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Overexpression of Citrate Synthase Increases Isocitric Acid Biosynthesis in the Yeast *Yarrowia lipolytica*

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Abstract: *Yarrowia lipolytica* is a non-conventional yeast producing valuable compounds, such as citric acids, from renewable raw materials. This study investigated the impact of citrate synthase overexpression on the biosynthesis of citric and isocitric acid in *Y. lipolytica*. Two transformants of *Y. lipolytica* A101.1.31 strain (efficient citric acid producer), overexpressing *CIT1* or *CIT2* gene (encoding proteins with citrate synthase activity), were constructed. The results revealed that overexpression of either of these genes enhances citrate synthase activity. Additionally, the *cit1* knockout strain was unable to use propionate as the sole carbon source, which proves that *CIT1* gene encodes a dual activity protein—citrate and 2-methylcitrate synthase. In the overexpressing mutants, a significant increase in isocitric acid biosynthesis was observed. Both *CIT1* and *CIT2* overexpressing strains produced citric and isocitric acid from vegetable oil in a ratio close to 1 (CA/ICA ratio for wild-type strain was 4.12).

Keywords: *Yarrowia lipolytica*; citrate synthase; 2-methylcitrate synthase; citrate; isocitrate

1. Introduction

Bulk chemicals such as organic acids can be readily produced with the use of microorganisms. These compounds are often intermediates of primary metabolism, as in the case of citric acid (CA), the Krebs cycle intermediate. Due to its natural origin, low toxicity and chemical properties, CA has found many applications in food and pharmaceutical industries as an acidifying and flavoring agent, antioxidant, preservative and stabilizer. This compound is also used as a cleaning agent in household chemicals as a replacement for environmentally hazardous, phosphate-containing cleaners. The global CA market was worth 2.5 billion USD in 2016 [1]. The demand for CA is constantly growing, especially for the food and beverages industry (57.9% of the CA market in 2016), where a CA growth rate of 5% from 2017 to 2025 is expected. This high demand can be met through its production in microbial-based processes. The well-established technology harnessing the fungus *Aspergillus niger* to produce CA from glucose or molasses provides high product yields [2]; however, it causes some environmental issues [3,4].

The yeast *Yarrowia lipolytica*, known for its ability to accumulate large amounts of lipids [5], is also an excellent CA producer [6–10]. This feature, combined with its ability to use a wide spectrum of substrates, such as *n*-alkanes, oils, hydrolyzed molasses and glycerol [11], makes CA production by *Y. lipolytica* an interesting alternative to processes with *A. niger*. Moreover, this yeast is non-pathogenic, and several processes, including yeast biomass and CA production, were classified as GRAS (Generally

Recognized as Safe) by the FDA (Food and Drug Administration, Silver Spring, MD, USA) [11,12]. Another advantage of *Y. lipolytica* over the fungus is its lower sensitivity to heavy metals or low oxygen concentrations and, more importantly, higher product yields [7,8,13,14].

Production of CA by *Y. lipolytica* is often accompanied by biosynthesis of isocitric acid (ICA) in various quantities, depending on the strain, composition of the growth medium and the carbon source used [13–16]. Although many researchers claim that ICA is an undesired byproduct of CA production (due to the difficulties in CA crystallization when the CA:ICA ratio reaches a threshold level), the commercial value of ICA has grown in recent years. This compound finds its applications as a marker for the detection of authenticity and quality of citrus juices [17] and in enzymatic assays [18]. Due to its distinctive structure, ICA can also be used as a chiral building block for pharmaceutical products [19–21] and as an anticoagulant in biological fluids [22]. According to several studies [13,21,23], potassium *Ds-threo*-isocitric acid could be used as an antioxidant and antihypoxic agent in sport medicine. ICA is the only metabolite able to unlock succinate dehydrogenase and therefore promote cell respiration, also during long and intense stress [24]. Several methods of ICA production exist, including chemical synthesis, extraction from plants (e.g., *Crassulaceae*) as well as microbial fermentation [13,21,23]. In chemical synthesis, ICA can be obtained as an inseparable mixture of four isomers, which makes this method futile [25,26]. Although retrieving ICA from plants is possible and is even used by the Merck chemical company, the most preferable ICA production process is its microbial biosynthesis. Biotechnological production has clear advantages, such as simplicity of microorganisms' culturing, no need to use land for farming and independence of changing seasons. The literature data report *Penicillium purpurogenum* [27], *Candida ravanii* [28], *Candida catenulate* [29] and *Y. lipolytica* as potential efficient ICA-producing microorganisms [13,20,23,25,30].

As mentioned above, *Y. lipolytica* is able to use wide spectrum of carbon sources. One of them, glycerol, is particularly interesting as it is a cheap byproduct of the biodiesel and soap industries. The process of biodiesel production generates vast amounts of waste glycerol, reaching annually up to 6.9 million tons [31]. Although pure glycerol is a valuable raw material used in the food, pharmaceutical and other industries, the compound derived from biodiesel production contains various numbers of impurities, which can be removed only using time and cost consuming processes, such as filtration, chemical additions and fractional vacuum distillation [32]. The contamination and refining processes' costs make biodiesel-derived glycerol unsuitable for the abovementioned industries. Fortunately, microorganisms, such as the yeast *Y. lipolytica*, are able to use crude glycerol to produce lipids, polyhydroxy alcohols and organic acids (including CA) [14,33,34], therefore providing a solution for waste glycerol management. This yeast is also able to use the triacylglycerols (TAGs) present in renewable vegetable raw materials, such as rapeseed oil. Due to the extracellular lipases, TAGs are hydrolyzed to glycerol and free fatty acids, which are further incorporated into the cellular metabolism of this yeast (Figure 1). Importantly, glycerol and free fatty acids derived from oils are consumed simultaneously [30], which makes TAGs a perfect substrate for efficient production of various compounds (e.g., CA and ICA) using *Y. lipolytica*.

To date, many studies devoted to CA production by *Y. lipolytica* from various, often renewable, substrates were conducted. Strategies for CA biosynthesis enhancement were based on optimizing culture conditions as well as by utilization of different carbon sources [7,8,30,35]. Some improvement was obtained also using mutagenesis by UV irradiation [36] and genetic engineering [6,9,30]. In contrast, studies devoted to ICA production are not very abundant [13,19–21,23]. One of the very important parameters describing the biosynthesis of both acids (CA and ICA) is their ratio. In the metabolism of *Y. lipolytica*, the ICA:CA ratio depends mainly on isocitrate lyase (ICL) and aconitase activities (Figure 1) [21,37]. While deletion of the *ICL1* gene in *Y. lipolytica*, encoding isocitrate lyase, enhanced ICA secretion only by 2–5% in cells growing on glucose or glycerol [6], overexpression of aconitase-encoding *ACO1* gene increased the ICA:CA ratio by up to 70% [37]. However, to the best of our knowledge, there was no attempt to manipulate CA and ICA biosynthesis in *Y. lipolytica* through the modification of citrate synthase activity (Figure 1). Furthermore, no reports identifying citrate synthase encoding

genes in *Y. lipolytica* were published. In this study, two genes (*CIT1* and *CIT2*) encoding proteins with citrate synthase activity were identified and two *Y. lipolytica* transformants overexpressing these genes, able to produce both CA and ICA from renewable raw materials, were characterized.

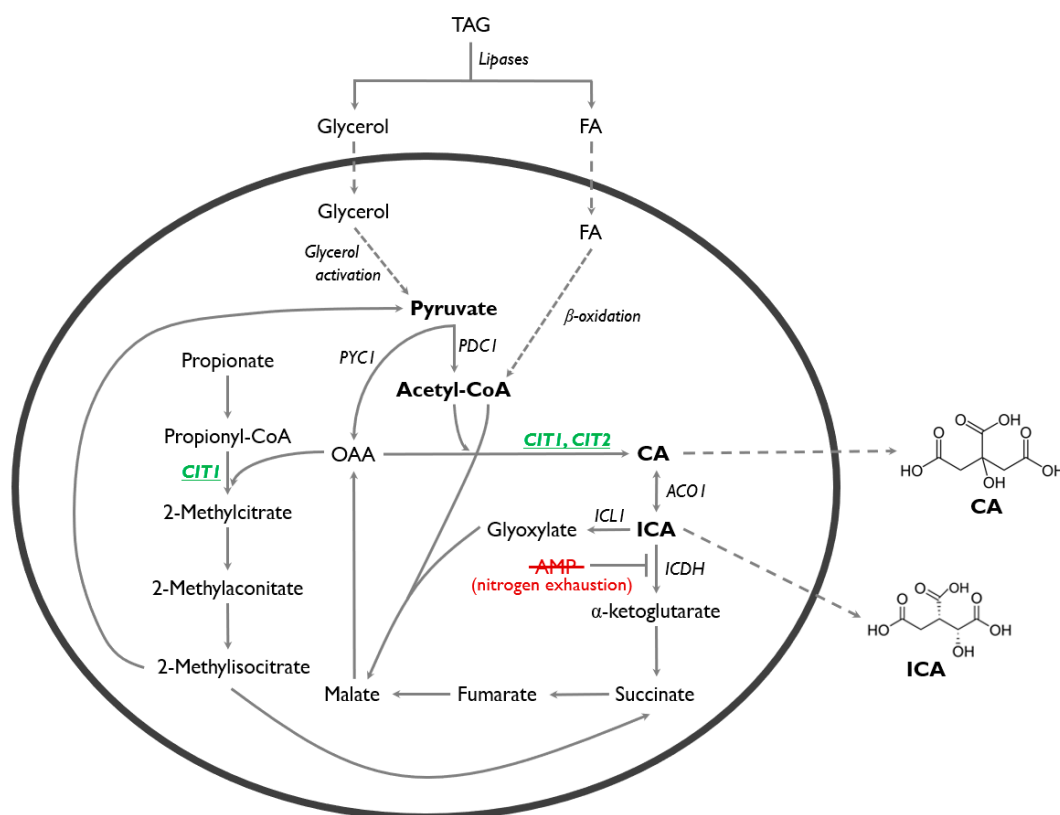


Figure 1. Schematic representation of pathways mentioned in this study. Abbreviations: TAG—triacylglycerols, FA—fatty acid, OAA—oxaloacetate, CA—citric acid, ICA—iscitric acid, AMP—adenosine monophosphate, *PYC1*—pyruvate carboxylase, *PDC1*—pyruvate dehydrogenase, *CIT1*—2-methylcitrate synthase, *CIT2*—citrate synthase, *ACO1*—aconitase, *ICL1*—isocitrate lyase, *ICDH*—isocitrate dehydrogenase.

2. Materials and Methods

2.1. Strains

The *Y. lipolytica* strains used in this study are listed in Table 1. All the targeted transformants were derived from the *Y. lipolytica* A101.1.31 strain, which is a UV mutant obtained from the wild-type *Y. lipolytica* A101 strain [36].

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

Strain	Genotype	Reference
A101	Wild type	[36]
A101.1.31	Acetate-negative UV mutant of A101	[7]
A101.1.31 U-	A101.1.31 <i>ura3-302</i>	
Δ cit1	A101.1.31 <i>cit1::URA3</i>	This study
Δ cit2	A101.1.31. <i>cit2::URA3</i>	
CIT1	A101.1.31 <i>CIT1-URA3</i>	
CIT2	A101.1.31 <i>CIT2-URA3</i>	

2.2. Growth Media and Conditions

E. coli strains were cultivated in 37 °C in LB medium consisting of 5 g dm⁻³ tryptone (Merck, Darmstadt, Germany), 10 g dm⁻³ yeast extract (Merck) and 10 g dm⁻³ NaCl. For plates, 2% agar was added. Solid LB medium with 0.1% kanamycin (Thermo Fisher Scientific, Waltham, MA, USA) was used for clone selection.

Rich (YPD) medium for *Y. lipolytica* cultures was prepared using 20 g dm⁻³ Bacto™ Peptone (Difco, Paris, France), 10 g dm⁻³ yeast extract (Merck) and 20 g dm⁻³ glucose (Merck). Minimal (YNB) medium was prepared using 1.7 g dm⁻³ yeast nitrogen base (without amino acids and ammonium sulfate, Sigma-Aldrich, St. Louis, MO, USA), 10 g dm⁻³ glucose and 5 g dm⁻³ NH₄Cl, and 50 mM phosphate buffer (pH 6.8) and 0.2 g dm⁻³ of uracil (Sigma-Aldrich) were used for selection of transformants. For plates, 2% agar was added. The solid medium for propionate utilization analysis was composed of 10 g dm⁻³ sodium propionate (Sigma-Aldrich) or glucose (control experiment), 1.7 g dm⁻³ yeast nitrogen base, 5 g dm⁻³ NH₄Cl, 50 mM phosphate buffer (pH 6.8) and 2% agar. All *Y. lipolytica* strains were routinely cultivated at 28 °C.

2.3. General Genetic Techniques, Plasmid Construction and Transformation

Standard molecular genetic techniques were adopted from Sambrook and Russell [38]. Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). Genomic DNA from yeast was extracted and purified as described in [39]. Nucleotide sequences of YALI0E00638g (*CIT1*) and YALI0E02684g (*CIT2*) genes were retrieved from GRYC database [40]. Genomic DNA from *Y. lipolytica* A101 wild-type strain was used as a template for PCR using primers listed in Table S1. The *Bcl*II or *Bgl*III and *Avr*II restriction sites were used for forward and reverse primers, respectively (underlined sequences). PCR was carried out using a Biometra T1 Personal thermal cycler (Biometra, Göttingen, Germany) using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The PCR products were verified by gel electrophoresis and the DNA fragments were recovered from the agarose gels using Gel-Out Kit (A&A Biotechnology, Gdynia, Poland). Amplified genes were cloned into JMP62URA plasmid digested with appropriate restriction enzymes and transformed into *E. coli* DH5α according to [38]. The obtained plasmids for gene overexpression contained a strong constitutive *pTEF* promoter, *URA3* gene for selection of *Y. lipolytica* transformants and KanR marker for *E. coli* selection. The deletion cassettes were prepared according to [41]. In brief, the promoter (P) and terminator (T) regions (~1kb) were amplified from *Y. lipolytica* A101 genomic DNA with the gene-specific P1/P2 and T1/T2 primers (Table S1). Additionally, P2 and T1 primers contained an extension bearing *I-sce*I restriction site. For the *CIT1* gene, primer pairs CIT1-P1/CIT1-P2 and CIT1-T1/CIT1-T2 and for CIT2—CIT2-P1/CIT2-P2 and CIT2-T1/CIT2-T2 were used. The obtained P and T regions were subsequently purified and used in a second round of PCR. The fused P-T fragments were ligated into pCR™-Blunt II-TOPO® vector (Invitrogen, Carlsbad, CA, USA). Lastly, the *URA3* marker was inserted at the *I-sce*I site yielding the constructs containing either CIT1-PUT or CIT2-PUT disruption cassette. Both the overexpression (random chromosomal integration) and deletion vectors (homologous recombination) were digested by *Not*I restriction enzyme to release the desired cassette. All *Y. lipolytica* transformations were performed using the lithium acetate procedure [42]. Positive *Y. lipolytica* transformants were verified by PCR and gel electrophoresis after genomic DNA extraction.

2.4. Bioreactor Studies

To test the ability of *Y. lipolytica* transformants to produce CA and ICA, as well as to determine the citrate synthase activity, bioreactor cultures were performed. Strains were maintained in 5-dm³ stirred-tank BIOSTAT B-PLUS bioreactors (Sartorius, Frankfurt, Germany) under the following conditions: working volume—2 dm³, temperature—28 °C, stirring rate—2.3 Hz and aeration rate—1 dm³ dm⁻³ min⁻¹. The CA production media consisted of 150 g glycerol, 3.0 g NH₄Cl, 0.2 g KH₂PO₄, 1.0 g

MgSO₄ × 7H₂O and 1.0 g yeast extract in 1 dm³ of tap water. pH was automatically maintained at 6.0 using 5 mol dm^{−3} NaOH solution. Medium for CA production using vegetable oil contained 200 g rapeseed oil (50 g at the beginning of the culture, 50 g after 24 h and 100 g after 48 h), 6 g NH₄SO₄, 2.0 g KH₂PO₄, 0.2 g Na₂HPO₄, 1.4 g MgSO₄ × 7H₂O, 0.8 g Ca(NO₃)₂ × 4H₂O, 0.5 g NaCl and 0.5 g yeast extract in 1 dm³ of tap water. pH was automatically maintained at 6.0 using 5 mol dm^{−3} NaOH solution. The inoculum (48 h) for all bioreactor cultures constituted 5% of the working volume and the initial optical density (OD₆₀₀) was adjusted to 0.5. Bioreactor cultures were performed in triplicate.

2.5. Analytical Methods

The analyses of substrate concentration, biomass and sum of citric acids during the bioreactor cultures were performed by taking two 10-cm³ samples from each culture 10 min after inoculation (time = 0); subsequent sampling was conducted at 12th, 18th, 25th, 44th, 60th and 72nd hours of the culture. Each sample was centrifuged for 5 min at 2700 RCF; the supernatants and cell pellets were collected and subjected to further analyses.

To determine biomass concentration, cell pellets were washed twice with distilled water and filtered under vacuum using membranes with a pore size of 0.22 µm. Biomass was determined gravimetrically after cell drying at 105 °C. Biomass was expressed as grams of cell dry weight per 1 dm³ (CDW g dm^{−3}).

The concentrations of glycerol and citric acids (sum) in the culture supernatants were measured using HPLC (Dionex-Thermo Fisher Scientific, UK) with a carbohydrate H+ column (Thermo Scientific, Waltham, MA, USA) coupled to UV (λ = 210 nm) and refractive index (RI) detectors (Shodex, Ogimachi, Japan). The eluate used was 25 mM trifluoroacetic acid (TFA). The conditions of the analyses were as follows: temperature—65 °C and flow rate—0.6 cm³ min^{−1}.

Isocitric acid was quantitatively determined using the fluorimetric Isoctirate Assay Kit from Sigma-Aldrich (MAK319) and Spark Multimode Microplate Reader (Tecan, Männedorf, Switzerland), following the manufacturer's instructions.

Citrate synthase activity assay using *Y. lipolytica* whole cell extracts (10 min of sonication using Sonics VCX500 sonicator, amplitude 100%, 0.5 s intervals) was performed with Citrate Synthase Assay Kit (Sigma-Aldrich, MAK193) using Evolution 300 UV-VIS spectrophotometer (Thermo-Scientific), according to the manufacturer's protocol. Total protein concentration was measured using Lowry's method [43]. All the assays were performed in triplicate.

2.6. Calculation

The mass yield coefficient of product formation (*Y*), expressed as grams of product per gram of substrate consumed, was calculated from the following equation:

$$Y = \frac{P}{S}$$

The specific productivity (*q*, grams of product per gram of biomass per hour) was calculated using

$$q = \frac{P}{X * t}$$

The volumetric productivity of citric acids (*Q*, grams per liter per hour) was calculated from

$$Q = \frac{P}{V * t}$$

where *P* is the total amount of CA or ICA in the culture medium at the end of cultivation [g], *S* is the total amount of substrate consumed [g], *V* is the initial volume of culture [dm^{−3}], *X* is the concentration of biomass in a bioreactor [g] and *t* represents the time of cultivation [h].

2.7. Bioinformatic Analysis

The nucleotide and amino acid sequences were retrieved from the Saccharomyces Genome Database (SGD) [44], NCBI [45] and GRYC [40] databases. The amino acid sequences were aligned using Clustal Omega [46] and visualized using Jalview 2.11.1.0 [47]. Conserved domains were identified using NCBI's conserved domain database.

3. Results and Discussions

3.1. Genes Encoding Citrate Synthases in *Y. lipolytica*

To identify citrate synthase genes in the yeast *Y. lipolytica*, amino acid sequences of *Saccharomyces cerevisiae* citrate synthases (ScCit1—YNR001C, ScCit2—YCR005C and ScCit3—YPR001W) retrieved from SGD were compared to the available protein sequences of *Y. lipolytica* CLIB122 using Protein BLAST [48]. It emerged that two sequences, annotated as YALI0E00638p (*YlCit1*, 54% amino acid sequence identity to ScCit1, 52% to ScCit2 and 50% to ScCit3) and YALI0E02684p (*YlCit2*, 76% amino acid sequence identity to ScCit1, 68% to ScCit2 and 46% to ScCit3) were found. The alignment of the amino acid sequences (Figure 2) showed substantial amounts of conserved motifs among the compared proteins. Interestingly, the *YlCit1* protein contains ScCit3-like domains, important for conversion of propionyl-CoA into 2-methylcitrate. This observation suggests that *YlCit1* may show 2-methylcitrate synthase activity.

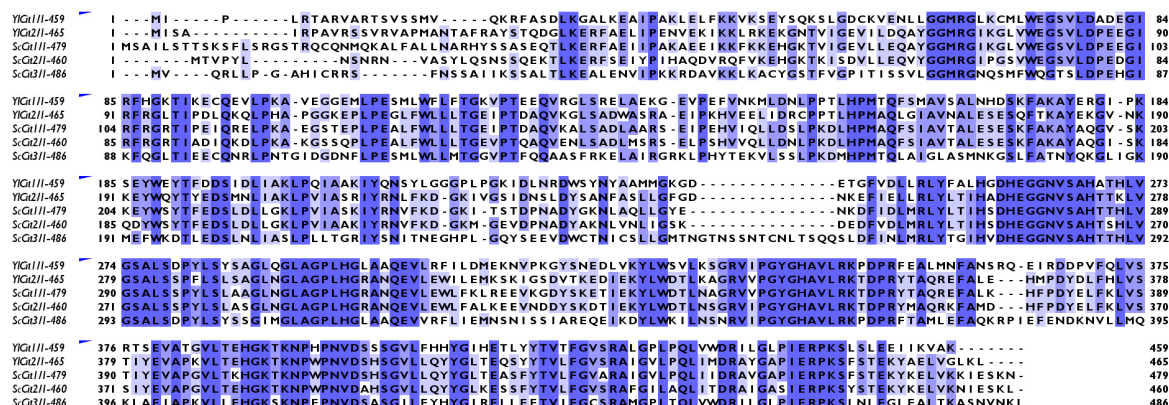


Figure 2. Alignment of the amino acid sequences of citrate synthases in *Y. lipolytica* and *S. cerevisiae*. The darker the color, the higher percentage of identity among sequences.

3.2. CIT1 and CIT2 Overexpression Enhances Citrate Synthase Activity

Citrate synthase activity was analyzed using whole cell extracts of the corresponding transformants grown in glycerol-based medium. The results presented in Figure 3 show that an additional copy of either the *CIT1* or *CIT2* gene in *Y. lipolytica* enhanced citrate synthase activity 3.93-fold (*CIT1*) and 6.34-fold (*CIT2*) at the 24th hour of culture compared to the control strain. The CA synthase activity in the control strain reached only 0.98 U/mg of protein, a value similar to the results demonstrated previously for *Y. lipolytica* VKM Y-2373 [30]. Notably, citrate synthase activity for both strains decreased over time (Figure 3).

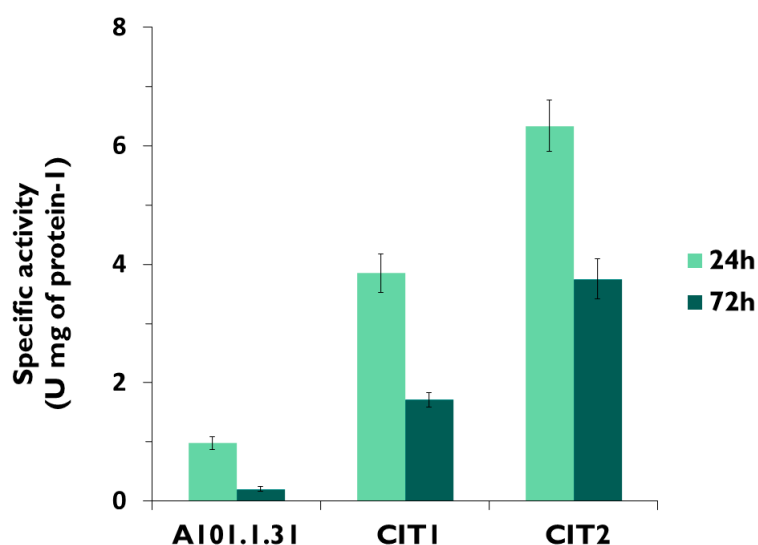


Figure 3. Specific activity of citrate synthase in *Y. lipolytica* strains grown in glycerol-based medium. The experiment was performed in three biological replicates.

3.3. CIT1 is Indispensable for Propionate Utilization in *Y. lipolytica*

In *S. cerevisiae*, the *CIT3* gene encodes the dual activity citrate and methylcitrate synthase [49]. To evaluate the methylcitrate synthase activity of proteins encoded by *CIT1* and *CIT2* genes in *Y. lipolytica*, deletion mutants of A101.1.31 with either $\Delta cit1$ or $\Delta cit2$ were constructed. Growth comparison in a media with 1% glucose and 1% propionate was evaluated (Figure 4). All tested strains exhibited usual growth on glucose; however, the $\Delta cit1$ transformant was unable to grow in the presence of propionate. This result clearly indicates that the *CIT1* gene is essential for propionate metabolism (Figure 1) and its product possesses the methylcitrate synthase activity which confirmed the prediction described above (Section 3.1).

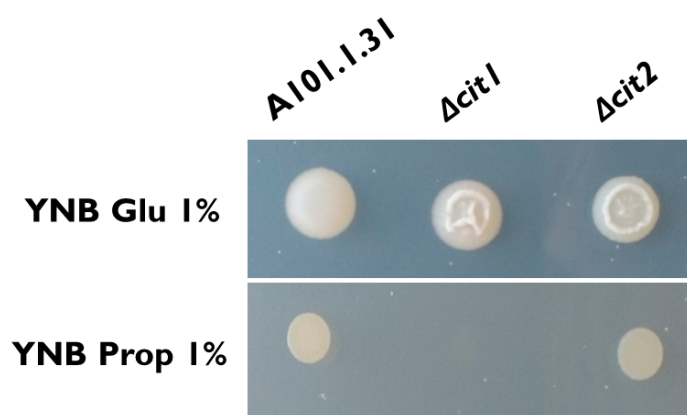


Figure 4. Growth of *Y. lipolytica* A101.1.31, $\Delta cit1$ and $\Delta cit2$ on YNB medium with 1% glucose (YNB Glu) and 1% propionate (YNB Prop).

3.4. Overexpression of CIT1 or CIT2 Increases ICA Biosynthesis

Y. lipolytica A101.1.31, an acetate negative mutant and CA overproducer [7,50], and its derivatives with overexpression of either *CIT1* or *CIT2* genes were cultivated in a glycerol-based medium promoting CA overproduction. Final biomass concentrations obtained by the transformants (18.35 g dm^{-3} and 18.80 g dm^{-3} for *CIT1* and *CIT2*, respectively) were slightly higher (8% and 10% higher for *CIT1* and *CIT2*, respectively) compared to the parental strain (Figure 5, Table 2).

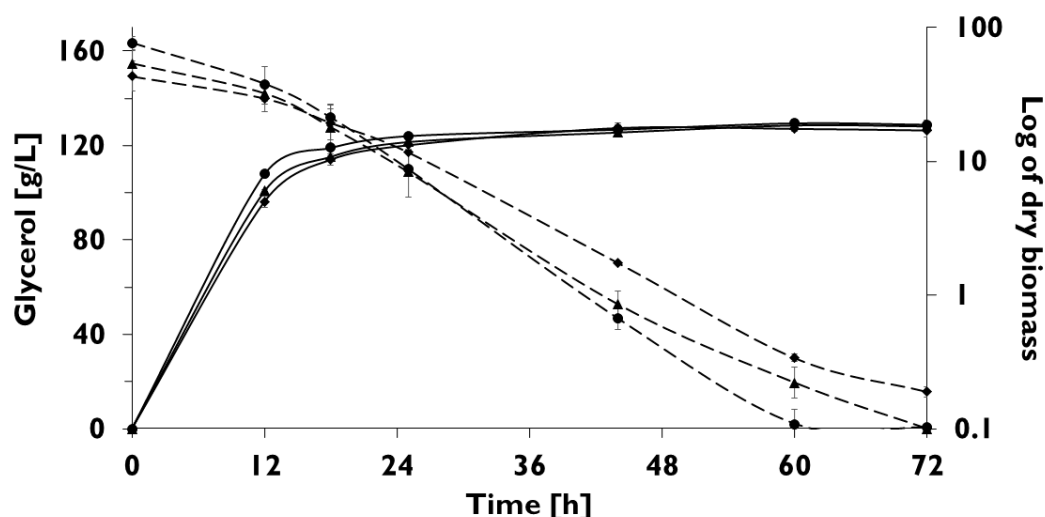


Figure 5. Growth and substrate consumption during bioreactor cultures of *Y. lipolytica* A101.1.31 (◆), CIT1 (▲) and CIT2 (●) strains in glycerol-based medium optimized for citric acid production. Solid lines represent changes in biomass, dashed lines represent glycerol utilization.

The total amount of citric acids at the end of the culture reached 10% and 23% higher values for CIT1 and CIT2 overexpressing transformants, respectively, compared to *Y. lipolytica* A101.1.31 (75.41 g dm^{-3}). However, only when a closer look at the concentrations of CA and ICA separately was taken into consideration was a better picture of the metabolism revealed (Figure 6, Table 2). CIT1 overexpression resulted in decreased CA biosynthesis and pushed the carbon flux towards ICA production. This was expressed by an almost 9.5-fold increase in ICA concentration compared to the parental strain. A similar, albeit not identical, situation was observed for the CIT2 transformant. In this case, the ICA amount at the end of the culture increased 6.8-fold, with a concomitant minor increase in CA concentration in comparison to A101.1.31. Therefore, the resulting CA to ICA ratios for both *Y. lipolytica* transformants were significantly lower and decreased from 29.8 for the parental A101.1.31 strain to 2.6 and 4.6 for CIT1 and CIT2 overexpressing strains, respectively. A high CA/ICA ratio for A101.1.31 cultivated on crude glycerol was previously described [14]. Additionally, Levinson and colleagues [51] reported CA/ICA ratios for wild strains of *Y. lipolytica* grown on glycerol ranging from 1.7 to 11.3.

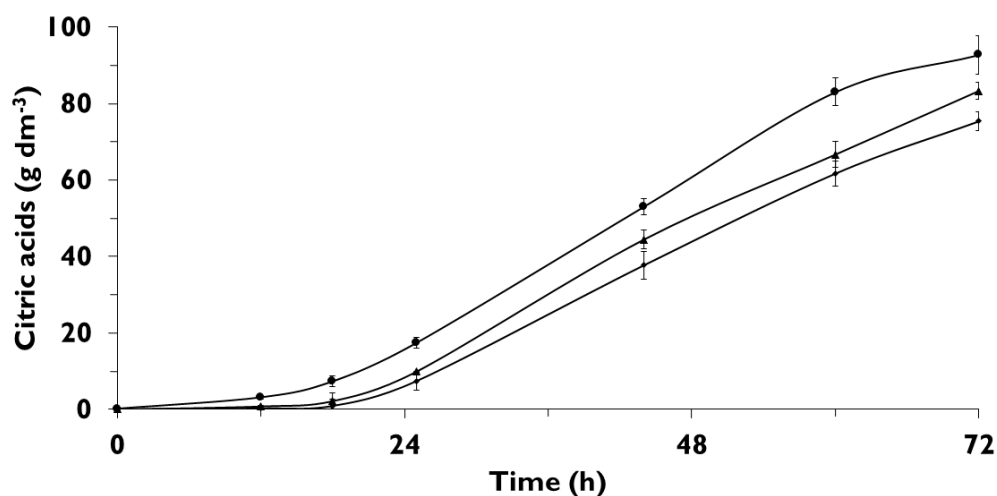


Figure 6. Biosynthesis of citric acids during bioreactor cultures of *Y. lipolytica* A101.1.31 (◆), CIT1 (▲) and CIT2 (●) strains in a glycerol-based medium.

Table 2. Parameters of bioreactor cultures of *Y. lipolytica* A101.1.31, CIT1 and CIT2 at 72nd hour of culture in a glycerol-based medium. Culture conditions were set up as follows: glycerol 150 g dm^{−3}, 28 °C, pH 6.0, aeration rate 1 dm³ dm^{−3} min^{−1} and stirring rate 2.3 Hz. All values are expressed as means derived from triplicate experiments. The SD did not exceed 5%. Abbreviations: X—biomass, Σ CA—sum of citric acids, CA—citric acid, ICA—isocitric acid.

	X	Σ CA	CA	ICA	CA/ICA	$Y_{\Sigma CA/X}$	$Y_{CA/X}$	$Y_{ICA/X}$	$Y_{\Sigma CA/S}$	$Q_{\Sigma CA}$	$q_{\Sigma CA}$	$Y_{CA/S}$	Q_{CA}	q_{CA}	$Y_{ICA/S}$	Q_{ICA}	q_{ICA}
	g dm ^{−3}	g dm ^{−3}	g dm ^{−3}	g dm ^{−3}		g g ^{−1}	g g ^{−1}	g g ^{−1}	g g ^{−1}	g dm ^{−3} h ^{−1}	g g ^{−1} h ^{−1}	g g ^{−1}	g dm ^{−3} h ^{−1}	g g ^{−1} h ^{−1}	g g ^{−1}	g dm ^{−3} h ^{−1}	g g ^{−1} h ^{−1}
A101.1.31	17.05	75.41	72.96	2.45	29.78	4.42	4.28	0.14	0.56	0.52	0.061	0.54	0.51	0.059	0.02	0.02	0.002
CIT1	18.35	83.30	60.33	22.97	2.63	4.54	3.29	1.25	0.54	0.58	0.063	0.39	0.42	0.046	0.15	0.16	0.017
CIT2	18.80	92.75	72.06	16.69	4.56	4.93	3.83	0.89	0.57	0.64	0.068	0.43	0.53	0.053	0.10	0.11	0.012

The volumetric and specific productivity reflect the results described above (Table 2). The highest volumetric productivity of CA was achieved by the CIT2 strain, while the lowest was achieved by the CIT1 overexpressing transformant. Inversely, this strain (CIT1) was the fastest ICA producer ($0.16 \text{ g dm}^{-3} \text{ h}^{-1}$). In terms of specific CA productivity, the highest result was achieved by the parental A101.1.31 strain.

The best CA-producing strain from one gram of glycerol was the *Y. lipolytica* CIT2 transformant; however, the parental A101.1.31 strain was characterized by similar performance. The CIT1 transformant was the least efficient CA producer, converting only 42% of available glycerol into CA. In contrary, the CIT1 strain was the most robust ICA producer (15% conversion), while A101.1.31 converted only 2% of glycerol into ICA. These data are in agreement with previous observations [7]. The parental strain, *Y. lipolytica* A101.1.31, did not consume all the available glycerol, leaving around 16 g dm^{-3} of the substrate in the medium at the end of the process (Figure 5). In contrast, the CIT1 overexpressing strain showed increased substrate consumption, which was reflected by the total utilization of glycerol during 72 h of the process. Similarly, overexpression of CIT2 resulted in a robust glycerol-utilizing phenotype. In the process using this strain, the substrate was depleted already in the 60th hour of the culture.

Taken together, overexpression of both genes (CIT1 and CIT2) does not increase CA but ICA biosynthesis for strains cultured in a medium containing glycerol as the sole carbon source.

3.5. ICA Production from Hydrophobic Substrate

Efficient biosynthesis of ICA from vegetable oil by *Y. lipolytica* was previously demonstrated [21]. Therefore, in the present study, the ICA-overproducing phenotype of *Y. lipolytica* transformants was analyzed also in a medium based on rapeseed oil optimized for ICA biosynthesis [21]. The A101.1.31 investigated during the first part of this study could not be used as a control strain, due to its inability to utilize even carbon fatty acids (Figure S1); this is why the parental strain of A101.1.31—*Y. lipolytica* A101 was used instead. The final biomass concentration reached by the transformants (26.9 g dm^{-3} for CIT1 strain and 30.9 g dm^{-3} for CIT2 strain) varied significantly compared to the value measured for the control strain (55 g dm^{-3} , Table 3).

All analyzed strains secreted higher amounts of citric acids at the end of the cultures (Table 3) compared to the processes in glycerol-based media (Table 2). In contrast to the glycerol-based cultures, the highest sum of citric acids was obtained by the CIT1 transformant (133.2 g dm^{-3}), while the CIT2 strain reached 109.9 g dm^{-3} , which represented the lowest value observed for cultures on rapeseed oil (Table 3). However, as mentioned above, it is difficult to assess the true effect of the analyzed genetic modifications without a closer look to the CA and ICA concentration. The titers of both citric acids at the end of the transformants' cultures were very similar, which resulted in a CA/ICA ratio close to 1 (1.04 for CIT1 and 0.95 for CIT2), whereas the final CA/ICA ratio for the control strain reached 4.12. A higher amount of ICA produced on hydrophobic substrates by *Y. lipolytica* A101 was also reported by Wojtatowicz and colleagues [50]. According to [21], similar results to these presented in the current study were reported—CA:ICA ratios equal to 1.06:1 and 1.14:1 for *Y. lipolytica* 710 and 68, respectively. However, the best described strain, *Y. lipolytica* 704-UV4-A/NG50, reached a CA:ICA ratio of 0.37:1 [21]. In the pilot-plant scale culture on refined sunflower oil, as demonstrated in [20], the wild-type *Y. lipolytica* EH59 produced 93 g dm^{-3} of ICA, with an ICA:CA ratio of 1.14:1.

Table 3. Parameters of bioreactor cultures of *Y. lipolytica* A101, CIT1 and CIT2 in a rapeseed oil-based medium. Culture conditions were set up as follows: rapeseed oil 200 g dm^{−3} (50 g at the beginning of the culture, 50 g after 24 h and 100 g after 48 h), 28 °C, pH 6.0, aeration rate 1 dm³ dm^{−3} min^{−1} and stirring rate 2.3 Hz. All values are expressed as means derived from triplicate experiments. The SD did not exceed 5%. Abbreviations: X—biomass, Σ CA—sum of citric acids, CA—citric acid, ICA—isocitric acid.

	Time	X	Σ CA	CA	ICA	CA:ICA	$Y_{\Sigma CA/X}$	$Y_{CA/X}$	$Y_{ICA/X}$	$Y_{\Sigma CA/S}$	$Q_{\Sigma CA_s}$	$q_{\Sigma CA}$	$Y_{CA/S}$	Q_{CA}	q_{CA}	$Y_{ICA/S}$	Q_{ICA}	q_{ICA}
	h	g dm ^{−3}	g dm ^{−3}	g dm ^{−3}	g dm ^{−3}		g g ^{−1}	g g ^{−1}	g g ^{−1}	g g ^{−1}	g dm ^{−3} h ^{−1}	g g ^{−1} h ^{−1}	g g ^{−1}	g dm ^{−3} h ^{−1}	g g ^{−1} h ^{−1}	g g ^{−1}	g dm ^{−3} h ^{−1}	g g ^{−1} h ^{−1}
A101	91	55	130.7	105.2	25.5	4.12	2.38	1.91	0.46	0.65	0.72	0.026	0.53	0.58	0.021	0.13	0.14	0.005
CIT1	94	26.9	133.2	67.8	65.4	1.04	4.95	2.52	2.43	0.67	0.71	0.052	0.34	0.36	0.027	0.33	0.35	0.026
CIT2	94	30.9	109.9	53.6	56.2	0.95	3.56	1.73	1.82	0.55	0.58	0.038	0.27	0.28	0.018	0.28	0.30	0.019

In contrary to glycerol cultures, however, according to the concentration of CA and ICA described above, the control A101 strain as well as the CIT1 transformant reached high volumetric productivity of citric acids during the processes with rapeseed oil (Table 3). The CIT2 transformant reached only $0.58 \text{ g dm}^{-3} \text{ h}^{-1}$. Both strains A101 and CIT1 presented also similar conversion of oil into citric acids. On the other hand, the highest specific production rate of citric acids was obtained by CIT1 and it was twice as high as the value reached by the control strain. This is due to the two times lower biomass concentration noted for the CIT1 transformant. All the analyzed parameters considering CA separately from ICA were higher for the A101 strain (Table 3). The CIT1 and CIT2 transformants reached similar values for all parameters regarding both CA and ICA.

It seemed plausible that overexpression of citrate synthase would cause an increased concentration of CA in the mitochondria, which would therefore be available in abundance for ICA-generating aconitase, leading in turn to high ICA biosynthesis. This compound cannot be further metabolized due to inactivation of ICDH by nitrogen-limiting conditions [52]. It is also important to note that overexpression of *citA* citrate synthase in the CA-producing fungus *A. niger* did not improve CA biosynthesis [53].

Both CA and ICA are valuable natural compounds. *Y. lipolytica* strains constructed in this study were able to produce these two acids in similar quantities from renewable substrates, e.g., glycerol and rapeseed oil. In combination with the abilities of *Y. lipolytica* to use waste glycerol from the biodiesel industry [33,54] and waste cooking oil [55,56] to produce various chemicals, it would be of great interest to culture the transformants using these substrates in order to obtain ICA and/or CA, which can be readily separated by esterification [20]. The amounts of ICA obtained in this study are very promising compared to the literature data [6,30,37]. Higher concentrations of ICA from glycerol were obtained by increasing the glycerol uptake through overexpression of the *GUT* genes [9] or from ethanol by supplementing the culture medium with zinc and itaconic acid [13]. Moreover, different cultivation modes (repeated batch fermentation) positively affect ICA biosynthesis [23]. None of this research, however, directly modified the Krebs cycle to prove the possibilities to improve ICA biosynthesis. Further enhancement of ICA biosynthesis by this peculiar yeast, except medium supplementation and/or the “push” strategy, represented by increased glycerol uptake, could be presumably reached by overexpression of *ACO1* gene encoding aconitase; however, this hypothesis remains to be verified.

4. Conclusions

The yeast *Y. lipolytica* is a highly appreciated industrial microorganism due to its wide range of applications in biotechnology. To our knowledge, this is the first study on the identification of genes encoding the citrate synthase in the yeast *Y. lipolytica*. We aimed to improve ICA biosynthesis by overexpressing either *CIT1* or *CIT2* genes. The activity assays showed that both genes encode proteins with citrate synthase activities; however, *YlCit2* proved to be the main citrate synthase, whereas the protein encoded by the *CIT1* gene possessed dual citrate and 2-methylcitrate synthase activity. The constructed *Y. lipolytica* transformants overexpressing either *CIT1* or *CIT2* genes produced noticeably more ICA in both glycerol- and vegetable oil-based media compared to the control strain. These results lay the foundation for future advancements in ICA biosynthesis by the means of genetic engineering and process optimization.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2071-1050/12/18/7364/s1>, Figure S1 (Comparison of sodium acetate and glucose utilization capabilities of the *Y. lipolytica* A101.1.31 strain and its *CIT* overexpressing mutants); Table S1 (Primers used in this study).

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