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Homologous Overexpression of Acyl-CoA Thioesterase 8 Enhanced Free Fatty Acid Accumulation in Oleaginous Fungus *Mucor circinelloides* WJ11

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Abstract: Thioesterases play an essential role in the metabolism of fatty acids since they are considered one of the key enzymes to change the total amount and composition of fatty acid in an organism's cells. Acyl-coenzyme A thioesterase 8 (ACOT8) exhibits substrate specificity mainly for short- to long-chain acyl-CoA. To identify and characterize the ACOT8 enzyme's superfamily in *Mucor circinelloides*, three genes were characterized and homologously expressed in *M. circinelloides* WJ11 which has been used as a model organism to investigate the mechanism of lipid accumulation. Multiple sequence alignment showed that McACOT8s had significant conserved motifs in the ACOT8 family. Experimental data indicated that the biomass of the three recombinant strains (McACOT8a, McACOT8b and McACOT8c) was slightly lower than that of the control strain Mc2075, but the total fatty acid (TFA) content was significantly increased by 30.3, 21.5 and 23.9%, respectively, and the free fatty acid production increased from 12.9% (control strain) to 19.2, 25.2 and 26.2% (recombinant types), respectively, with a maximum increase of 103.1% in McACOT8c. The three strains did not show any significant improvement in fatty acid composition compared to the control strain. However, in terms of fatty acid composition of free fatty acid, McACOT8b and McACOT8c contained γ-linolenic acid (C18:3), which was not detected in the control strain or in McACOT8a, indicating that ACOT8b and ACOT8c had substrate specificity for C18:3. These results displayed that ACOT8 can increase TFA accumulation and that it may be an important target of genetic manipulation for microbial oil production.

Keywords: Acyl-CoA thioesterase 8; free fatty acid; homologous overexpression; *Mucor circinelloides*; total fatty acid

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1. Introduction

Thioesterases are a large superfamily of enzymes catalyzing the cleavage of thioester bonds and releasing free fatty acids, both medium- and long-chain fatty acids. These thioester bonds are present in a variety of activated fatty acid acyl-coenzyme A (acyl-CoA) substrates, acyl-acyl carrier proteins (acyl-ACPs), acyl-glutathione and other cellular molecules [1]. At present, the importance of the enzymatic activity of acyl-CoA/ACP thioesterases can be identified on the basis of the acyl-CoA/ACP esters and free fatty acids involved, in addition to the genetic manipulation of thioesterases to increase lipid content and alter fatty acid composition, which is a common technique for enhancing microbial lipid production [2].

Acyl-ACP thioesterases are an important enzyme in fatty acid synthesis, and the fatty acids synthesized in the plastid are hydrolyzed into free acyl-CoA/ACP under its

action, thereby terminating the fatty acid carbon chain extension [3]. Although the fungal fatty acid synthase complex was more efficient kinetically and had a unique structure, no thioesterase activity was detected. However, in bacterial and plant FAS complexes, thioesterase preference is a key metabolic control for fatty acid chain length. By increasing the activity of TesA, which is natural ACP-type I thioesterase in *Escherichia coli*, its binding to specific substrates was enhanced, resulting in dodecanoic acid (C12) levels of up to 49% and octadecanoic acid (C8) levels of up to 50%, exceeding natural levels of total free fatty acid [4]. In order to study the function of acyl-ACP thioesterase, plant and bacterial thioesterases have been heterologously expressed in *E. coli* [5]. The composition of free fatty acids produced by elm (*Ulmus americana*) FatBl increased the chain termination rate, mainly from tetradecanoic acid (C14)-hexadecanoic acid (C16) to C8-decanoin acid (C10) in *E. coli*, indicating that there was an alternative mechanism for the evolution of medium-chain production [3]. What is more, five different acyl-ACP thioesterases from *Anaerococcus tetradius* (GenBank Accession No: KR180390), *Cuphea hookeriana* (GenBank Accession No: KR180391), *Cuphea palustris* (GenBank Accession No: KR180392), *Clostridium perfringens* (GenBank Accession No: KR180393) and *Umbellularia californica* (GenBank Accession No: KR180394) with specificity for medium-chain acyl-ACP molecules are expressed in *Yarrowia lipolytica* to form C8 and C10 acids. These new fatty acid products were found to account for 40% of the total cellular lipids. Furthermore, the reduction in chain length resulted in a twofold increase in specific lipid production in these engineered strains [6].

CoA-binding substrates are intermediates in many cellular biosynthetic pathways, including acyl-CoA and malonyl-CoA, which have important roles in β-oxidation and fatty acid synthesis [7]. Acyl-CoA thioesterase 8 (ACOT8) is capable of hydrolyzing short- to long-chain acyl-CoA, and may be involved in regulating coenzyme A-SH (CoASH) levels in peroxisome [8]. Further kinetic characterization of ACOT8 in mice revealed two interesting features [9,10]. First of all, an unusually wide range of acyl-CoA substrate specificity on all acyl-CoAs with ACOT8 was active, from two to twenty carbon atoms, saturated and unsaturated fatty acids, methyl-branching fatty acids as well as bile-acid intermediates, which had the highest activity. Another feature that was not seen in other types of thioesterases was the strong inhibition of activity by CoASH. The sensitivity to CoASH and the very wide substrate specificity suggests that this enzyme plays a vital role in regulating acyl-CoA/CoASH levels in peroxidase (by sensing CoASH levels) to optimize the flux of fatty acids through the β-oxidation system, thereby releasing free fatty acids and accumulating more fatty acid content. Knockout of the ACOT8 gene leads to stunted cell growth, which can be rescued with the addition of non-esterified myristic acid [11]. Overexpression of human ACOT8 in mammalian human and mouse cell lines as well as in transgenic mice results in the accumulation of lipid droplets [12] and the bacterial homolog acyl-CoA thioesterase hydrolyses medium- to long-chain acyl-CoA [13]. The ACOT8 homolog ACH2, which is a putative peroxisomal acyl-CoA thioesterase, was present in *Arabidopsis thaliana* and, after detailed characterization results, showed that it hydrolyzes C12- arachidic acid (C20) saturated and unsaturated acyl-CoA and was insensitive to feedback inhibition by CoASH [14], indicating that acyl-CoA thioesterase can significantly hydrolyze polyunsaturated long-chain fatty acid. In haploid *Saccharomyces cerevisiae*, the proportion of extracellular unsaturated fatty acids accumulated by the strains produced by replacing the FAA1 and FAA4 genes encoding two acyl-CoA synthases with the ACOT8-encoding *Mus musculus* peroxisomal ACOT was higher than that of the wild-type strain [15].

Mucor circinelloides WJ11, as a typical oil-producing fungus, is widely used as a model organism to study the mechanism of lipid accumulation. Currently, advances in metabolic and genetic engineering have allowed researchers to construct more efficient strains, specifically by modifying genes involved in lipid synthesis pathways to produce valuable fatty acids; moreover, its fatty acid profile suggests that it is mainly formed from long-chain fatty acids [16,17]. Although many researchers have studied the role of key enzymes in the lipid synthesis pathway, the study of how endogenous acyl-CoA thioesterases in

lipid metabolism plays a role in stopping fatty acid chain elongation has not been reported. Therefore, we identified and studied the role of ACOT8 in the intracellular lipid metabolism of *M. circinelloides* WJ11, and three genes, ACOT8a, ACOT8b and ACOT8c, were homologously overexpressed in *M. circinelloides* to investigate the role of the genes in lipid accumulation in this microbial fungus.

2. Materials and Methods

2.1. Microorganisms, Cultivation and Transformation Conditions

M. circinelloides WJ11 (CCTCC no. M2014424) was used as the source of three genes (ACOT8a, ACOT8b and ACOT8c). *M. circinelloides* M65 (a urea-assisted strain derived from WJ11), which has similar oil-producing properties to the wild-type strain, was used as a background strain for all transformation experiments to overexpress these three genes. The cultures were cultured at 28 °C in complete yeast extract-peptone glycerol (YPG), minimal microbial microdroplet culture (MMC) and yeast nitrogen base media (YNB), which were supplemented with uracil at 600 mg/L when needed [18]. *E. coli* Top10 was grown in lysogeny broth (LB) at 37 °C with shaking at 220 rpm and used to maintain and propagate the recombinant plasmids in all cloning experiments [19].

2.2. Construction of ACOT8 Overexpressing Strains of M. circinelloides

Total RNA of *M. circinelloides* WJ11 was isolated using Trizol reagent (Takara, Dalian, China) according to standard protocols and reverse transcribed into cDNA. The cDNA was used as a template to obtain gene fragments of McACOT8a, McACOT8b and McACOT8c via PCR using the corresponding primers, as shown in Table S1. These fragments were cloned into the expression vector pMAT2075, which was constructed and preserved by our laboratory using the restriction endonucleases *Xho*I and *Nhe*I to produce a recombinant plasmid pMAT-ACOT8a, pMAT-ACOT8b and pMAT-ACOT8c. The plasmids expressing McACOT8a, McACOT8b and McACOT8c were transfected into *M. circinelloides* using the electroporation-mediated procedure described by Torres-Martinez et al. [20]; the CarRP gene associated with pigment synthesis was replaced and the transformants were color screened until all colonies turned white, and a mutant strain carrying the empty vector pMAT2075 was used as a negative control.

2.3. Fermentation of M. circinelloides Transformants

Approximately 10^6 spores of ACOT8 overexpression and the control strains were initially inoculated into 100 mL of Kendrick and Ratledge (K&R) medium [21] in 500 mL baffled flasks and cultured in a rotating shaker at 130 rpm, 28 °C for 24 h, and then 200 mL medium was placed in a 1.0 L baffled triangular flask and placed in a constant temperature shaker with 150 rpm and 28 °C to select the appropriate transformants. Finally, fermentation was carried out in a 3.0 L fermenter with 1.5 L medium maintained at 28 °C with 700 rpm agitation and 1.0 vvm aeration. Automatic addition of 2 M sodium hydroxide was used to maintain the pH at 6.0. Fermentation broth (1.0 L) consisted of 80.0 g glucose, 1.5 g $MgSO_4 \cdot 7H_2O$, 2.0 g diammonium tartrate, 7.0 g KH_2PO_4, 2.0 g Na_2HPO_4, 1.5 g yeast extract, 0.1 g $CaC1_2 \cdot 2H_2O$, 8.0 mg $FeC1_3 \cdot 6H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $Co(NO_3)_2 \cdot 6H_2O$ and 0.1 mg $MnSO_4 \cdot 5H_2O$.

2.4. Analysis of Cell Dry Weight (CDW) and Culture Supernatant

Mycelia of overexpressing strains were cultured in a fermenter for 4 days, filtered through a Buchner funnel under reduced pressure and then washed with distilled water. The collected mycelium was placed in a centrifuge tube after weighing for freeze-drying, and the CDW of mycelium in the centrifuge tube was determined using the differential weight method. The residual glucose concentration in the medium was determined using a glucose oxidase kit (Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China) and the residual ammonium concentration was determined using the indophenol test [22]. The ratio of CDW to time yielded the growth rate. The ratio of the mass of generated cells

to the mass of consumed substrate was substrate cell yield (Yx/s). The specific growth rate (μ) was calculated using the following formula; N_2 and N_1 are the CDW at t_2 and t_1, respectively.

$$\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)\ (/h) \quad (1)$$

2.5. Total Fatty Acid (TFA) Content and Lipid Composition Analysis

After freeze-drying treatment, cells of mycelium were cleaved using hydrochloric acid (4 mol/L) with the addition of pentadecanoic acid (C15:0) as the internal standard; chloroform/methanol (2:1, *v*/*v*) was added to extract TFA [23], and then fatty acid methyl ester (FAME) was prepared by adding 10% (*w*/*w*) methanol hydrochloride at 60 °C for 3 h.

Lipid composition of overexpressed and control strains was determined using thin layer chromatography (TLC): chloroform/methanol/water (2:1:1, *v*/*v*/*v*) was added for extraction. Lipids in the organic phase were dissolved with ethyl ether, then dropwise added to the silica gel plate and placed in a chromatographic chamber with the developing agent (*n*-hexane/ethyl ether/acetic acid, 50:50:1). The silica gel plate was placed in an iodine chamber for color development. The gray scanning software ImageJ was used to process the developing strip and obtain the gray peak area. The stained bands were scraped off and methylated with 10% (*w*/*w*) methanol hydrochloride at 60 °C for 3 h.

FAME were extracted with *n*-hexane, determined using gas chromatography with an Agilent 123-3232 DB-Waxetr column (30 m × 320 μm × 0.25 μm) column, and then the procedure was as follows according to the previous study [24]: 120 °C for 3 min, ramp to 200 °C for 5 °C/min, ramp to 220 °C for 4 °C/min, hold for 2 min. The peaks were identified using standard sample of FAME (FAME mixed label from Sigma-Aldrich, St. Louis, MO, USA).

2.6. Gene Expression and RT-qPCR Analysis

Genetically modified strains of *M. circinelloides* were cultured in fermenters and mycelium was collected at 6, 12, 24 and 96 h. After grinding the mycelium under liquid nitrogen, total RNA of *M. circinelloides* was extracted using Trizol and reversed to cDNA using a qPCR SYBR Green Master Mix kit (Accurate Bio-Medical, Changsha, China). Relative expression of mRNA was analyzed with real-time quantitative polymerase chain reaction (RT-qPCR) using the $2^{-\Delta\Delta C}$ method [25], with mRNA expression of each overexpression strain at 6 h being one. Three independent biological replicates were analyzed.

2.7. Statistical Analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$ and very significant when $p < 0.01$.

3. Results

3.1. Identification and Protein Sequence Analysis of ACOT8 from M. circinelloides WJ11

According to the annotated genome of oleaginous fungus *M. circinelloides* WJ11, three putative ACOT8 sequences, McACOT8a (gene ID: evm. model. scaffold00002.31), McACOT8b (gene ID: evm. model. scaffold00050.52) and McACOT8c (gene ID: evm. model. scaffold00233.10) were found. McACOT8a, McACOT8b and McACOT8c, which are 338, 352 and 349 amino acid residuals in length, respectively, shared the same domains and subfamilies. The analytical results using the conserved domain database of the national center for biotechnology information (NCBI) showed that McACOT8a, McACOT8b and McACOT8c possessed the same superfamily domain. Multiple sequence alignment of four other identified ACOT8 genes and three ACOT8 from *M. circinelloides* WJ11 was performed (Figure 1), indicating that the ACOT8s identified in this species are homologous to genes of the already-identified family, suggesting that ACOT8a-c belongs to the thioesterase 4 family and is able to hydrolyze the acyl-CoA bond.

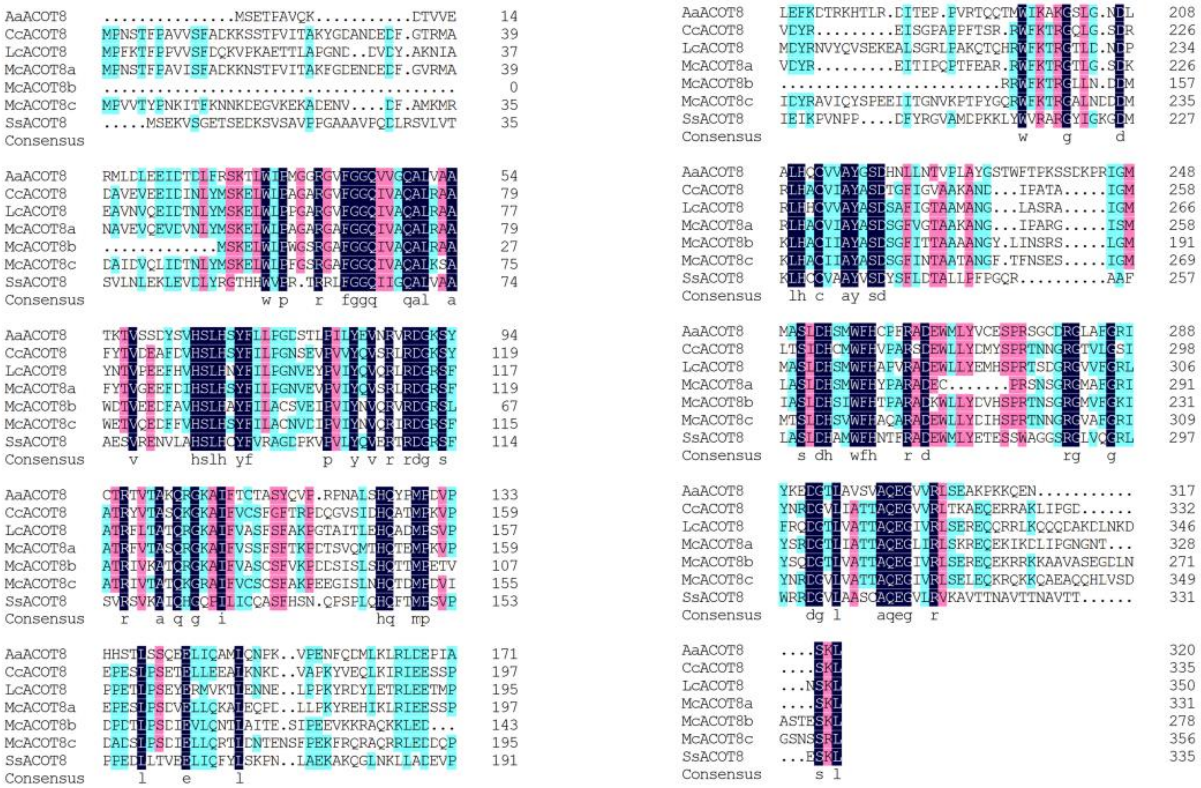

Figure 1. Protein sequence alignment of McACOT8a, McACOT8b and McACOT8c with ACOT8s from five organisms. All the protein sequences were obtained from GenBank and the IDs were as follows. Aa, *Actinomortierella ambigua* (KAG0262595.1); Cc, *Choanephora cucurbitarum* (OBZ86625.1); Lc, *Lichtheimia corymbifera* (CDH57968.1); Mc, *Mucor circinelloides*; Ss, Salmo salar (ACN10909.1).

3.2. Construction of Plasmids and Screening of Transformants

Transformation of *M. circinelloides* WJ11 was carried out using homologous recombination to verify whether the target genes were associated with fatty acid accumulation. Transformants with fragmented empty vectors were used as a negative control to obtain control strain Mc2075, and transformants with fragments of the uracil synthesis gene (*pyrF*), promoter (*Pzrt1*) and ACOT8a-c were used as positive transformants to obtain different overexpression strains McACOT8a, McACOT8b and McACOT8c (Figure 2A). Genomic DNA from the transformants was extracted and used as a template for PCR validation using the primer CarRP-up-F/CarRP-down-R (sequence in Table S1). The band sizes of the transformants McACOT8a, McACOT8b and McACOT8c after amplification were 6372 bp, 6213 bp and 6447 bp, and Mc2075 was approximately 5361 bp (Figure 2B). As shown in Figure 2C,D, by measuring the growth of three groups of transformants (two per group) with biomass and fatty acid content, it was evident that homologous overexpression of ACOT8 in *M. circinelloides* WJ11 had no significant effect on the biomass in the fungus, but had a significant promotional effect on the TFA content. The TFA content of the engineered strains exceeded the control strain Mc2075 (20.8%), and finally, three overexpression strains McACOT8a-1 (25.4%), McACOT8b-2 (26.9%) and McACOT8c-2 (29.3%) were selected as late fermentation strains based on the results of the TFA content.

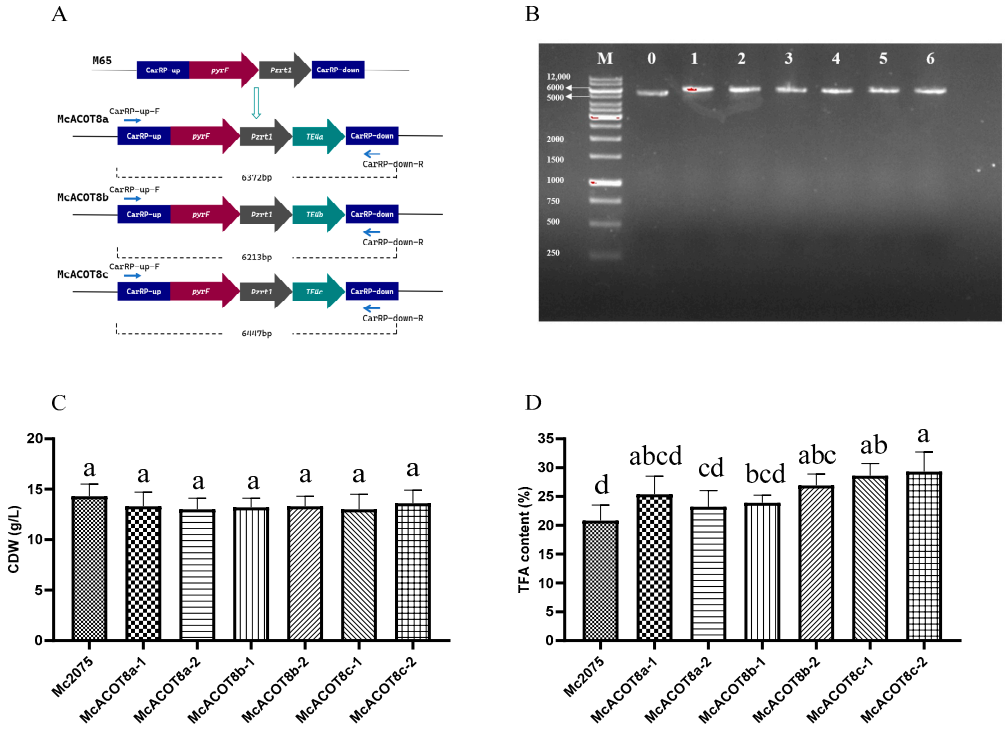

Figure 2. Overexpression of McACOT8a, McACOT8b and McACOT8c in oleaginous *M. circinelloides* WJ11. (**A**) McACOT8a, McACOT8b and McACOT8c expressed under the promotor *Pzrt1* and inserted into the genome using homologous recombination. The *pyrF* gene encodes orotidine-5′-monophosphate decarboxylase, allowing *pyrF* deletion mutants to grow in uracil-restricted medium. CarRP-down is a terminator. (**B**) Polymerase chain reaction amplification of the genome of the control strain Mc2075 (0) and McACOT8a-c-expressing transformants with genomic DNA as the template. M, GeneRuler DNA Ladder Mix; 0, Mc2075; 1, McACOT8a-1; 2, McACOT8a-2; 3, McACOT8b-1; 4, McACOT8b-2; 5, McACOT8c-1; 6, McACOT8c-2; (**C**) CDW of the control strain Mc2075 and McACOT8a-c-expressing transformants; (**D**) TFA of the control strain Mc2075 and McACOT8a-c-expressing transformants. Duncan's multiple range test was conducted and data with different letters indicate statistically significant differences among groups at $p < 0.05$.

3.3. Effect of ACOT8's Overexpression on Cell Growth in the Fermenter

The effects of McACOT8a-1, McACOT8b-2 and McACOT8c-2 overexpression on growth were analyzed by measuring CDW, residual glucose and ammonium in the culture of each expression strain during the whole fermentation process (Figure 3). Generally, all overexpression strains showed a similar and typical growth profile to the control. The biomass of McACOT8a, McACOT8b and McACOT8c (18.0, 17.6 and 17.5 g/L) was significantly lower than that of the control (18.7 g/L) after 4 days of growth. Cell growth and lipid accumulation require the supply of carbon and nitrogen sources. In the first 12 h, cells grow, at which point nitrogen sources are completely consumed. In 12 h to 24 h, cells are in a state of adjustment, so there is a short plateau. By calculating the growth rate, specific growth rate and Yx/s data of fungi cell biomass in Figure S1, the maximum rate of the four strains was in 12 h, and the growth rate decreased significantly thereafter. After 40 h growth, the consumption rate of glucose as a carbon source slowed down and the growth rate of oleaginous fungus cells decreased. Under this culture condition, the TFA accumulation of the strain was saturated, so the TFA accumulation rate of the strain slowed down after 40 h. As shown in Figure S1D, the accumulation of TFA yield in the cells was significantly increased, and TFA yield in McACOT8a-1 cells reached 5.9 g/L at 96 h, while that in the control strain was only 4.7 g/L. The glucose consumption trend of these strains was almost similar. Glucose was consumed rapidly before 48 h, and then the cells grew slowly after 48 h and the glucose consumption rate slowed down, generating

a slower TFA accumulation rate in the strains. Finally, glucose remained above 30 g/L at the end of fermentation (Figure 3C). The medium provided sufficient carbon sources for the growth of *M. circinelloides*, but it was limited by the space of the fermentation tank and the oxygen concentration. *M. circinelloides* did not fully utilize these carbon sources. The organic nitrogen content in the medium was complicated and difficult to be extracted and detected. The consumption of the nitrogen source can be explored by measuring the change in the content of ammonium in the medium. There was no difference in ammonium consumption between the four strains. At 6 h, the consumption of nitrogen source was fast (Figure 3D) and completely exhausted at 12 h.

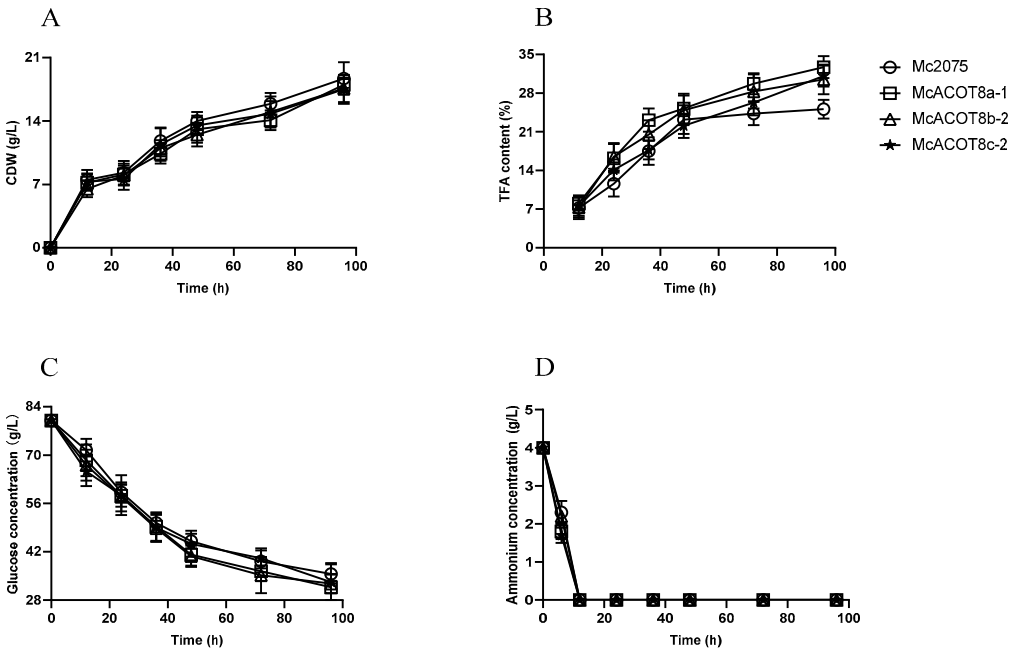

Figure 3. Cell growth and TFA accumulation of engineered strain McACOT8a-1, McACOT8b-2 and McACOT8c-2 and Mc2075 cultivated for 96 h in 3 L fermenter in 1.5 L modified K&R medium. (**A**) CDW; (**B**) TFA content; (**C**) residual glucose concentration; (**D**) residual ammonium concentration. Samples taken from the fermenter at the indicated times. Values are means of three independent replicates with standard error bars.

3.4. Effect of ACOT8's Overexpression on TFA Accumulation and Composition in M. circinelloides WJ11

All of the strains consumed nitrogen rapidly in the early stages of growth and division, and TFA began to accumulate in the cells when the nitrogen source was depleted. The three recombinant strains McACOT8a-1, McACOT8b-2 and McACOT8c-2 all had higher TFA contents than the control strain Mc2075. McACOT8a-1 and McACOT8b-2 showed the same growth trend as the control strains, with rapid growth before 48 h and slow growth after 48 h (Figure 3B). TFA accumulation increased by approximately 21.5 and 23.9% in both McACOT8b-2 and McACOT8c-2 overexpression strains compared with that of the control (from 25.1% in the control to 30.1 and 31.1% in McACOT8b-2 and McACOT8c-2, respectively). Throughout the fermentation process, the TFA mass of the McACOT8a-1 overexpression strain (32.7%) was higher than that of the other two engineered strains and increased by 30.3% compared with the control (25.1%). This result confirms that the enzymes of ACOT8 have a significant effect on TFA accumulation in *M. circinelloides* WJ11. In terms of TFA composition, as shown in Table 1, McACOT84a-1 showed an increase in C16:0 and C18 unsaturated fatty acids and a relative decrease in C18:0 compared with the control strain; McACOT8b-2 showed an increase in C18 unsaturated fatty acids and a decrease in C16:0 and C18:0, and McACOT8c-2 showed only minor changes in fatty acid composition compared with the control strain.

Table 1. Fatty acid content (% of the total) in TFA fractions of Mc2075, McACOT8a-1, McACOT8b-2 and McACOT8c-2.

	TFA Content (%)						
Strains	**C14:0**	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
Mc2075	1.5 ± 0.3 ^{a}	23.5 ± 0.2 ^{cd}	2.2 ± 0.5 ^{b}	7.0 ± 0.2 ^{a}	38.3 ± 0.4 ^{b}	14.7 ± 0.2 ^{a}	12.9 ± 0.4 ^{ab}
McACOT8a-1	1.5 ± 0.3 ^{a}	25.2 ± 0.7 ^{a}	1.2 ± 0.7 ^{d}	4.8 ± 0.5 ^{c}	39.2 ± 0.5 ^{a}	15.0 ± 0.8 ^{a}	13.1 ± 0.5 ^{ab}
McACOT8b-2	1.5 ± 0.2 ^{a}	22.6 ± 0.1 ^{d}	1.5 ± 0.3 ^{c}	6.7 ± 0.2 ^{a}	39.4 ± 0.3 ^{a}	15.1 ± 0.4 ^{a}	13.2 ± 0.2 ^{a}
McACOT8c-2	1.6 ± 0.9 ^{a}	24.1 ± 1.3 ^{b}	2.7 ± 0.8 ^{a}	5.8 ± 0.6 ^{b}	39.7 ± 1.2 ^{a}	13.8 ± 0.7 ^{b}	12.3 ± 0.3 ^{b}

Duncan's multiple range test was conducted and data with different letters indicate statistically significant differences among groups at $p < 0.05$.

The distance traveled on the thin silica gel plate in TLC varied with the mobility (Rf) in the unfolding agent, with triglycerides (TAG), free fatty acids, 1,3-diglycerides (1,3-DAG), 1,2-diglycerides (1,2-DAG) and monoglycerides (MAG) increasing in polarity and decreasing in distance from the origin, in that order (Figure 4A). After coloration development on TLC silica gel plates, it was found that the lipid product of *M. circinelloides* WJ11 had the highest proportion of TAG in the total lipid content, with its band appearing at the top of the thin silica gel plate; followed by free fatty acids, 1,3-DAG and 1,2-DAG near each other; and the lowermost band near the spot sample was glycerol monoester, which was less abundant. The area of the gray scale peaks obtained after processing the developed bands with the gray scale scanning software ImageJ was plotted to scale (Figure 4B). Compared with the control strain Mc2075, all three overexpression strains showed a significant decrease in TAG content, but an increase in free fatty acid content of 48.8, 95.4 and 102.3%, respectively (from 12.9% in the control to 19.2, 25.2 and 26.1% in McACOT8a-1, McACOT8b-2 and McACOT8c-2, respectively), and the fractions of the three overexpressing strains differed considerably in the composition of free fatty acid, as shown in Table 2. Compared with the control strain, McACOT8a-1 showed a significantly higher C16:0 in free fatty acid, while in McACOT8b-2, the ratio of C18:3 was increased. Most notably, free fatty acid of overexpression strains McACOT8b-2 and McACOT8c-2 were able to accumulate C18:3, which was not present in Mc2075, and the presence of C18:3 was not detected after overexpression of ACOT8a. 1,3-DAG content in McACOT8b-2 showed an advantage of 11.3%, an increase of 54.8% compared with 7.3% in the control strain Mc2075.

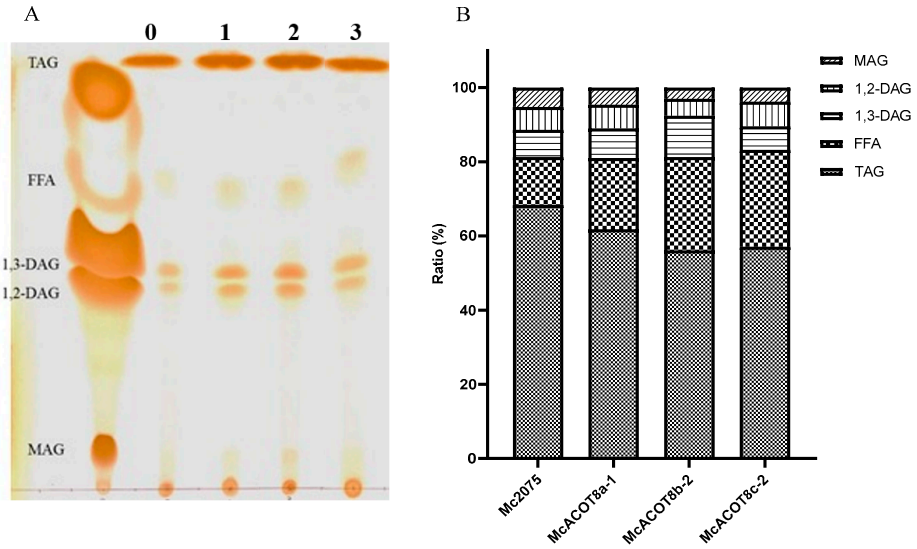

Figure 4. Thin layer chromatography (TLC) analysis. (**A**) TLC silica plates of control strain and overexpressing strains: 0, Mc2075; 1, McACOT8a-1; 2, McACOT8b-2; 3, McACOT8c-2. (**B**) Fatty acid analysis of different components of McACOT8a-c and Mc2075.

Table 2. Fatty acid content (% of the total) in free fatty acid fractions of Mc2075, McACOT8a-1, McACOT8b-2 and McACOT8c-2.

	Free Fatty Acid Content (%)						
Strains	**C14:0**	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
Mc2075	2.9 ± 0.3^{a}	40.6 ± 3.2^{b}	3.1 ± 0.5^{a}	14.2 ± 0.2^{c}	34.0 ± 1.7^{b}	5.2 ± 0.2^{a}	ND
McACOT8a-1	2.5 ± 0.9^{a}	45.2 ± 5.7^{a}	2.1 ± 0.7^{b}	15.8 ± 1.5^{b}	31.5 ± 3.5^{c}	2.9 ± 0.4^{b}	ND
McACOT8b-2	2.4 ± 0.2^{a}	38.1 ± 4.1^{b}	2.1 ± 0.3^{b}	13.0 ± 1.2^{d}	36.4 ± 3.3^{a}	6.3 ± 0.4^{a}	1.7 ± 0.2^{b}
McACOT8c-2	2.8 ± 0.5^{a}	45.9 ± 4.3^{a}	2.3 ± 0.8^{b}	17.9 ± 1.6^{a}	25.3 ± 2.2^{d}	3.4 ± 0.7^{b}	2.9 ± 0.3^{a}

Duncan's multiple range test was conducted and data with different letters indicate statistically significant differences among groups at $p < 0.05$.

3.5. Expression Levels of Different ACOT8 Genes in the Overexpressing Strains of M. circinelloides WJ11

The mRNA levels of three recombinant strains were analyzed with RT-qPCR at 6, 12, 24 and 96 h of growth in fermenters (Figure 5). Taking the mRNA expression level of McACOT8a-1, McACOT8b-2 and McACOT8c-2 at 6 h as 1, the expression of all three strains was significantly higher at 12 and 24 h compared with this value, and increased the most at 12 h, by 32.9-, 42.2- and 26.5-fold, respectively. Although it increased rapidly from 6 to 12 h, it tended to decrease with increasing incubation time after 12 h until 96 h, when McACOT8a-1 and McACOT8b-2 were only marginally expressed, while McACOT8c-2 was also expressed 19.84-fold at 96 h, similar to 24 h of the fermentation process. The mRNA in ACOT8a-c remained high throughout incubation, confirming that it was overexpressed in each of the three recombinant strains.

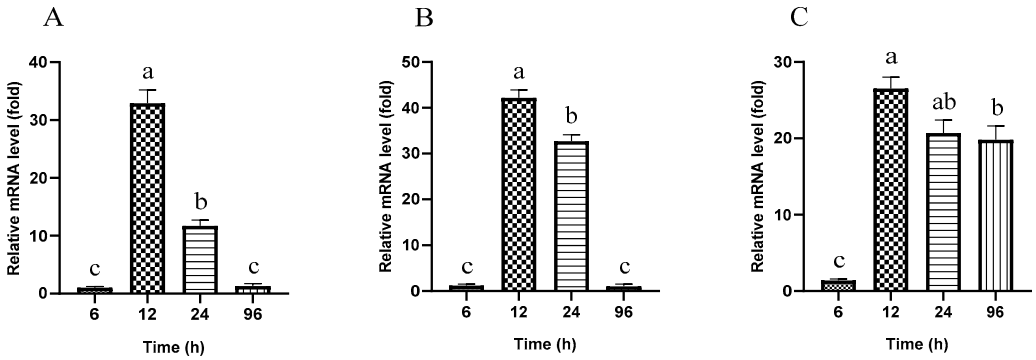

Figure 5. The ACOT8 mRNA expression levels of McACOT8a-1, McACOT8b-2 and McACOT8c-2 gene determined with RT-qPCR. (**A**) mRNA expression of McACOT8a-1; (**B**) mRNA expression of McACOT8b-2; (**C**) mRNA expression of McACOT8c-2. Duncan's multiple range test was conducted and data with different letters indicate statistically significant differences among groups at $p < 0.05$.

4. Discussion

Thioesterases play a key role in lipid accumulation and metabolism, they are widely found in prokaryotes and eukaryotes and are an important processor of various products. The largest proportion of thioesterase is still acyl-CoA/ACP thioesterase, and a variety of thioesterases perform multiple biologically important functions by enabling the cleavage of thioester bonds in a wide range of substrates. Therefore, lipoyl thioesterases genes are often genetically modified to enhance fatty acid production in oil-producing microorganisms [26]. Acyl-CoA thioesterases are critical and essential in the lipid synthesis pathway of microorganisms. As shown in Table 3, it was found that homologous overexpression of acyl-CoA thioesterase (*tesA*) and two genes encoding acyl-CoA synthetase (*fadD5* and *fadD15*) in *Corynebacterium glutamicum* create a cyclic route between acyl-CoAs and fatty acids; and the resulting *fadDs*-disrupted and *tesA*-amplified strains increased their production by 72% and produced fatty acids consisting primarily of oleic acid, palmitic acid and stearic acid [27]. Overexpression of thioesterase ACOT8I in the oleaginous fungus *Mortierella*

alpina enhanced the transformation from acyl-CoA to free fatty acids, increased the production of free fatty acids and adjusted the fatty acid type of lipid species [2]. Further, in the engineered *E. coli* strain containing an endogenous thioesterase, at least 50% of the fatty acids produced can be present in free-acid form [28]. The acyl-CoA thioesterase of *M. musculus* was expressed in *S. cerevisiae*, and the unsaturated fatty acid content in the final engineered strain was higher [15]. Clearly, overexpression of acyl-CoA thioesterases can increase fatty acid production in oil-producing microorganisms.

Thioesterase not only plays a role in the accumulation of microbial lipid content, but also has specificity to fatty acids of different substrates. According to previous studies, ACOT8 in its active state is substrate-specific for all acyl-CoA substrates, from two to twenty carbon atoms, saturated and unsaturated fatty acids, and the experiment proved it. In the specific hydrolysis substrate of acyl-CoA, there is a significant difference with acyl-ACP. The eukaryotic homolog of ACH2 identified in *A. thaliana* is peroxisome acyl-CoA thioesterase, which is up-regulated during the period of increased fatty acid oxidation, and studies have shown that ACH2-6His hydrolyzes medium- to long-chain fatty acyl-CoA, but has the highest activity for long-chain unsaturated acyl-CoA [14]. Four ACOTs were identified in *A. thaliana*, of which two are likely peroxisomes (AtACH1 and AtACH2, with C-terminal PTS1 signals of eAKL and eSKL, respectively) and two others, AtACH4 and AtACH5, were located in the endoplasmic reticulum [29]. Of these, ACH2 was characterized in detail and shown to hydrolyze long-chain saturated and unsaturated acyl-CoA [14]. That ACOT8 is located in the peroxisome and has broad activity against long-chain acyl-CoAs, with the greatest activity levels for CoA esters of primary bile acids choloyl-CoA and chenodeoxycholoyl-CoA, suggests that ACOT8 is a regulator of peroxisomal lipid metabolism [9]. These data suggest that McACOT8 may be involved in lipid biosynthesis and play an important role in free fatty acid accumulation and fatty acid alteration in *M. circinelloides*.

Table 3. Different sources of Acyl-ACP/CoA thioesterases were overexpressed in different microbial species on lipid or TFA accumulation and fatty acid composition.

Acting Substrate	Sources	Gene Origin	Alterations in Lipids	References
Acyl-CoA	*C. glutamicum* ATCC 13032	*C. glutamicum* ATCC 13032	showed a 72% increase in production and produced fatty acids consisting mainly of oleic acid, palmitic acid and stearic acid	[30]
Acyl-CoA	*S. cerevisiae*	*M. musculus*	accumulated more extracellular free fatty acid with higher unsaturated fatty acid	[15]
Acyl-CoA	*M. alpina* ATCC 32222	*M. alpina* ATCC 32222	free fatty acid content increased by about 3-fold and the linoleic acid content in free fatty acid increased from 1.3% to 9.0%	[2]
Acyl-CoA	*M. circinelloides* WJ11	*M. circinelloides* WJ11	TFA content was significantly increased by 30.3, 21.5 and 23.9%, respectively, and free fatty acid production increased with a maximum increase of 103.1%	this study

Numerous experiments and studies have reported that thioesterases can promote the production of TFA or alter the composition of fatty acids in organisms, suggesting that thioesterases are very important targets in the genetic modification of microbial oil synthesis. This is due to the CoASH sensitivity and very broad substrate specificity of this type of thioesterase, which plays an important role in regulating acyl-CoA/CoASH levels in peroxidase, optimizing the flux of fatty acids through the β-oxidation system, thereby accumulating more fatty acid content. As a high oil-producing strain, *M. circinelloides* WJ11 has become a model organism for the study of oil accumulation mechanisms due

to the application of its complex genetic engineering tools and the high production of polyunsaturated fatty acids from its genome sequence [12]. Although the role of key enzymes in the lipid synthesis pathway has been studied by many researchers at home and abroad, the study of endogenous thioesterases has not yet been reported. The present experiment was designed to investigate the functions of three acyl-CoA thioesterases in *M. circinelloides* WJ11, deepening the exploration of the lipid synthesis pathway in *M. circinelloides* WJ11, enriching the study of key enzymes in lipid synthesis and the gene pool of the thioesterase family, providing a basis for future research on the specific production of certain fatty acid products, offering further possibilities for the construction of *M. circinelloides* cell factories and thus making the conversion of *M. circinelloides* to biodiesel more efficient. This will provide a further possibility for the construction of a *M. circinelloides* cell factory and, thus, make the conversion of *M. circinelloides* to biodiesel more possible and practical.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9060545/s1, Table S1: Primers of CarRP-up-F/CarRP-down-R and ACOT8a-c in *M. circinelloides* WJ11; Figure S1: The growth rate (A), $Y_{X/S}$ (B), specific growth rate (C) TFA yield (D) and TFA growth intensity (E) of control and engineering strains.

Author Contributions: F.X. and M.G. contributed equally to this work and are considered the first authors. F.X. and M.G. designed the experiment, performed the experimental work and wrote the original draft. R.W. and Y.C. were involved in the fermentation testing. Z.X., W.D. and H.Y. performed the data curation. Y.Z. carried out the result interpretation. Y.S., C.R. and H.Z. conceived the study, contributed to designing the research protocol and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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