

Oleaginous yeast *Yarrowia lipolytica* mutants with a disrupted fatty acyl-CoA synthetase gene accumulate saturated fatty acid

Jinjing Wang^{a,b}, Borun Zhang^a, Shulin Chen^{b,*}

^a The Laboratory of Molecular Genetics and Breeding of Yeasts, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China

^b BBEL, Department of Biological Systems Engineering, Washington State University, Pullman, WA 99163, USA

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ABSTRACT

Fatty acyl-CoA synthetases are critical enzymes involved in lipid metabolism. The oleaginous yeast *Yarrowia lipolytica* is currently generating interest in biofuel research due to its ability to convert raw materials into value-added end products. In this study, the putative acyl-CoA synthetase gene *YAL1* in *Y. lipolytica* was interrupted with the copper resistance (*CRF1*) gene to allow selection without antibiotics to facilitate industrial applications. Deletion of *YAL1* led to reduced acyl-CoA synthetase activity. Furthermore, the fatty acid profile and lipid content of the mutant were different from the wild-type strain. The ratio of saturated to unsaturated fatty acids increased 6-fold, and the total lipid production of the mutant strain increased to 1.47-fold of the wild-type strain. The results indicate that *YAL1* in *Y. lipolytica* is involved in fatty acid elongation and desaturation, whereas the homologous, highly conserved *FAA1* gene from *Saccharomyces cerevisiae* was shown to be responsible for fatty acid activation. The increased ratio of saturated to unsaturated fatty acids would result in a higher combustion value and better oxidative stability for biofuel products obtained from the fatty acids from the engineered *Y. lipolytica* mutant.

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1. Introduction

Biofuels as alternatives for petroleum fuel have generated great interest in recent years. As alternative lipid sources, oleaginous microbes are being used as feedstock of biofuel [10,11,26]. Various oleaginous yeast species have a high lipid content (40–70% of dry biomass) [22]. Among them, *Yarrowia lipolytica* is one of the most extensively studied “unconventional” microbes. Metabolism in *Y. lipolytica* is directed toward lipid accumulation. Moreover, the unique ability of this yeast to efficiently use hydrophobic substrates (HS) makes this microorganism a prime candidate for use in the production of bio-oils [1]. Genetic and metabolic engineering have been used to modify the strains for the production of a higher amount of lipids and alternative novel chemicals and fuels [1]. Several enzymes including acyl-CoA oxidase (Aox), glycerol-3-phosphate dehydrogenase (G3PDH), diacylglycerol acyltransferase, and sterol acyltransferase were shown to be involved in lipid accumulation pathways [1]. It was reported that deletion of the *POX1-6* genes coding for six Aox-related enzymes in β -oxidation pathways [18] and eliminating the *GUT2* gene coding for G3PDH of the glycerol pathway triggered lipid accumulation [2]. It has been concluded that genes involved in every step of fatty acid metabolism,

including carbon flux toward triglycerides (TAG) and fatty acid β -oxidation, could affect or control lipid accumulation [1,2,18].

Fatty acyl-CoA synthetases (E.C. 6.2.1.3) are present in most organisms and are involved in the utilization of fatty acids. Fatty acids are channeled through fatty acyl-CoA derivatives into the β -oxidation pathway for degradation (Fig. 1); the activation of the CoA molecule is catalyzed by the enzyme fatty acyl-CoA synthetase. Several genes encoding acyl-CoA synthetases in *Saccharomyces cerevisiae* were identified, including *FAA1*, *FAA2*, *FAA3*, *FAA4*, and *FAT1*. Studies have shown that these enzymes are involved in fatty acid trafficking, recycling, and intracellular utilization. Michinaka et al. [17] and Scharnewski et al. [23] also demonstrated that *S. cerevisiae* deficient in acyl-CoA synthetase could secrete fatty acids. Interestingly, a number of studies in adipocytes suggest that different isoforms of acyl-CoA synthetase are involved in lipid storage and possibly channel fatty acids into TAG synthesis rather than into β -oxidation and energy production [21,25]. However, the function of the fatty acyl-CoA synthetase of the oleaginous yeast *Y. lipolytica*, which is comprised of 15 different genes located on several chromosomes, remains largely unstudied in terms of its role in lipid metabolism. Located on chromosome D, the *YAL1* gene was reported to have similar functions as the *FAA1* gene encoding the acyl-CoA synthetase in *S. cerevisiae* [5]. Thus, this study was designed to understand the role of acyl-CoA synthetase in fatty acid metabolism in the oleaginous yeast *Y. lipolytica*. The *YAL1* gene was deleted via homologous recombination to understand how it

* Corresponding author. Tel.: +1 509 335 3743; fax: +1 509 335 2722.
E-mail addresses: chens@wsu.edu, jj851014@hotmail.com (S. Chen).

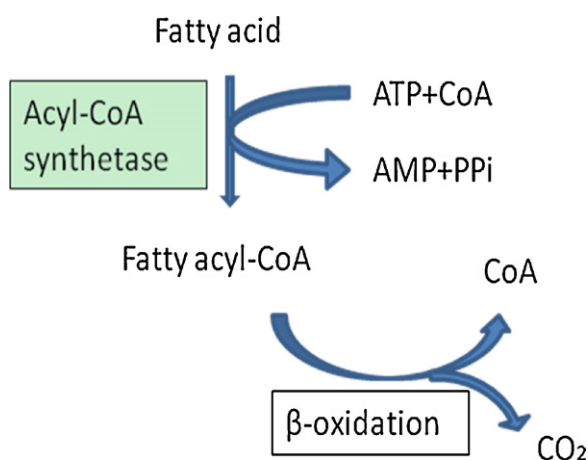


Fig. 1. Fatty acid present in the cytoplasm first reacts with CoA catalyzed by acyl-CoA synthetase and then enters the β -oxidation pathway for fatty acid degradation.

Adapted from Beopoulos et al. [1].

affects lipid content and fatty acid composition, and its potential to produce energy-efficient biofuel products.

2. Materials and methods

2.1. Strains, media, and growth conditions

The yeast strains used are shown in Table 1. Yeasts were grown at 30 °C on YPD medium [2% (w/v) glucose, 2% (w/v) peptone, and 1% (w/v) yeast extract]. Recombinant strains were selected on YPD plates containing various amounts of CuSO₄. *Escherichia coli* cells were grown at 37 °C on LB medium [1% (w/v) tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract] containing 50 μ g/ml ampicillin when necessary. 1.5% agar was added to produce solid media. Cells were harvested at specified time points by centrifugation and the cell pellets were resuspended in deionized water to achieve desired cell densities (OD₆₀₀ in the range of 1.2–1.5).

2.2. Construction of plasmids

Yeast genomic DNA was prepared as described by Burke et al. [4]. The *YAL1* gene, whose sequence was used for homologous recombination, was amplified from W29 genomic DNA by PCR with the primers YAL1-F (CGAATTCGCATCCACAATCTTCTTCGG (EcoRI))/YAL1-R (AAGGATCCACACATTTACGCCAGACCT (BamHI)) (Invitrogen). The PCR product was subsequently sub-cloned into a pGEM-T Easy vector to generate the plasmid pTA1. The *CRF1* gene conferring copper resistance was amplified from W29 genomic DNA by PCR with the primers CRF1-F (CGAGATCTGATGTGAGCCGTTATTCG (BglII))/CRF1-R (CCCTCGAGAAGAAACGCACATCTGTAATCC (XhoI)) and sub-cloned into a pGEM-T Easy vector to generate the plasmid pTCRF. Plasmid pTA1 was digested by *BglII* and *XhoI*, and the 2.9-kb fragment containing the *CRF1* gene from pTCRF coding for copper resistance was ligated into the *BglII/XhoI* site of pTA1. The resulting plasmid bearing the expression cassette YC (*yal1* Δ :*CRF1*) was designated as pTAC (Fig. 2a).

2.3. Yeast transformation and confirmation of recombinant strains

Plasmid pTAC was digested by *ScaI* and the linearized fragment containing cassette YC was transformed into *Y. lipolytica* W29 by electroporation using the Gene Pulser Xcell™ Electroporation System (Biorad). Recombinant strains bearing the deleted genes were selected on YPD plates with 31 mM CuSO₄ as a selection marker. Genomic DNA was extracted using a yeast DNA extraction kit (Thermo) and PCR was performed to confirm the gene disruption, which results from the homologous recombination between the introduced linearized DNA and the homologous regions of the genome. The 50 μ l assay volume contained 25 μ l *Taq* master mix (NEB) and 0.2 μ M primers; the PCR cycling parameters were 30 s at 94 °C, 45 s at 48–55 °C, and 3 min at 72 °C for a total of 30 cycles, followed by a final extension for 15 min at 72 °C. PCR products were sent for sequencing to confirm the disruption of *YAL1* gene.

2.4. Acyl-CoA synthetase assay

The acyl-CoA synthetase extraction protocol was modified from the approach described by Fægeman et al. [8]. Once the cultures had reached the logarithmic phase, they were centrifuged for 4 min at 3000 \times g and 4 °C. The pellet, which contained the yeast cells, was washed with cold distilled water and re-suspended in a lysis buffer composed of 200 mM Tris-HCl (pH 8.0), 5 mM β -mercaptoethanol,

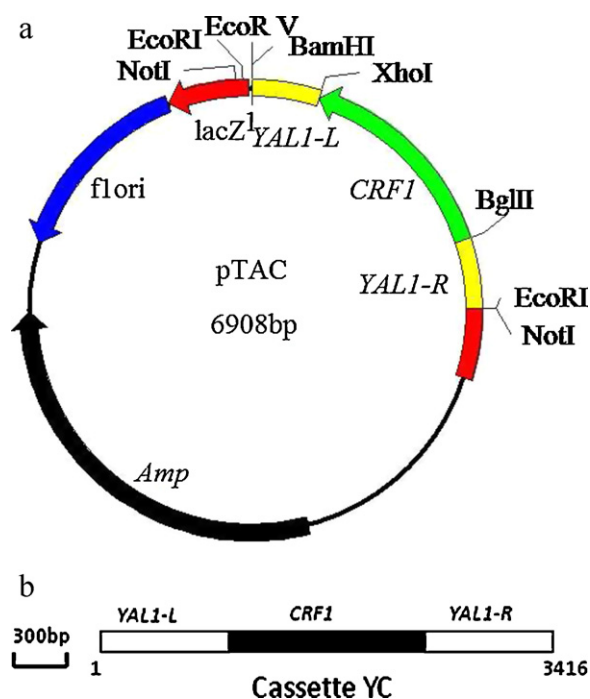


Fig. 2. (a) Construction of plasmid pTAC and (b) cassette YC used for homologous recombination.

4 mM EDTA, 10% glycerol, 0.01% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by a vigorous vortex of the cell suspension containing glass beads with five 1-min cycles at 4 °C. After centrifugation for 5 min at 1500 \times g and 4 °C, the supernatants were used for the acyl-CoA synthetase activity assay. The amount of protein was determined by the Coomassie Brilliant Blue method [3]. The acyl-CoA synthetase activity was determined as described by Ichihara et al. [24]. Briefly, reactions (0.2 ml) contained 0.15 M MOPS-NaOH (pH 7.7), 1 mM DTT, 0.25 mM CoA Na₃, 4.5 mM ATP Na₂, 10 mM MgCl₂, 1% methanol, 1.5 U acyl-CoA oxidase, 2 KU catalase, 0.2 mM potassium salt of fatty acid, 0.55 mM Triton X-100, and fractions prepared from *Y. lipolytica* (up to 100 μ g protein). The reaction was performed at 30 °C for 30 min. During the reaction, synthesized acyl-CoA was converted into *trans*-enoyl-CoA and H₂O₂ by the action of acyl-CoA oxidase *in situ*, and then formaldehyde was produced from the H₂O₂ and methanol by catalase. The sequential reactions were terminated by adding 0.2 ml of 2 M KOH at 0 °C. The color-producing reagent for aldehydes, 0.2 ml of 0.6% 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole in 0.5 M HCl, was then added to the chilled alkaline solution. The mixture was incubated at 37 °C for 10 min, and then 0.5 ml of 1% NaIO₄ was added. The molar absorption coefficient of the resulting purple dye was 29,200/M/cm at 550 nm. One unit of acyl-CoA synthetase was calculated as the amount of enzyme required to form 1.0 nmol of acyl-CoA per minute at 30 °C.

2.5. Preparation of yeast cells and fatty acid analysis

Yeast cells and supernatant were separated by centrifugation at 8000 \times g for 5 min. The cell pellet was washed in 1 ml of distilled water and centrifuged at 8000 \times g for 3 min. The fatty acid concentrations of cell pellet and supernatant were measured using the gas chromatography (GC) method described by O'Fallon et al. [19]. For biomass determination, cells were washed with distilled water and dried at 80 °C until a constant weight was reached (approximately 24 h).

3. Results and discussion

3.1. Genetic manipulation of *Y. lipolytica*

Plasmid pTAC (Fig. 2a) was successfully constructed and confirmed by enzyme digestion and PCR. The linearized *ScaI* fragment from pTAC was transformed into *Y. lipolytica* W29. Recombinant strains were selected on 31 mM CuSO₄, as the host strain could only grow on less than 22 mM CuSO₄. Seven different transformants were selected for resistance to higher copper concentrations (31 mM CuSO₄) and sequentially named using the nomenclature TY1 up to TY7. The copper-resistant gene served as a selection

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Genotype	Source
Strains		
<i>Escherichia coli</i> Top 10	F- <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74recA1araD139Δ(<i>ara-leu</i>)7697<i>galUgalK</i>rpsL(Str^R)<i>endA1nupG</i></i>	Invitrogen
<i>Yarrowia lipolytica</i> W29	Wild-type	ATCC 20460
Plasmids		
pGEM-T Easy	Cloning vector, <i>amp</i>	Promega
pTA1	Cloning vector, <i>amp</i>	This work
pTAC	Recombinant plasmid	This work

marker because this oleaginous yeast is widely used for biofuel production by simultaneous bioremediation of industrial polluted wastewater for biofuel production [14,20]. Unlike the antibiotic resistance genes, the expression of *CRF1* gene in *Y. lipolytica* would not prevent the use of this yeast in an industrial setting, which might be the case an exogenous antibiotic tag was introduced. In addition, higher copper resistance could broaden the industrial use of *Y. lipolytica*.

3.2. *YAL1* gene disrupted reduces acyl-CoA synthetase activity

Genomic DNA was extracted from mutant strains and wild-type strain and PCR was performed to analyze the disruption of the *YAL1* gene. As Fig. 3 shows, when using the primer pair *YAL1*-F/*CRF1*-F, the sizes of the PCR products from mutant strains were 3.1 kb, while no fragment was obtained from wild-type strain W29. Moreover, the sizes of the PCR products from mutant strains using the primer pair *YAL1*-F/*YAL1*-R were 3.4 kb, which aligns with the expression cassette *YC* (*yal1* Δ ::*CRF1*) (Fig. 2b), while the sizes of the PCR products from the wild-type strain were 1.7 kb. This indicated that the *YAL1* gene was disrupted in mutant strains. The sequencing results of the PCR products also confirmed the disruption of *YAL1* gene in the mutant strains. However, acyl-CoA synthetase activities were still detected in mutants (Fig. 4), whereas the activities measured in mutants were lower than wild-type strain to different degrees. The acyl-CoA synthetase enzyme has multiple functions in fatty acid metabolism [8,23] and plays a crucial role in intermediary metabolism by catalyzing the formation of fatty acyl-CoA through a two-step process. It is also reportedly involved in the regulation of various cell functions by catalyzing the production of long chain fatty acyl-CoA esters (e.g. protein transporters, enzyme activation, protein acylation, cell signaling, and transcriptional regulation) [9].

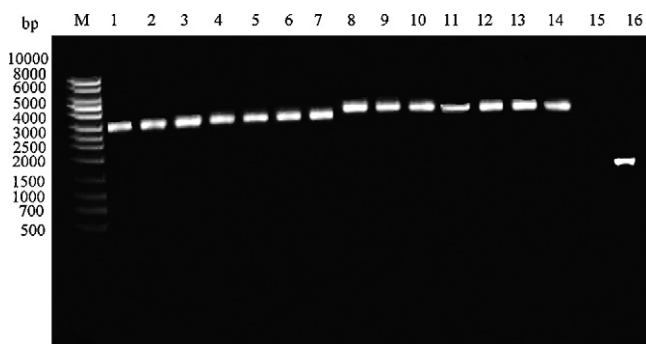


Fig. 3. PCR analysis of recombinant strains using different primer pairs. Lane M: 1 kb marker; lane 1–7: mutant strains TY1–TY7 (primer pair *YAL1*-F/*CRF1*-F); lane 8–14: mutant strains TY1–TY7 (primer pair *YAL1*-F/*YAL1*-R); lane 15: wild-type strain w29 (primer pair *YAL1*-F/*CRF1*-F); lane 16: wild-type strain w29 (primer pair *YAL1*-F/*YAL1*-R). For mutant strains, the sizes of PCR products using primer pair *YAL1*-F/*CRF1*-F were 3.1 kb and the PCR products using *YAL1*-F/*YAL1*-R were 3.4 kb; for the wild-type strain, no product was obtained using *YAL1*-F/*CRF1*-F, and PCR products using *YAL1*-F/*YAL1*-R were 1.7 kb.

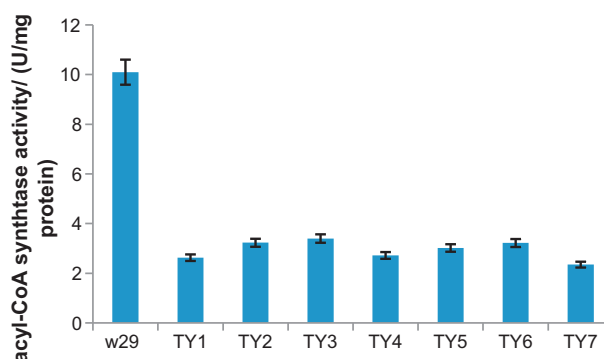


Fig. 4. Measurement of acyl-CoA synthetase activities of mutant strains versus the wild-type strain (column). Measurements are done in three biological replicates, and in every biological replicate three technical replicates are applied.

As shown in Fig. 4, when compared with the wild type strain, which has an activity of 10.10 U/mg protein, all mutant strains exhibited lower activities (2.35–3.23 U/mg protein). According to the ANOVA result, though the enzyme activities of all mutants were different, the difference was not significant ($p > 0.05$) (Table 2). However, we still chose TY7 for further study because it had the lowest enzyme activity.

3.3. Growth of *Y. lipolytica* mutants containing an interrupted *YAL1* gene

To examine whether *YAL1* gene disruption has any effect on cell growth, cell growth curves were determined. *YAL1* gene disruption slightly influenced recombinant strain growth. Defective growth was fully restored by reintroducing the *YAL1* gene to mutant strains (Fig. 5a). One explanation for decreased cell growth in the mutant strains is the altered ratio of unsaturated to saturated fatty acid when acyl-CoA synthetase expression was decreased, which caused changes in the physical properties of membranes and subsequently affected growth properties.

Table 2
ANOVA of acyl-CoA synthetase activities of mutant strains (mean \pm SD, $n = 3$).

Strains	Enzyme activity (U/mg protein)		
	Replicate #1	Replicate #2	Replicate #3
TY1	2.63 \pm 0.01	2.77 \pm 0.02	2.49 \pm 0.01
TY2	3.23 \pm 0.01	3.39 \pm 0.01	3.05 \pm 0.00
TY3	3.40 \pm 0.02	3.57 \pm 0.01	3.23 \pm 0.02
TY4	2.72 \pm 0.01	2.86 \pm 0.00	2.59 \pm 0.01
TY5	3.02 \pm 0.02	3.18 \pm 0.03	2.89 \pm 0.02
TY6	3.22 \pm 0.03	3.38 \pm 0.04	3.01 \pm 0.02
TY7	2.35 \pm 0.02	2.47 \pm 0.03	2.23 \pm 0.03
F-Value	1.127		
p-Value	0.346		

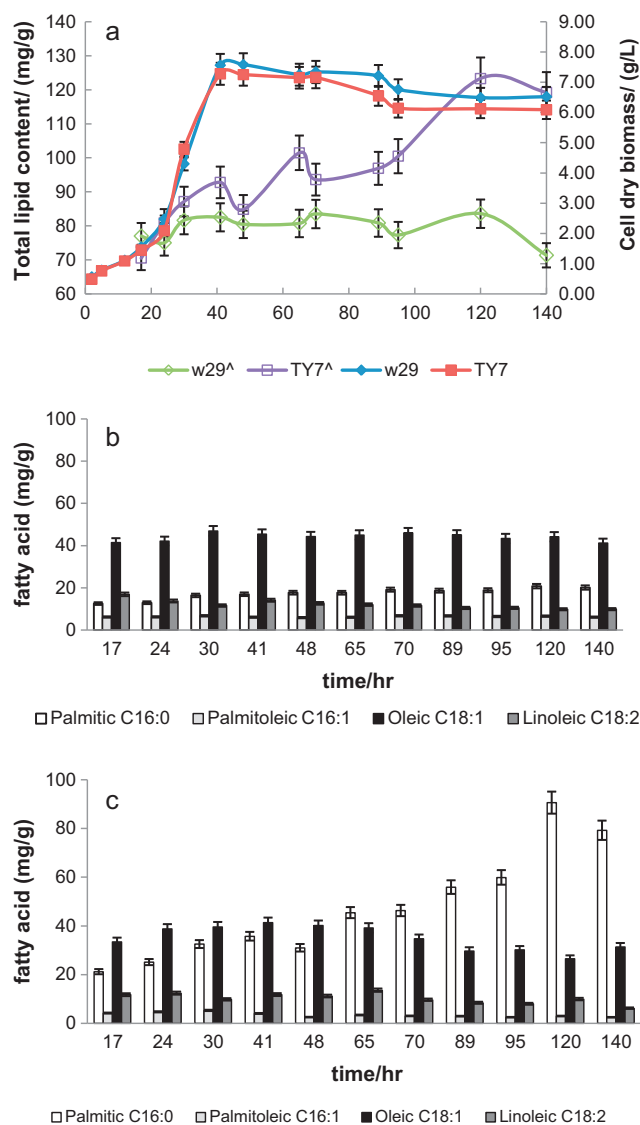


Fig. 5. Fatty acid analysis of the W29 host strain and the TY7 mutant strain. (a) Growth curve and total lipid content of W29 (diamond) and TY7 (square). (b) Relationship between fatty acid concentration and culture stage of W29. (c) Relationship between fatty acid concentration and culture stage of mutant TY7. Data for total lipid content. Measurements are done in three biological replicates, and in every biological replicate three technical replicates are applied.

3.4. Fatty acid analysis

Fatty acids were not detected in the supernatant of recombinant strains. However, the recombinant strain TY7 accumulated more lipid than the host strain. The maximum lipid production of TY7 (123.34 ± 0.64 mg/g dry weight, $p < 0.01$) increased 1.47-fold compared to that of the host strain (83.58 ± 1.19 mg/g dry weight, $p < 0.01$) (Fig. 5a). Previous studies in *S. cerevisiae* indicated that a deficiency in acyl-CoA synthetase activity might result in induced fatty acid transport across the cell membrane into the media [8,17,23]. However, because *S. cerevisiae* is not a strain that normally stores lipid in the cytoplasm, it is possible that attenuation of acyl-CoA synthetase activity elicits an active export of fatty acids that might be necessary for cellular homeostasis [8]. However, oleaginous yeast can accumulate large amounts of lipid in the cytoplasm and does not need to excrete fatty acids to maintain cellular homeostasis. Such an explanation could account for the fact that no fatty acids were detected in the supernatant of the mutant strains.

Table 3

Fatty acid profiles from *Y. lipolytica* host strain W29, mutant strain TY7^a and the recovered strain TY7^{+b} (mean \pm SD, $n = 3$).

Fatty acids	% of total fatty acids in strains		
	W29	TY7	TY7 ^{+b}
Palmitic C16:0	25.51 \pm 0.86	65.15 \pm 6.76	28.61 \pm 1.33
Palmitoleic C16:1	8.00 \pm 0.01	1.06 \pm 0.04	6.84 \pm 0.04
Oleic C18:1	54.30 \pm 0.53	22.44 \pm 1.00	45.95 \pm 1.86
Linoleic C18:2	12.11 \pm 0.20	3.93 \pm 1.00	10.24 \pm 0.44
Saturated/unsaturated fatty acid ratio	1:3	2.4:1	1:2.9

^a Fatty acid was analyzed from cells growing for 120 h by gas–liquid chromatography. The results are mean values from three independent experiments.

^b The complementary strain of TY7 was named as TY7⁺.

Instead, the amount of total lipid accumulation increased 1.47-fold in a recombinant strain compared to that of the host strain.

The four most prevalent fatty acids in *Y. lipolytica* cells when glucose was used as carbon source were palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), and linoleic acid (C18:2). In the wild-type strain, the concentration of the four fatty acids remained constant during culture (Fig. 5b). Unsaturated fatty acids accounted for 74.41% of the total fatty acids present. The predominant unsaturated fatty acid was oleic acid, which accounted for 54.30% of the total fatty acids. However, in the recombinant strain TY7, the amount of saturated palmitic acid (C16:0) increased from 25.51% to 65.15% (Table 3). Therefore, the ratio of saturated to unsaturated fatty acids increased during the later phase of culture (from 1:3 to 2.4:1 (Table 3, Fig. 5c)), which also happened in other mutant strains (e.g. s/u fatty acid ratio in TY3 was 2.1:1, and s/u fatty acid ratio in TY5 was 2.3:1). After reintroducing the *YAL1* gene to the mutant strain, the profile of fatty acids measured from the cell was similar to the wild-type strain (Table 3), indicating that the disruption of the *YAL1* gene was the reason for the changes in fatty acid profiles. Thevenieau et al. [27] reported that after activation by the fatty acyl-CoA synthetase specific for long chain fatty acids localized in the endoplasmic reticulum or cytosol, fatty acids could either be used directly for lipid biosynthesis upon desaturation and elongation or transported into peroxisomes to be degraded by the β -oxidation pathway. In this study, the *YAL1* gene was disrupted, resulting in a deficient fatty acyl-CoA synthetase that blocked the process of desaturation and elongation, which could explain an increased amount of C16:0 fatty acid and an increased ratio of saturated to unsaturated fatty acid.

As the only *FAA* homologous gene in *Y. lipolytica*, the *YAL1* gene was reported to function most similarly to *FAA1* gene, which accounted for most of the acyl-CoA synthetase activity among the five genes encoding the acyl-CoA synthetase in *S. cerevisiae* (*FAA1* to *FAA4*, and *FAT1*) [5]. The acyl-CoA synthetase encoded by *FAA1* actively imported fatty acids with a preference for C12:0–C16:0 chain lengths. This is pertinent for this study in that the *YAL1* gene encodes the acyl-CoA synthetase that catalyzed palmitic acid to palmitic acyl-CoA. Therefore, after disruption of the *YAL1* gene, the amount of palmitic acid (C16:0) increased dramatically because palmitic acid could not be transferred to next step of fatty acid metabolism. To explain the reason for the reduction in longer-chain fatty acid (C18) concentrations, studies focusing on acyl-CoA synthetase-1 (encoded by the *ACSL1* gene) in adipocytes revealed that *ACSL1* is involved in the fatty acid elongation process [15]. To understand the role of these enzymes further, we compared the genes encoding acyl-CoA synthetase in different organisms. The phylogenetic tree of genes related to acyl-CoA synthetase was constructed (Fig. 6). The gene most similar to *YAL1* was found in *Lachancea thermotolerans* with 59% homology and similar functions to *FAA1* in *S. cerevisiae*. *YAL1* showed 53% identity with *FAA1* and 51% identity with *Faa1p* protein from *S. cerevisiae*. Meanwhile, *YAL1*

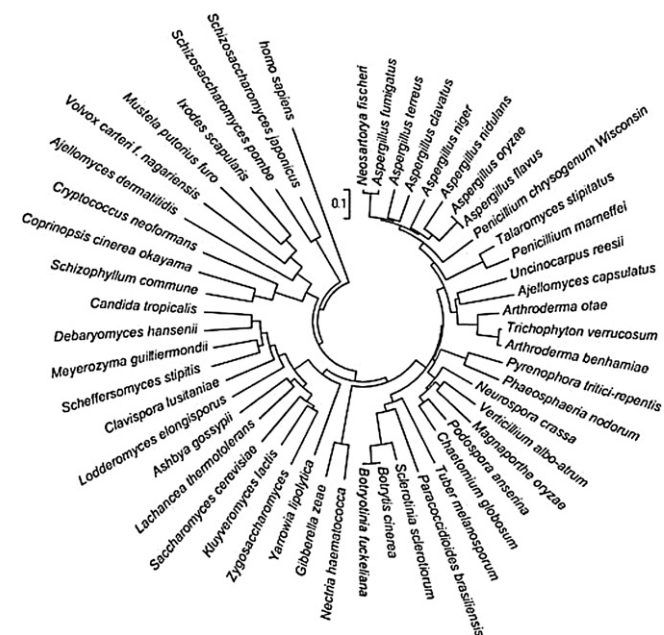


Fig. 6. Phylogenetic tree of genes encoding acyl-CoA synthetases in different organisms. Scale length indicates 0.1 substitutions per site. Incomplete sequences are not represented for the sake of reliability of the phylogenetic distribution.

showed 43% identity with *ACSL1* and 31% identity with the primary structure of the homologous protein from *Mus musculus*. Therefore, the *YAL1* gene could have functions related to both *FAA1* and *ACSL1*. In studies involving *S. cerevisiae* genetic modifications, disruption of a single gene can affect the functions of multiple genes [28]. Because the function of many genes of oleaginous yeast remain unclear, it is reasonable to hypothesize that these yeasts could have interrelated gene functions to allow for precise adaptation to environmental changes.

For the production of biofuel, oxidative stability has been the subject of considerable research. The percentage of unsaturated fatty acid will affect the oxidative stability of the final product [7] and affects the quality of biofuel during extended storage [6,13]. The yeast *Y. lipolytica* has been recognized as a very good candidate for biotechnological uses due to its capability to accumulate high amounts of lipids [1]. This yeast is able to grow on industrial waste and agro-industrial by-products to produce large amounts of non-specific oils, which could be used as starting materials for biofuel synthesis [16]. The increased ratio of saturated to unsaturated fatty acids in the *YAL1* mutant could increase oxidative stability, which could help prolong the storage life of biofuel made from this organism. In addition, a higher amount of saturated fats could help increase the cetane numbers (CN) of produced biofuel, which is directly related to a better combustion value. A low unsaturated fatty acid concentration could improve the CN because unsaturated fatty acids are low-cetane compounds that have a negative effect on biofuel combustion values [12]. In contrast, the methyl ester of palmitic acid (C16:0) is solid at room temperature and thus biodiesel cannot contain a large percentage of methyl palmitate. The ideal biodiesel is made from methyl esters from unsaturated palmitoleic (C16:1) and oleic (C18:1) acids.

4. Conclusion

The *YAL1* gene in *Y. lipolytica* was interrupted by the copper-resistant (*CRF1*) gene via homologous recombination. As a result, more fatty acids accumulated in the mutant strains, leading to a significant increase in the percentage of C16:0 and an increased

ratio of saturated to unsaturated fatty acids. Although *YAL1* did not show activity in transport of fatty acids across the cell membrane, the results revealed a function of *YAL1* in fatty acid metabolism during the fatty acid elongation process, with a preference for a C16 chain length. The findings from this study are potentially useful in synthetic biology research involving the production of short chain fatty acids for applications such as the production of aviation fuel.

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