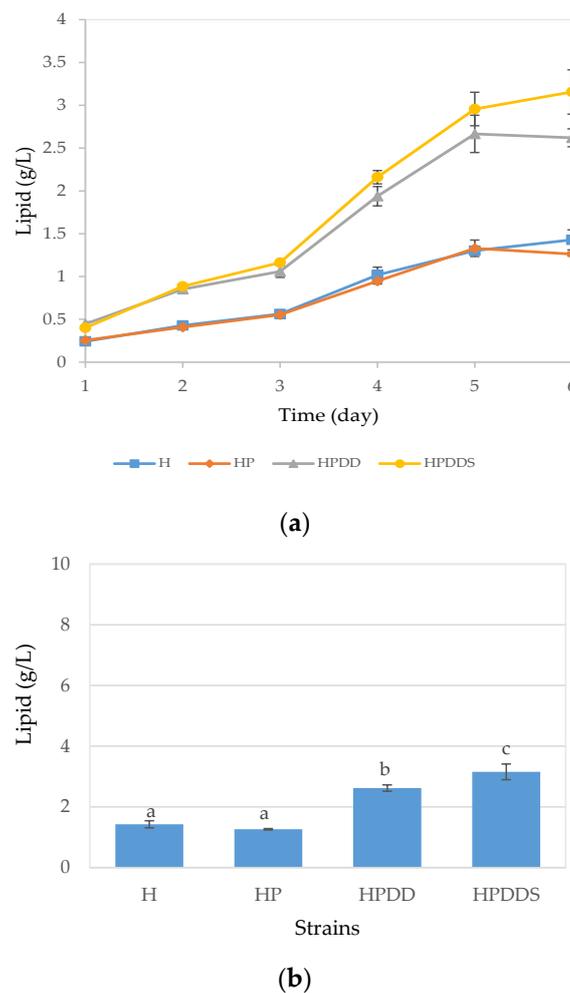
**Figure 3.** Comparative citric acid production by four strains during 6-day shake flask cultivation (a) and on the last day (b) at  $28 \pm 1$  °C under nitrogen limiting conditions (C/N = 60). Error bars represent standard deviation of  $n = 3$ . H: H222 wild-type strain, HP: H222  $\Delta$ POX1-6, HPDD: H222  $\Delta$ POX1-6  $\Delta$ LEU2+*DGA1 DGA2*, HPDDS: H222  $\Delta$ POX1-6  $\Delta$ LEU2 + *DGA1 DGA2*  $\Delta$ SNF1. abc Columns with dissimilar letters at the top are significantly different ( $p < 0.05$ ).

Our method could not distinguish between two isomers, citric acid and isocitric acid. Therefore, our reported concentration corresponds to the sum of these two acids. The results shown in Figure 3 indicate that all strains produced citric acid (a by-product of lipid biosynthesis) at different levels. It is interesting to note that the citric acid production was followed by citric acid degradation by the H and HP strains due to the exhaustion of glycerol, an extracellular carbon supply. In fact, *Y. lipolytica* is not only capable of citric acid production, but also use of it as a carbon and energy source [33]. Both the HPDD and HPDDS strains produced significantly ( $p < 0.05$ ) more citric acid, ranging from 32 to 35 g/L, as the by-product of lipid biosynthesis. Consumption of citric acid was not observed

for these two strains. This can be due to the availability of glycerol as a substrate during the 6-day shake flask fermentation. The HPDDS strain devoid of *SNF1* produced the highest level of citric acid during this period under nitrogen-limiting conditions. The maximum peak of citric acid was obtained at the end of incubation for the resting cells when the final pH was in the range of 2.3–2.5. One study suggested that the citric acid production occurs mainly during the stationary phase and is minimal at pH 3.0 [34]. Subsequently, we selected the best citric acid-producing strain, HPDDS, for further studies in the bioreactor.

### 3.1.4. Lipid Production

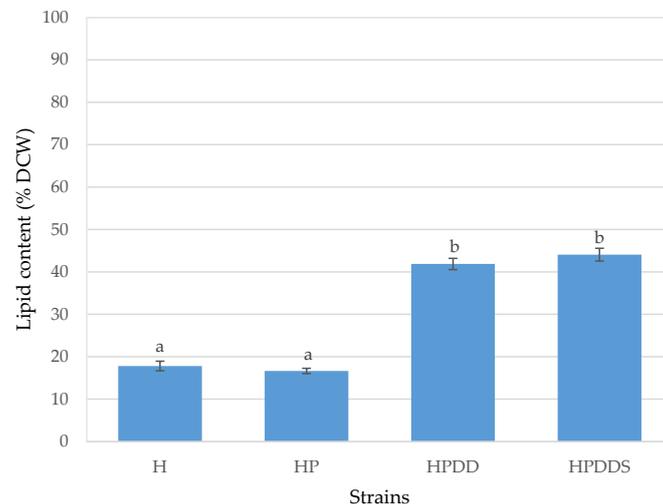
The results of lipid production by all four strains in the 6-day shake flask cultures are presented in Figure 4.



**Figure 4.** Comparative lipid production by four strains during 6-day shake flask cultivation (a) and on the last day (b) at  $28 \pm 1$  °C under nitrogen limiting conditions (C/N = 60). Error bars represent standard deviation of  $n = 3$ . H: H222 wild-type strain, HP: H222  $\Delta$ POX1-6, HPDD: H222  $\Delta$ POX1-6  $\Delta$ LEU2 + *DGA1 DGA2*, HPDDS: H222  $\Delta$ POX1-6  $\Delta$ LEU2 + *DGA1 DGA2*  $\Delta$ SNF1. abc columns with dissimilar letters at the top are significantly different ( $p < 0.05$ ).

Lipid accumulation in the wild-type strain and the strain with the inactive  $\beta$ -oxidation degradation pathway was limited to 1.3–1.4 g/L, representing 17 to 18% of DCW under the nitrogen-limiting conditions. This observation accords with the low level of lipid accumulation (less than 1 g/L accounting for 3 to 20% of DCW) obtained by growing *Y. lipolytica* on biodiesel-derived

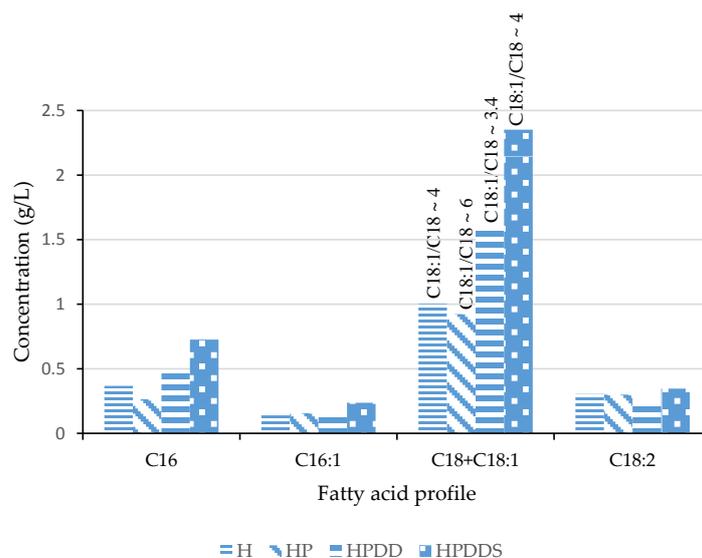
glycerol under nitrogen-limiting conditions [35]. Lipid content can be enhanced through optimization of culture conditions [20] or through genetic manipulation. Our genetic engineering significantly ( $p < 0.05$ ) increased the total fatty acid content to 2.6 g/L (42% of DCW) in the HPDD strain and to 3.15 g/L (44% of DCW) in the HPDDS strain in the 6-day shake flask cultivations. We observed an improvement of 2.47-fold in lipid content over the wild-type strain. The variation of lipid content percentages among all four strains is also notable (see Figure 5).



**Figure 5.** Comparative lipid content of four strains at the end of 6-day shake flask cultivation at  $28 \pm 1$  °C under nitrogen limiting conditions (C/N = 60). Error bars represent standard deviation of  $n = 3$ . H: H222 wild-type strain, HP: H222  $\Delta$ POX1-6, HPDD: H222  $\Delta$ POX1-6  $\Delta$ Leu2+DGA1 DGA2, HPDDS: H222  $\Delta$ POX1-6  $\Delta$ Leu2 + DGA1 DGA2  $\Delta$ SNF1. ab columns with dissimilar letters at the top are significantly different ( $p < 0.05$ ).

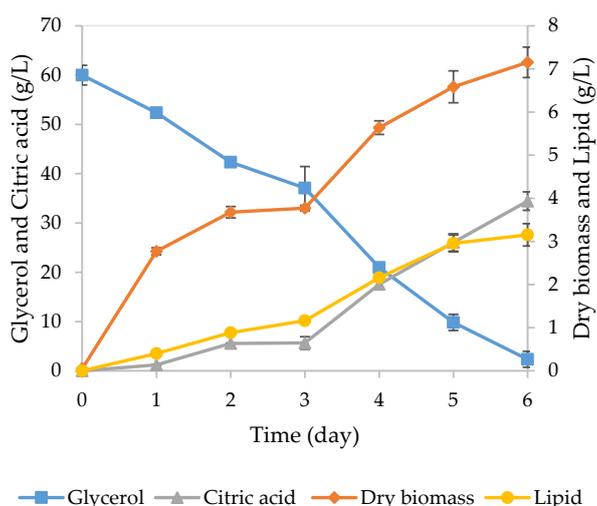
In comparison, another study coupled *DGA2* overexpression with *SNF1* deletion, resulting in a lipogenic phenotype for an engineered *Y. lipolytica* with a lipid content of over 76% using acetate as a carbon source [36]. Our titers are higher than those reported by Poli et al., who achieved 4.9 g/L biomass and 1.48 g/L lipid (30% of DCW) by growing *Y. lipolytica* QU21 on 100 g/L glycerol and NH<sub>4</sub>SO<sub>4</sub> as a nitrogen source in a shake flask study [26]. Figure 5 indicates that HPDDS is the best lipid-producing strain compared to other strains under our study conditions (C/N of 60 and leucine supplementation of 100 mg/L). However, the lipid content of this strain is not significantly different ( $p > 0.05$ ) from the HPDD strain. The HPDDS strain showed an increase in the lipid production of over 120% compared to the wild-type strain. Figure 6 presents the major fatty acid content of the strains. We optimized our GC-FID method for analysis of the fatty acid content. However, our final method and the corresponding GC-FID column could not efficiently separate C18 from C18:1 fatty acid. Therefore, we reported the sum of these two fatty acids together, with an approximate ratio of C18:1/C18 based on their corresponding peak heights.

The analysis of the fatty acid profile among different strains is presented in Figure 6. Oleic acid (C18:1) and palmitic acid (C16) were the predominant fatty acids, with a concentration that varied by strains. Oleic acid had the highest concentration, ranging from 44% to 51% in the strains. The predominance of oleic acid is in accordance with the 47% and 59% oleic acid reported by Papanikolaou et al. (2013) for the wild and engineered strains grown on 90 g/L of glycerol [37]. The other minor fatty acids, stearic acid (C18), linoleic acid (C18:2), and palmitoleic acid (C16:1), underwent smaller changes as a result of our genetic modification.



**Figure 6.** Comparative fatty acid profile of four strains at the end of 6-day shake flask cultivations at  $28 \pm 1 \text{ }^\circ\text{C}$  under nitrogen limiting conditions ( $C/N = 60$ ). H: H222 wild-type strain, HP: H222  $\Delta POX1-6$ , HPDD: H222  $\Delta POX1-6 \Delta LEU2 + DGA1 DGA2$ , HPDDS: H222  $\Delta POX1-6 \Delta LEU2 + DGA1 DGA2 \Delta SNF1$ .

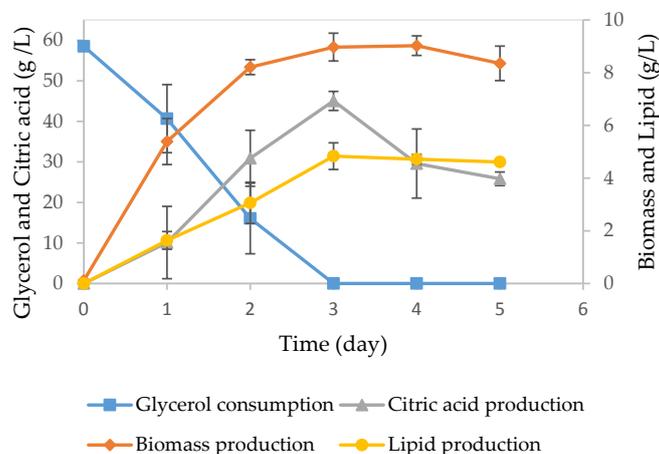
Figure 7 re-presents the time-course of glycerol consumption and metabolite production for the HPDDS strain. This strain produced citric acid and lipid at a yield of about 0.59 g/g and 0.05 g/g of consumed glycerol, respectively. Therefore, citric acid is considered to be the major bioproduct of this engineered strain. Previous research has reported citric acid production at a yield of 0.93 g/g of glucose hydrol from this yeast [38]. Another study reported a mass yield of 0.90 g citric acid from each gram of glycerol containing waste [34]. Similarly, the lipid titer, content, and yield of 2.82 g/L, 0.39 g/g, and 0.1 g/g of glucose, respectively, were also reported for the *SNF1*-deleted strain overexpressing the *DGA2* gene [36]. That study also reported a synergistic effect between the *SNF 1* deletion and *DGA2* overexpression when combined in the same strain.



**Figure 7.** Time course study of glycerol consumption and metabolite production by the HPDDS strain during 6-day shake flask cultivations at  $28 \pm 1 \text{ }^\circ\text{C}$  under nitrogen limiting conditions ( $C/N = 60$ ). Error bars represent standard deviation of  $n = 3$ . HPDDS: H222  $\Delta POX1-6 \Delta LEU2 + DGA1 DGA2 \Delta SNF1$ .

### 3.2. Fermentation Study

Figure 8 presents data from the time course study of the feedstock consumption and the metabolite production from the best strain (HPDDS) in the 1-L bioreactor. The average of the two batch fermentation rounds is shown.



**Figure 8.** Time course study of glycerol consumption and metabolite production by HPDDS strain during 5-day batch fermentation at 28 °C under nitrogen limiting conditions (C/N = 60). Data from 2 rounds of batch fermentation. HPDDS: H222  $\Delta$ POX1-6  $\Delta$ LEU2+DGA1 DGA2  $\Delta$ SNF1.

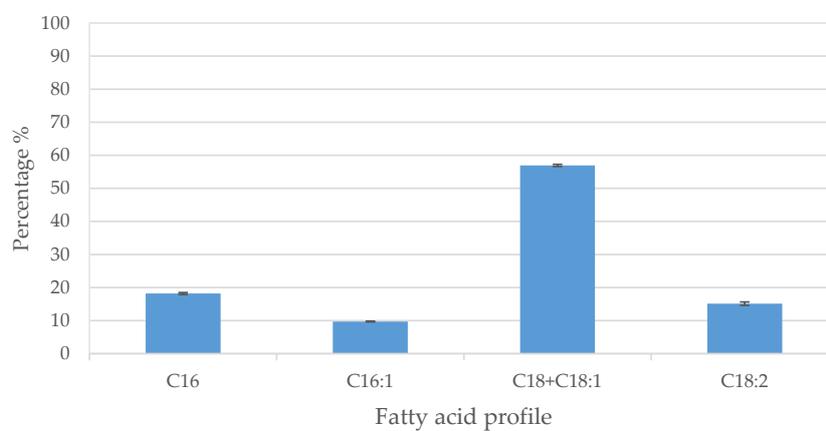
The engineered strain completely utilized 60 g/L glycerol within the three days of fermentation. After that, the strain began to use the secreted citric acid as the carbon source. This observation can explain citric acid reduction during the remaining days of fermentation. The maximum biomass production significantly ( $p < 0.05$ ) increased, from 7.15 g/L in the shake flask to 9 g/L in the bioreactor for the same strain. A slight reduction in biomass was observed on the last day of the fermentation. This may be due to biomass loss during the precipitation of cells containing a relatively large amount of intracellular lipids (over 50%).

Fermentation in the bioreactor ( $p < 0.05$ ) significantly enhanced citric acid and lipid production over the shake flask culture. Citric acid reached the concentration of up to 45 g/L with a yield of 0.75 g/g. The productivity of citric acid was also increased from 0.39 g/L·h to 0.63 g/L·h. Similarly, citric acid was obtained at a productivity of 0.4 g/L·h by growing this yeast in an unbuffered medium with glucose under nitrogen-limiting conditions in flasks [13]. In that study, citric acid was produced at a titer and yield of 35 g/L and 0.43 g/g, respectively, from high initial raw glycerol (80 g/L). In another study, citric acid was obtained at a volumetric productivity of 0.89 to 1.14 g/L·h from *Y. lipolytica* when grown on pure or impure glycerol in a fermenter [34]. In fact, this product was less stable in our bioreactor cultivation over the shake flask fermentation due to a faster glycerol consumption rate and perhaps more control over aeration conditions. Our results show that scale-up of the engineered HPDDS strain in a 1-L bioreactor also enhanced lipid yield and productivity. The peak of lipid production was reached during three days of fermentation at 4.8 g/L, accounting for 53% of DCW, with a productivity of about 0.07 g/L·h. Fontanille et al., used glycerol at a concentration of 80 g/L with a C/N ratio of 62 in batch bioreactor experiments [39]. They reported biomass, lipid titer, and content of 42 g/L, 16 g/L, and 38%, respectively. In another study, crude glycerol with 60% impurity was added to an industrial effluent at a concentration of 4%. Utilization of the feedstock resulted in a lipid titer of 2.21 g/L, which was increased to 2.81 g/L after scale-up [40].

It is not surprising that we observed the constitutive lipid accumulation phenotype simultaneously with biomass propagation. This was due to the inactivation of *SNF1*. The highest biomass, citric acid, and lipid content was reached within the three days of the bioreactor fermentation. This was faster than the shake flask cultivation, in which peaks were reached in six days. Likewise, a constitutive fatty

acid production was reported in both growth and oleaginous media after deleting *SNF1*, a negative regulator of lipid accumulation [5]. Another study introduced an approach for constitutive lipid accumulation and citric acid secretion by deleting the *PHD1* gene (YALI0F02497g) encoding the synthesis of 2-methylcitrate dehydratase [37]. The researchers reported a citric acid titer and yield of 57.7 g/L and 0.91 g/g of waste glycerol, respectively, under nitrogen-limiting conditions. However, their maximum lipid content was obtained under nitrogen excess conditions at a titer of 0.98 g/L (accounting for up to 31% of DCW) from 60 g/L waste glycerol. Generally, the *de novo* production of both lipid and citric acid is biochemically equivalent under nitrogen-limiting conditions [37]. The predominance of one occurs at the expense of another depending on the strain. For instance, citric acid was secreted as a major bio-product when the lipid content was less than 22% [13,41].

We also examined the fatty acid composition of accumulated lipid. The results are shown in Figure 9.



**Figure 9.** Fatty acid composition of accumulated lipid in HPDDS strain from the bioreactor fermentation. HPDDS: H222  $\Delta$ POX1-6  $\Delta$ LEU2 + *DGA1 DGA2  $\Delta$ SNF1*.

In this study, the major accumulated fatty acids were, in order of abundance, oleic, palmitic, linoleic, stearic, and palmitoleic acid. The ratio of C18:1/C18 was about 8, based on the associated peak heights. Similarly, oleic acid and palmitic acid accounted for 44% and 36% of total fatty acids when glycerol was used in the production medium [40]. Papanikolaou et al. (2013) reported that oleic acid and palmitic acid constitute 52% and 21% of total fatty acids in the engineered strain devoid of *PHD1* gene using glycerol at a concentration of 60 g/L under nitrogen-limiting conditions [37].

### 3.3. Comparative Study of *LEU2* Expression and Leucine Addition

We also examined the effect of *LEU2* expression over leucine supplementation, and the results are listed in Table 4. In order for *LEU2* expression to occur, we transferred the low-copy shuttle vector pGR12 carrying the leucine marker into the HPDDS strain and finally grew the transformants on YNB-Leu (C/N = 60) for six days. We used the shuttle vector-based expression since the *LEU2* locus on the chromosome was used for the site-specific integration of the *DGA 1, 2* genes. For leucine supplementation, we grew the HPDDS uracil<sup>+</sup> leucine<sup>-</sup> on the YNB-Ura (C/N = 60) medium. This defined medium contained 1.92 g/L of Drop-out Synthetic Mix minus Uracil supplement, with about 20% leucine content.

A higher rate of glycerol consumption was observed for the leucine supplementation treatment over the *LEU2* expression treatment. The level of citric acid and lipid production was also significantly ( $p < 0.05$ ) higher for the former treatment. In the case of leucine supplementation, the lipid content reached 46%, while it was 30% for the *LEU2* expression treatment. This highlights the stimulatory role of leucine in directing carbon flux toward citric acid and lipid production under nitrogen-limiting conditions in the HPDDS strain. We postulate that the concentration of exogenous leucine that was

added to the media is higher than those endogenous one, which was synthesized by the *LEU2* carrying low-copy replicative vector. Higher leucine concentration can create a stronger signaling effect for metabolite production in the strain.

**Table 4.** The effect of leucine supplementation over *LEU2* expression on biomass and metabolite production by the HPDDS strain. HPDDS: H222  $\Delta$ *POX1-6*  $\Delta$ *LEU22+DGA1 DGA2*  $\Delta$ *SNF1*. a, b Values superscripted with dissimilar letters in same column are significantly different ( $p < 0.05$ ).

Strain and Culture Medium	Residual Glycerol	DCW (g/L)	Citric Acid (g/L)	Lipid (g/L)
HPDDS <i>ura</i> <sup>+</sup> <i>leu</i> <sup>-</sup> , YNB-Ura	4.93 ± 0.74 <sup>a</sup>	7.75 ± 0.89 <sup>a</sup>	28.36 ± 4.36 <sup>a</sup>	3.6 ± 0.18 <sup>a</sup>
HPDDS <i>ura</i> <sup>+</sup> pGR12-leucine, YNB-Leu	2.84 ± 0.45 <sup>b</sup>	6.73 ± 0.24 <sup>a</sup>	14.21 ± 1.12 <sup>b</sup>	1.99 ± 0.15 <sup>b</sup>

#### 4. Discussion

The metabolism of glycerol to citric acid [42] and lipogenesis pathway [43] in *Y. lipolytica* has been studied before. Glycerol is assimilated into the cell via facilitated diffusion and is subsequently phosphorylated [41]. *Y. lipolytica* has a unique glycerol metabolism that is dedicated to glycerol-3-phosphate (G3P) and TAG synthesis [44]. Biosynthesis of TAG requires G3P backbone that is acylated by fatty acids [45]. The *de novo* synthesis of fatty acids uses starting units of acetyl-CoA and malonyl-CoA, as well as the cofactor and energy in the form of NADPH and ATP [46]. In *Y. lipolytica*, acetyl-CoA carboxylase (*ACC1*) catalyzes the first committed step of fatty acid synthesis. This involves the conversion of acetyl-CoA to malonyl-CoA precursor. A constant supply of this precursor is required for biosynthesis of fatty acids and other secondary metabolites in yeast [47]. The saturated fatty acids released from fatty acid synthetase complex (FAS), in the form of acyl-CoA [48], are transferred to the endoplasmic reticulum (ER). Fatty acids may undergo further elongation and desaturation before being incorporated into complex lipids through the Kennedy pathway [3]. In the final step of the TAG synthesis pathway, *DGA1*, YALI0E32769g on the lipid droplet (LD) membrane, and *DGA2*, YALI0D07986g in the ER, play prominent roles in acylation of diacylglycerol to produce TAG, which is stored in LDs especially during the stationary phase [49–51]. Therefore, the overexpression of *DGA1* and *DGA2* results in enhanced lipid accumulation [3,52–59]. TAG synthesis and remobilization is a dynamic process. The latter is carried out by the action of lipases and hydrolases [60] in response to the change of cellular or environmental conditions. Deletion of one or two intracellular lipases, *YITGL3* and *YITGL4* led to a two-fold increase in the capacity of cell to accumulate lipid [61]. Degradation of released fatty acids take places in the peroxisome via  $\beta$ -oxidation pathway. Intracellular lipid degradation (turn over) can occur along with citric acid secretion in stationary phase [37]. Studies show that nitrogen-limiting conditions also promotes citric acid secretion [62]. In fact, lipid synthesis and citric acid production compete for acetyl-CoA precursors [34].

In our study, glycerol was utilized by all four strains for biomass, lipid, and citric acid synthesis under nitrogen-limiting conditions. The results show that our genetic engineering strategies negatively affected biomass production to different degrees. Inactivation of *SNF1* regulatory gene compensated for part of the biomass loss by increasing the lipid content. The inactivation of *SNF1* gene can enhance the activity of *Acc1* and creates larger pool of malonyl-CoA [63]. This generates a push that when combined with the *DGA1* and *DGA2* overexpression, can result in promotion of lipid accumulation accounting for some biomass recovery.

In terms of citric acid production, the citric acid producer H222 wild-type strain showed a citric acid peak of 5 g/L under nitrogen-limiting conditions. Prior study showed that citric and isocitric acids occurs and were excreted by the *Y. lipolytica* control strain at a concentration of 9 g/L [5]. Generally, in wild-type strains, citric acid can also be the product of lipid degradation. However, the availability of glycerol as an external carbon source can lessen the contribution of this phenomenon to citric acid production by reducing internal lipid turnover. In fact, fatty acid remobilization rate decreases in response to a large amount of glycerol [37]. Our engineered strains of HP, HPDD, and HPDDS

showed significantly ( $p < 0.05$ ) higher citric acid production compared to the wild strain. However, the exhaustion of glycerol was followed by reduction in citric acid due to its consumption by the cells. Our best-engineered strain, HPDDS, produced citric acid at the titer and yield of more than 45 g/L and 0.75 g/g of pure glycerol in both the flask and bioreactor. The citric acid production at a titer of more than 35 g/L was reported in the literature using a high concentration of industrial glycerol (80 and 120 g/L) [13]. Another study screened tens of strains for citric acid production. They reported a citric acid production range of 71 to 98 g/L by *Y. lipolytica* strains when they were fed with pure or impure glycerol under the nitrogen-limiting conditions [34]. The citric acid production can be further enhanced by adjusting the pH and dissolved oxygen [16]. However, it should be noted that the control over oxygen did not significantly affect citric acid production from *Y. lipolytica* grown on glycerol, as sole source of carbon [64]. Regarding the effect of our genetic engineering on lipid content, inactivating the *POX* genes did not significantly ( $p > 0.05$ ) increase lipid content. This was most likely due to the preference of the strain for utilizing glycerol as carbon source over the intracellular lipids [37]. However, this is a beneficial genetic modification for stable lipid accumulation, particularly after exhaustion of the carbon source [45]. In fact, peroxisomal lipid degradation is still active in cells grown on non-fatty acid feedstock during the stationary phase [65]. In terms of impaired peroxisomal degradation, fatty acids undergo activation by cytosolic YIFaa1p and are re-stored in LD [66]. Disruption of  $\beta$ -oxidation is also a good strategy for creating positive synergism with other complementary modifications to the lipid pathway. Disruption of this pathway is often carried out by deleting *POX* genes [44,45]. Overexpression of diacylglycerolacyltransferases upon integration into the genome generates a pull towards the lipid biosynthesis to accommodate more acyl-CoA pool in the lipid droplets. Thus, further diversion of carbon flux into the lipid droplet was achieved by *DGAs* overexpression. Overexpression of these two diacylglycerolacyltransferases in the HPDD strain that was devoid of active  $\beta$ -oxidation increased lipid accumulation to more than 41% of DCW compared to 17% in the wild-type strain. Similarly, the overexpression of diacylglycerol acyltransferase increased the lipid content from 13% to the range of 39–53% of the DCW in the strain without active *PEX10* [58]. Other studies have attempted *DGA1* or *DGA2* overexpression strategy to enhance lipid content in this yeast [3,54–57]. Overall, overexpression of *DGA* genes increased lipid content while decreasing biomass formation in the HPDD strain due to *LEU2* deletion. Our best-engineered strain, HPDDS, produced lipid at a titer of 3.15 g/L in the shake flask, rising to 4.8 g/L in the batch bioreactor cultivation. A lipid titer of 2.6 to 6.5 g/L was obtained by engineered *Y. lipolytica* from 60 grams of sugar using batch fermentation [51,56]. The coupling of *DGA1,2* overexpression and *SNF1* deletion enabled a maximum lipid content of nearly 44% in the best lipid producing strain, HPDDS. This increased to 53% in the bioreactor. In the same fashion, overexpression of *DGA1* in a  $\Delta$ *SNF2* background led to a 2.7 fold increase in lipid content of *S. cerevisiae* from 11% to 27% [67]. They suggested the strategy using high-copy number plasmid for *DGA1* overexpression and supplementing the media with leucine for enhancing lipid accumulation in the  $\Delta$ *SNF2* disruptant. In the same way, deletion of *SNF1* in combination with *ACC1* overexpression had synergistic effect on enhancing production of fatty acid derivative [68]. Similar synergism was also observed by deletion of the *SNF1* gene and overexpression of *DGA2* in *Y. lipolytica* [36]. The effect of *SNF1* deletion on lipid accumulation in *Y. lipolytica* has also been studied. This deletion resulted in constitutive lipid accumulation phenotype and 2.6-fold higher fatty acid content [5]. This may have been achieved by down-regulating the  $\beta$ -oxidation and enhancing the *ACC1* expression level. *Snf1* is a global regulator of cellular energy and contributes to fatty acid regulation at various points. Its deletion can cause overexpression of fatty acid synthases (*FAS1* and *FAS2*) and glyceraldehyde-3-phosphate dehydrogenase (*GPD*) expression [36]. A protein kinase *Snf1* also contributes to other signaling pathways, including amino acid metabolism regulation [69]. Inactivation of *Snf1* regulator under nitrogen-limiting conditions can promote citric acid and lipid production as carbon overflow metabolites, while low pH and dissolved oxygen values, and *DGA* genes overexpression can favor lipid production in the resultant phenotype. In fact, pH reduction due to acidification imposes physiologic stress for initiation of lipid accumulation in *Y. lipolytica* [70].

Additionally, longer incubation time may result in reduction of citric acid. This is due to its utilization as carbon source, mainly for cellular maintenance.

In our study, the deletion of *SNF1* gene in the HP strain with deficient  $\beta$ -oxidation did not increase lipid accumulation. Therefore, we did not proceed with that mutant strain. Deletion of *SNF1* in the context of inactive  $\beta$ -oxidation can impose a feedback inhibitory effect of acyl-CoA on *Acc1* [36]. It is notable that saturated fatty acids can provoke a feedback inhibitory effect on some biosynthetic enzymes of the fatty acid biosynthesis pathway [71]. Moreover, a study reported that the knockout of *SNF1* results in transcriptional pattern that differed from one on lipid accumulation devaluing the important role of this gene in lipid accumulation [69]. One alternative solution is to use hyperactive mutant *Acc1* for higher malonyl-CoA production and consequently higher TAG accumulation, comprised of longer chain fatty acids, without need for *snf1* inactivation [72]. This can contribute to a carbon flux re-direction from citric acid to lipid production. The *Snf1* kinase has multiple regulatory roles under different conditions [5], so its preservation can prevent unintended metabolic consequences. Thus, we deleted *SNF1* gene after the integration of the *DGA1* and *DGA2* expression cassette in the HPDD strain to create a synergistic effect to improve metabolite production of TAG.

Overall, the disruption of peroxisomal fatty acid degradation pathway, overexpression of *DGA1* and *DGA2* genes, and inactivation of *SNF1* resulted in the prevention of lipid degradation, up-regulation of TAG biosynthesis pathway, a higher supply of malonyl-CoA and carbon flux towards lipid and citric acid production. Other studies also reported the great performance of *Y. lipolytica* for *de novo* lipid production. An engineered strain overexpressing *ACC1* and *DGA1* produced 28.5 g/L biomass with a lipid content of 61.7% DCW during 5 days of fermentation using 90 g L<sup>-1</sup> of glucose [54]. This was further enhanced by overexpressing delta-9 stearoyl-CoA desaturase (*SCD*) to reach biomass, lipid titer, and productivity of 80 g/L, 55 g/L, and 1 g/L·h, respectively, from 150 g/L of glucose [73]. Optimization of the genotype and phenotype resulted in 20 g/L of biomass, 15 g/L lipid, and lipid content of 75% DCW [52]. Combination of evolutionary engineering method with float-based screening resulted in biomass, lipid content and productivity of 45 g/L, 87% and 0.51 g/L·h [74].

We obtained constitutive accumulation of lipid and a high yield of citric acid (0.75 g/g of pure glycerol) from our engineered strain in the bioreactor. The H222 strain used in this study has shown promise for production of organic acids mainly in the form of citric acid and isocitric acid (up to 12%) from different feedstock, including glycerol [75]. We tested some culture media compositions and fermentation conditions for our strains. Fermentation using glycerol under controlled conditions in the bioreactor enhanced lipid productivity from 0.02 to 0.07 g/L·h. In fact, bioreactor cultivation enabled higher citric acid and lipid productivity.

Our genetic engineering strategy noticeably increased the C16 and C18:1 fatty acid contents. Generally, long chain saturated fatty acids are stored in LDs, while short and unsaturated ones are mainly utilized in other anabolic activities [76]. Monounsaturated fatty acyl-CoAs are better substrates for acylation over saturated ones [73]. This may be due to their higher reactivity or toxicity against the host compared with the saturated fatty acids [77]. In fact, TAG synthesis creates buffering capacity to detoxify excess unsaturated fatty acids [77].

We also tested the effect of leucine on biomass and metabolite production at two levels, 380 and 980 mg/L, using YNB-Ura media (data not shown). We did not observe any significant difference ( $p > 0.05$ ) caused by leucine in the foregoing concentration range for the H and HPDDS strains. The stimulatory role of this amino acid was notable in the range of 1–100 mg/L for the leucine-auxotrophic HPDDS strain, and this is due to the compensatory role of this supplementation for the *LEU2* deletion. Previously, it was reported that increasing the leucine supplementation from 100 mg/L to 1600 mg/L enhances lipid accumulation by about six times, and results in genotypic complementation in the leucine<sup>-</sup> strains without active  $\beta$ -oxidation pathway [52]. It was suggested that this is due to the signaling role of this amino acid and its degradation to acetyl-CoA precursors, which can be subsequently diverted to the lipid biosynthesis pathway. This amino acid plays a regulatory

role in lipid metabolism [52]. Studies show that an increased level of leucine may promote lipid metabolism in *Y. lipolytica* through down-regulation of amino acid biosynthesis and deviation of flux from that [69]. A recent study showed a correlation between lipid accumulation and regulation of amino acid biosynthesis [69]. It is plausible that the increased leucine as an important sensor molecule induces transcriptional response [52]. This branch-chain amino acid plays a part in synthesis of fatty acids in adipocytes [78]. The possible role of Snf1, protein kinase, in amino acid metabolism regulation [69] can also explain the interaction of this gene manipulation with leucine metabolism.

The nitrogen-limiting conditions can result in the prevention of biomass proliferation and promotion of lipid accumulation and citric acid secretion to deal with the excess carbon flux. In fact, nitrogen-limiting conditions leads to down-regulation of ribosome structural genes [79]. This also results in degradation of intracellular AMP to release NH<sub>4</sub><sup>+</sup> ions. Depletion of AMP interferes with the TCA cycle, leading to the accumulation of citric acid in mitochondria and eventually in the cytoplasm [21,37]. Part of citric acid is subsequently broken down by ATP: citrate lyase (ACL) to acetyl-CoA that serves *de novo* synthesis of fatty acid [80]. Although excess availability of carbon can induce lipogenesis in engineered strains [52], it can also trigger citric acid secretion into the growth medium [37]. Thus, it is important to adjust glycerol concentration together with the C/N ratio for optimum biomass and lipid production.

In this study, we achieved constitutive lipid accumulation and citric acid secretion under nitrogen-limiting conditions. Papanikolaou et al., observed similar results by the deleting 2-methylcitrate dehydratase-coding gene. However, they used two different conditions of nitrogen limited and nitrogen excessive conditions for higher citric acid and lipid production, respectively [37].

In summary, our engineered strain shows promise for the simultaneous accumulation of lipid and secretion of citric acid under nitrogen-limiting conditions. The generally recognized as safe (GRAS) status of this yeast justifies the suitability of the major bio-products for delivery to food and pharmaceutical industries. It needs to be pointed out that medium optimization was beyond the scope of our study. Further optimizing the production medium in terms of carbon, nitrogen, and leucine contents is suggested to reach a higher biomass, and subsequently a higher production level. Additionally, the direct use of crude glycerol or pretreated feedstock at larger scale fed-batch fermentation can provide further validation for the performance of the platform developed.

## 5. Conclusions

In this study, we developed an approach for simultaneous lipid accumulation and citric acid secretion using engineered *Y. lipolytica* in batch fermentation. The combination of deleting the fatty acid degrading pathway, overexpressing key TAG synthesizing genes, and manipulating the lipid regulatory system led to the constitutively accumulation of lipid and secretion of citric acid into the media under the nitrogen-limiting conditions. A relatively high yield of citric acid was achieved along with lipid accumulation from glycerol. This engineered yeast biorefinery platform can be refined and integrated in a fed-batch or continuous system for valorization of a glycerol waste stream into citric acid and lipid.

**Supplementary Materials:** The following are available online at [www.mdpi.com/2311-5637/3/3/34/s1](http://www.mdpi.com/2311-5637/3/3/34/s1).

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**Author Contributions:** Ali Abghari developed the concept, designed the experiments, performed the experiments, analyzed the data, and drafted this paper. Shulin Chen revised the manuscript and approved the final version for publication. All authors read and approved the final manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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